



NIOSH

**PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES**

MARCH 22-23, 1984

**BETHESDA MARRIOTT HOTEL
BETHESDA, MARYLAND**

The papers included in these Proceedings were printed as they were submitted.

Authors who did not submit papers were sent transcripts of their presentation and the appropriate portions of the questions and discussion sessions for editing. Their styles of editing varied, as could be expected. To the extent possible, we have attempted to arrive at a consistent format.

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Thursday Morning, March 22

INTRODUCTORY REMARKS

Richard H. Adamson, Ph.D.
Director, Division of Cancer Etiology
National Cancer Institute

INTRODUCTORY REMARKS

Richard H. Adamson, Ph.D.
Director, Division of Cancer Etiology, NCI

It gives me great pleasure to welcome you to this Third NCI/EPA/NIOSH Workshop. An important function of the NCI is to collaborate and exchange information with other governmental and non-governmental research institutions and with Federal regulatory agencies. The NCI/EPA/NIOSH Collaborative Program on Environmental and Occupational Cancer Studies is a good example of this activity; the Program Workshops, which are held on a regular basis, play an important role in information dissemination and coordination.

The collaborative program with NIOSH initially began in 1976 as a result of a House Committee report. NCI has continued this collaborative program with NIOSH even though there has been no Congressional directive to do so for several years. In Fiscal Year (FY) 1982, it became NCI policy to bring individual research projects before the Division of Cancer Cause and Prevention (now called Division of Cancer Etiology) Board of Scientific Counselors; thus, these projects began receiving outside peer review in 1982. The funding level on the pass-through dollars for FY 1982 was about \$1.2 million, with NCI supporting about an equal amount through direct funding. In FY 1984, the pass-through amount is budgeted at \$950,000. NCI is funding approximately the same amount through the collaborative agreement with NIOSH. It should be noted that the NCI is supporting \$17.6 million for occupational carcinogenesis in FY 1984. The decrease in the amount for the pass-through dollars is a result of budgetary decreases, better quality concept review by the Board and an attempt by the Project Review Group, which now includes a member of the Division of Cancer Etiology Board of Scientific Counselors, to focus only on projects of high priority to both agencies. Virtually all approved projects are currently being funded. Examples of projects currently being funded by the pass-through dollars include: Cytogenetic Assays and Analysis of Occupationally Exposed Workers; Mortality and IH Evaluation of Workers Exposed to Lead Chromate Paints; Mortality Study of Workers Exposed to Hallowax; Case Control Study of Lung Cancer and Diesel Fumes; An Inventory of Union Records Systems; An Assessment of Worker Exposure in the Newsprint Industry; and A Mortality Study of Workers Exposed to Toluene Diisocyanate. We anticipate that the NCI/NIOSH collaborative agreement will begin now to place emphasis on large-scale epidemiologic studies, especially those involving interactions with smoking.

The NCI/EPA Collaborative Program on Environmental Cancer formally constituted in FY 1978. The program included collaboration with the EPA Office of Toxic Substances as well as the EPA Office of Research and Development. The importance of this collaboration was indicated in both the House and Senate reports of the FY 1978 budget. The House report acknowledged that as much as 80 to 90 percent of all cancers can be attributed to environmental factors associated with cancer. The Senate similarly noted that there should be "increased overall, as well as interagency, activity as a result of heightened interest in environmental causes of cancer" and especially urged collaboration with EPA and NIOSH.

There has been no Congressional directive since 1980 concerning this collaboration. Nevertheless, NCI has maintained a collaboration with EPA, and for FY 1984 the total collaborative agreement is estimated to be \$1.9 million: \$950,000 is currently budgeted for pass-through funds to EPA; approximately the same amount (\$950,000) is budgeted for direct funding by NCI. While the number of projects and total dollars have decreased over the years due to budget constraints for both agencies, the quality of the research projects has greatly improved. As noted earlier, for the collaboration with NIOSH, each NCI/EPA project is concept reviewed by the Division of Cancer Etiology Board of Scientific Counselors, and a member of the Board has been appointed to the NCI/EPA Project Review Group. The NCI/EPA projects are emphasizing applications of new technology in terms of measuring exposure and determining risk. Examples of projects being supported with the EPA pass-through funds include: The Application of Explant Culture Techniques to Human-Animal Comparative Biochemical Studies in Chemical Carcinogen Risk Assessment; Development of a National Data Base on Body Burden of Toxic Chemicals Including Carcinogens; The Characterization and Validation of Carcinogen-Induced Transformation of Human Cells In Vivo; and Body Burden of Toxic Substances in Support of the Total Exposure Assessment Methodology (TEAM) Study. The NCI is also funding a project concerned with biochemical, pharmacologic, and tumorigenic studies on a composite of drinking water carcinogens and mutagens utilizing aquatic animals as the bioassay animal.

In 1981 the NCI transferred the Carcinogenesis Testing Program to the National Institute of Environmental Health Sciences (NIEHS), an action that provides for direct management by the National Toxicology Program (NTP) of the NIH components of the program. Therefore, both NIOSH and EPA should, in future years, interact more closely with the NTP, especially for studies relating to testing of chemicals, to assist in designing studies more relevant to risk assessment and for development and validation of testing methods.

NCI intends to continue its collaboration with NIOSH and EPA through this collaborative program and also by utilizing other mechanisms. It is hoped that in the future collaborative projects will take advantage of the expertise of each agency and, in particular, utilize the expertise NCI has in epidemiology.

I would like to conclude by thanking several individuals who have been involved in the program and in putting together these workshops over the past four years. The NCI effort has been headed by Drs. Kraybill, Cameron, and Kelsey. They have been ably assisted by Ms. Blackwood and Ms. Olbrich. Dr. Bridbord, representing NIOSH, has been associated with the collaborative program for many years. He has transferred to the Fogarty Center, and Dr. Roy Fleming has taken his place. I would also like to thank Drs. Farland and Ulvedal, from EPA, for their efforts.

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University of North Carolina

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PRESENTATION:

Retrospective Cohort Mortality Study of Underground Gold Mine Workers

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RETROSPECTIVE COHORT MORTALITY STUDY OF
UNDERGROUND GOLD MINE WORKERS

Presented at the 3rd NCI/EPA/NIOSH Collaborative Workshop: Progress on
Joint Environmental and Occupational Cancer Studies

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ABSTRACT

In a retrospective cohort mortality study of 3328 gold miners who worked in full-time underground jobs for at least one year between 1940 and 1965, there were 861 observed versus 765 expected deaths overall. The primary exposures were to amphibole minerals in the cumingtonite-grunerite series and silica. No association, as measured by length of employment underground, dose (total dust x time), or latency was apparent with lung cancer mortality (43 observed vs 43 expected).

Exposure to silica as measured by total dust was strongly associated with mortality due to respiratory tuberculosis (36 observed vs 9.9 expected) and non-malignant respiratory disease (53 observed vs 19 expected).

Other important findings include an excess in mortality due to accidents, other than transportation (58 observed vs 17.5 expected), chronic renal disease (8 observed vs 5.0 expected), and leukemia/aleukemia (10 observed vs 5.9 expected).

ACKNOWLEDGEMENTS

We wish to thank the staff of the NIOSH, Industrywide Studies Branch, and the staff of the Stanford Research Institute (SRI) International for their assistance in obtaining, coding and editing the data required for this study. In particular, the NIOSH clerical staff under the direction of Clorinda Battaglia, Edith Dodd, Pauline Bischak, Judy Edelbrock and Mary Hogan; and the SRI staff directed by Mac Kaplowitz should be acknowledged. Dr. William Gaffey of SRI contributed a great deal to the initial phases of this study and the Homestake Mining Company especially Mr. Langan Swent provided a great deal of assistance throughout the study. Finally, we wish to thank Diane Manning, Marsha Gran, Janet Graydon and Nancy Rosenberger for preparation of the manuscript.

I. Introduction

The Homestake population lends itself well to the examination of several important epidemiologic questions which have been addressed in this study:

- A. Of primary concern and the reason for initiating this study is the hypothesis that exposure to the amphibole mineral fibers found in the ore of the Homestake gold mine is associated with asbestos-related disease, such as lung cancer, asbestosis and mesothelioma.
- B. Since silica dust has been a significant exposure at the mine, questions relating to this exposure have also been addressed. In particular, it has recently been suggested that silica exposure is associated with an increased risk of lung cancer,¹ and that it may be related to chronic renal disease.² Silica related nonmalignant respiratory diseases such as silicosis and silicotuberculosis have also been examined in some detail.
- C. As in most mining populations, mortality due to accidents is a potential problem. This was also examined in the study population.
- D. Since the analytic program used in this study is capable of evaluating 89 different categories of mortality, data on other causes of death are presented but not analyzed in any detail.

II. Description of the Facility and Environmental Aspects

The mining practices at Homestake have improved continually since the early 1900's. The changes in mining practices have resulted in lower occupational exposures to total dust which is probably directly or indirectly correlated with most other specific exposures. Dry drilling techniques were employed until 1919 when wet drilling was initiated. Changes in blasting were introduced in 1939, and then again in 1950; these changes substantially reduced exposure to dust and smoke. Also in 1950, automatic water valves were added to all drills, a change which further reduced exposure to dust.

Ventilation in the mine, as measured by exhaust air rate, has also undergone vast improvements since the early 1900's. From 1918 to 1923, the ventilation rate was estimated to be 77,000 cubic feet per minute (cfm). By successive improvements the rate in 1975 had been increased to 720,000 cfm. Additional improvements in dust control have resulted from secondary ventilation techniques using progressively larger fans to deliver clean air to work sites.³

Another factor which reduced miners' exposures to many of the contaminants in the mine was the reduction in the number of hours spent underground per week. The number of work hours per week has decreased steadily from 52 hours in 1920, to the present low of 40 hours. Prior to 1920, the number of work hours per week was greater than 52 hours.

Based on data collected during environmental surveys conducted at the Homestake Mine the following contaminants were thought to have the greatest potential for causing adverse health effects: quartz (free silica), amphibole mineral *fibers (or fragments) classified in the cumingtonite-grunerite (CG) mineral series, arsenic (arsenopyrite), and radon daughters.³⁻⁶ Other airborne contaminants such as trace metals, carbon monoxide, carbon dioxide, methane, nitrogen dioxide, total hydrocarbons, benzo(a)pyrene, and total aldehydes were identified in the mine but only in negligible quantities.

Based on examination of historical survey data, it appears that mean exposures to arsenic and radon daughters were within the current occupational standards. During an industrial hygiene survey³ conducted in 1977, the geometric mean arsenic exposure for all miners grouped together was 1.17 ug/m^3 (95% confidence limits = 0.77 and 1.77 ug/m^3). The arithmetic mean for all miners was 2.03 ug/m^3 which was half the average exposure found by Mining Enforcement and Safety Administration (MESA)⁶ in 1974. Only one worksite in the 1977 survey was found to exceed the Occupational Safety and Health (OSHA)⁷ standard of 10 ug/m^3 . The NIOSH⁸ recommended arsenic standard of

* Since the fibers conform with the NIOSH definition of asbestos fibers they will be referred to as "fibers" throughout the remainder of the report. The fibers do not conform to other definitions, e.g. U.S. Bureau of Mines, of asbestos which are based on strict mineralogical properties of asbestos.

2 ug/m³ for any 15-minute period was exceeded in 16 of the 51 samples. Based on a recent quantitative risk assessment by OSHA (47 FR 15358, April 9, 1982; 29 CFR 1910.1018) the levels of arsenic exposure experienced by the Homestake workers may have resulted in a slight excess in lung cancer mortality.

In the 1977 survey,³ radon daughter levels ranged from 0 to 0.169 work levels (WL). In a survey conducted by MESA⁹ in 1977, levels were reported to range from 0.01 to 0.130 WL. In a U.S. Bureau of Mines⁴ survey conducted in 1960 the average exposure was 0.006 WL. In 1968 and 1975 MESA^{10,11} found levels around 0.01 WL. The exposures found prior to 1977 were probably low because there was no attempt to identify sources of exposure in relation to ventilation systems. The standard¹² for radon daughters is 4 working level months (WLM)/year. Therefore, the levels found in these surveys at Homestake were considerably below the accepted standard.

Based on electron microscopy analysis of airborne samples collected during the 1977 survey,³ 84 percent of the airborne fibers were identified as amphiboles, while the remaining 16 percent were either unidentified (ambiguous) or not amphibole minerals. Sixty-nine percent of the amphiboles were characterized as CG, 15 percent as tremolite-actinolite, with the remaining 16 percent identified as fibrous hornblende minerals. The CG fibers had a geometric mean diameter of 0.43 microns (95% confidence limits of 0.39 and 0.47) with a

geometric mean length of 3.3 microns (95% confidence limits of 3.0 and 3.6); the tremolite-actinolite fibers had a geometric mean diameter of 0.27 microns and a geometric mean length of 4.1 microns. Twenty-four percent of the CG fibers and 32 percent of the tremolite-actinolite fibers had lengths greater than 5 microns.

Based on optical microscopy, personal exposures to fibers, as measured during the 1977 survey, for most job categories were below the current OSHA and Mine Safety and Health Administration (MSHA) time-weighted averages (TWAs) asbestos standard of 2 fibers (greater than 5 microns in length) per cubic centimeter. The geometric mean TWA exposure for all miners was 0.44 fibers (greater than 5 microns in length) per cubic centimeter. A summary of these findings is given in Table 1.

Prior to the early 1950's the dust levels and free silica content of the dust (39 percent in settled dust and 13.1 percent in *respirable dust) were sufficiently high to cause silicosis. After the early 1950's the overall dust levels dropped substantially to a point where the exposure to free silica was less likely to cause pneumoconiosis. This conclusion is based on the various standards which have been

* The silica content of respirable dust was not measured until gravimetric sampling was developed in the mid-1970's.

adopted for occupational exposure to free silica. The American Conference of Governmental Industrial Hygienists (ACGIH)¹³ threshold limit Value (TLV) in terms of millions of particles per cubic foot (mppcf) is $300/\%SiO_2 + 10$ mppcf. Since the percent free silica found in the settled dust is 3%, the TLV is equal to $300/39 + 10 = 6.1$. Table 2 gives a summary of the average dust level by year and job category. Prior to 1952, the levels of total dust were above the TLV for silica, whereas, beginning in 1952, most of the dust levels have been substantially below the TLV.

In order to determine whether or not cause-specific mortality among Homestake miners is associated with exposure to total dust, an index of exposure was calculated. This index is based on the yearly average total dust exposure for specific job categories (Table 2) and on the yearly average time spent (per shift) underground. The average time spent underground was estimated for each year, where the maximum time spent underground occurred prior to 1920 and was reported to be 3600 hours per year. This was assigned a weighting factor of 10. For each year thereafter, the average time spent underground has declined. Based on a factor of 10 for 3600 hours/year, a factor was calculated for each year corresponding to the average number of hours worked for that year (e.g., for 1933, when the yearly average was 2642 hours the weighting factor was calculated to be $2624 \text{ hours}/3600 \text{ hours} \times 10 = 7.3$). All full-time underground jobs were assembled into five major groups based on similarity in job function and exposure to total dust.

One additional category was used to group all jobs not considered full-time underground. The index of exposure was calculated for each job category for each year by multiplying the dust level by the weighting factor for time underground. The indices of exposure are given in Table 3 for each year and for each job grouping. For the analyses of this study, index of exposure represents an approximate intensity of exposure so as to examine dose-response relationships.

III. Methods

The epidemiologic study was designed as a retrospective cohort mortality analysis. The study cohort was defined so as to include all white, male Homestake workers who were employed full-time underground in the mine department for one year or more between January 1, 1940 and December 31, 1964. Their employment could have begun prior to 1940. All of the mine records were initially screened to select the study cohort, and company personnel records were used to supplement the data when necessary. For each member of the final cohort, the jobs held at the Homestake Mine were coded into the six categories described above.

All workers who were known to have mining experience in uranium mines were excluded from the cohort; this procedure resulted in the exclusion of 68 workers.

An attempt was made to determine the vital status of each worker as of June 1, 1977. For all those workers identified as deceased, the

underlying cause of death was coded by a nosologist according to the International Classifications of Diseases (ICDa) revision in effect at the time of death. Workers with an unknown vital status were considered alive as of 6/1/77 for purposes of analysis. This procedure typically results in slight underestimation of the specific mortality risks. If a miner was known to be deceased but a death certificate was not obtained, he was considered dead, cause unknown. If a worker died subsequent to the closing date, he was considered alive in the analysis of this study.

The mortality analysis was conducted using a person-years life table computer program described by Waxweiler, et al.¹⁴ In the analysis, person-years (PY) at risk of dying were calculated for each worker, with PYs starting to accumulate after the worker had qualified for the study cohort, or in other words, after he had worked one year in full-time underground mining jobs after January 1, 1940. The PYs for each worker were accumulated until the closing date (June 1, 1977) or until his date of death, whichever occurred first. The PYs for all workers were then distributed into 5-year age groups and 5-year calendar-time periods and were multiplied by the corresponding U.S. white male mortality rate to yield the number of deaths expected. For certain causes of death an analysis of mortality was conducted by latency (time since first employment in a full-time underground job), by duration of employment in full-time underground jobs, and by accumulated exposure or dose (dust-days).

For the analysis by latency and by length of underground employment, time spent in jobs that were not full-time underground was ignored. When calculating latency and length of underground employment, work history prior to 1940 was included. For the analysis by dose, PYs were distributed into categories of cumulative dose. Again, for this analysis PYs did not begin to accumulate until the employee worked for at least one year after January 1, 1940, even though, cumulative dose was based on total work history including employment at the mine prior to 1940. Cumulative dose was calculated by multiplying the index of total dust exposure (Table 3) by time worked underground, yielding dose units equal to the index of dust exposure multiplied by days (dust-days).

Estimates of risk for specific causes of death were calculated as standardized mortality ratios (SMR) -- observed deaths/expected deaths x 100.

IV. Results

A. Cohort Description

The total cohort consists of 3,328 white males and 79,123 person-years. The results of vital status follow-up are given in Table 4. Only 5.6% of the cohort was lost to follow-up (unknown vital status), with a fairly uniform distribution of unknowns by date first employed.

A distribution of the cohort by length of underground employment and by year first employed underground is shown in Table 5. From this distribution, it can be observed that a large influx of new workers began employment in the ten years after World War II (1945-1954), and that 64 percent of the cohort was first employed in underground jobs after 1944. This influx of workers is due to a resumption of full-scale operations after the War. From an epidemiological viewpoint, this observation is important because it yields two sub-cohorts, one first employed before the War and a second first employed after the War. The former group is older and is therefore a selected surviving cohort; their maximum latency is longer and their exposures were relatively higher than those of the latter group.

B. Overall Mortality

Table 6 lists the observed and expected deaths for many of the causes which were examined. The SMR for all causes was greater than normal and considerably greater than that seen for most working populations where the "healthy worker effect (HWE)" is seen. This increase in overall mortality is due mainly to increases over expectation in deaths from nonmalignant respiratory disease, tuberculosis, and accidents. In fact, examination of the mortality due to circulatory disease (SMR = 85; p less than 0.05), which is the cause of death usually most affected by the HWE, indicates that the HWE is in fact a factor in the mortality experience of this population.

C. A Priori Hypotheses

Lung Cancer

The overall risk for lung cancer (including malignancies of the trachea, bronchus, and lung) in this population is almost exactly that expected based on U.S. mortality rates, 43 observed vs. 42.9 expected. When analyzed by latency and length of employment (Table 7), there are no apparent trends in lung cancer mortality with increasing latency or with an increase in length of underground employment. There is a non-statistically significant excess in mortality for those workers employed for 15-19 years; however, the risk decreases after 20 years of employment in full-time underground jobs. This particular excess may, therefore, be a statistical fluctuation.

In Table 8 the mortality analysis is stratified by latency and dose. Again, there is no demonstration of an increase in lung cancer risk with an increase in dose as measured by dust-days.

Nonmalignant Respiratory Disease (NMRD) and Respiratory Tuberculosis (RTB)

Because of the heavy exposures to silica prior to 1952, it was hypothesized that mortality from nonmalignant respiratory disease (NMRD) and respiratory tuberculosis (RTB) would be associated with

employment at the gold mine. Overall, there was a highly statistically significant risk (p less than 0.01) for both NMRD (53 observed vs. 19.00 expected) and RTB (36 observed vs. 9.94 expected). Mortality for NMRD by latency and length of employment underground is given in Table 9. When examined by latency (column total) a statistically significant risk occurs after 25 years, and the risk for subsequent time periods remains fairly constant -- at a SMR of approximately 300. Except for slight fluctuations, there is a positive risk gradient with an increase in length of underground employment (row total). Further evidence for a dose-response relationship is seen in Table 10 where the mortality for NMRD by latency and dose (measured by total dust-days) is given. Again, there is a general increase in risk with an increase in dose. It should be noted that dose in this analysis is heavily weighted by length of underground employment so the results are not totally independent of those presented in Table 9.

The mortality due to RTB by latency and length of underground employment is presented in Table 11. In contrast to NMRD, where the SMR leveled off at about 300 after 25 years of latency, there is an SMR of 258 (4 observed vs. 1.55 expected) for RTB during 20-24 years of latency but the SMR increases to 1683 (17 observed vs. 1.01 expected) after 35 years. The risk by length of underground employment for RTB is clearly parallel to that seen for NMRD. There is a general increase in risk with an increase in length of

underground employment. The pattern of mortality for RTB by latency and dose is given in Table 12, where it can be seen that the excess risk is restricted to the last two highest dose categories (or at a dose greater than 320000 dust-days).

Since NMRD and RTB both reflect silica and dust-related disease (silicosis and silicotuberculosis) and due to the fact that there may have been misdiagnosis between the two diseases, especially in the earlier years,¹⁵ the pattern of mortality with these two causes combined were examined in Table 13 (latency and length of employment underground) and Table 14 (latency and dose). Considered as one disease, there is a doubling of risk beginning at 20 years of latency and the risk levels off at an SMR of approximately 400 after 25-years. There is a time-related gradient of risk with an increase in length of employment underground, where the statistically significant excess begins during the 10-14 year period. Except for one category (160000-320000 dust-days) there is also a dose-related gradient in risk.

Temporal Considerations

The mortality patterns for diseases relevant to the a priori hypotheses were examined by subcohorts defined by date first employed. The results of this analysis are given in Table 15.

(0.40 deaths were expected), there were 4 deaths due to NMRD (1.40 deaths were expected), there were 3 deaths due to lung cancer (4.76 deaths were expected) and there were 21 deaths due to accidents (4.23 deaths were expected). Although the number of deaths is small, it appears that the pattern of mortality by latency and length of underground employment for NMRD is similar to that seen in the entire cohort. That is, there is an increase in risk after 20 years of latency (SMR = 667, 3 observed vs. 0.45 expected) and the risk for those with greater than 5 years of underground employment is elevated (SMR = 548, 4 observed vs. 0.73 expected).

D. Other Causes of Interest

Chronic Renal Disease

During the course of this study, mortality due to chronic renal disease became of interest because of observations which indicated that mine workers may be experiencing an increased risk from this cause of death.² In this study the cause of death category -- "Chronic and unspecified nephritis and other renal sclerosis" was examined in response to this interest. The results for this analysis can be found in Table 6. There is a non-statistically significant excess (SMR = 160, 8 observed vs. 5.0 expected). When examined by length of employment (Table 16) there appears to be an

Much of the excess risk in the cohort for overall mortality occurred in the subcohort of workers who were first employed prior to 1930. In fact, when this subcohort is excluded, the resulting SMR for all causes is reduced to 102 (526 observed vs. 517 expected).

More specifically, 32 of the 36 deaths due to RTB occurred in the sub-cohort first employed prior to 1930, resulting in an SMR of 792. For NMRD, 33 of the 52 deaths (SMR = 510) occurred in this sub-cohort. Smaller but still elevated risks for NMRD persisted in many of the subsequent sub-cohorts. For lung cancer there was an elevated risk (15 observed vs. 12.02 expected; SMR = 125) for the subcohort first employed prior to 1930, all other subcohorts are near or below normal. The risk for accidents among those first employed prior to 1930 is no higher than in subsequent years; in fact, the highest risks (SMR's greater than 400) occurred after 1945.

One additional analysis was performed on a subcohort of all workers first employed after 1951. The purpose of this analysis was to determine cause-specific mortality risks associated with a substantial decrease in total dust levels after 1951 (Table 15). The results from this analysis are limited since there were only a total of 109 deaths, which was significantly more than expected (109 observed vs. 92.66 expected; SMR = 118, p less than 0.05). In terms of specific diseases of interest, there were no deaths due to RTB

increase in risk with an increase in length of employment, however, this is based on very small numbers of deaths in each employment category. The mortality for this cause of death by dose (dust-days) is illustrated in Table 17. Although the numbers are small, there appears to be an indication of a positive dose-response, and it appears that the increased risk is confined to those with 320000 or more dust-days where there are 6 observed and 2.10 expected deaths.

Malignant Neoplasms of the Lymphatic and Hematopoietic System

It has been hypothesized that exposure to asbestos may be related to an excess risk due to mortality from lymphatic and hematopoietic cancer.¹⁶⁻¹⁷ Furthermore, an increase in lymphatic tumors has been noted in animals exposed to silica.¹⁸ Therefore, an analysis of this category of disease was of interest in this study. For the whole disease category combined, a non-statistically significant excess was observed (19 observed vs. 13.9 expected deaths). For leukemia and aleukemia in particular, the SMR was 170 (10 observed vs. 5.9 expected deaths).

V. Discussion

This study of mine workers at Homestake offers important epidemiologic information regarding the lung cancer risk from short, non-commercial amphibole fibers, and silica exposure. In comparison to other

studies¹⁹ of asbestos exposure which clearly demonstrate an excess lung cancer risk associated with asbestos, this study indicates there exists only a slight excess in mortality after 30 years of latency; 29 deaths were observed in that category and 22.5 were expected. However, there is no apparent evidence of a positive dose-response for lung cancer, whether the dose is measured by length of employment or by dust-days.

When mortality for lung cancer is examined by date first employed there is a slight excess mortality in those workers first employed before 1930. Although exposures to total dust were generally higher during that time period, the higher exposures do not explain the higher risk, since this is not consistent with the results of the dose-response analyses. It should be noted that the risk for nonmalignant respiratory disease especially among those employed prior to 1930 is very high (65 observed vs. 10.5 expected, SMR = 620; for RTB and NMRD combined). It is possible that this high risk for nonmalignant respiratory disease could have competed with the risk for lung cancer; that is, those with heavy exposure to total dust (and thus, to CG fibers) may have died prematurely from nonmalignant dust-related disease before the malignant disease was able to become manifest. To eliminate this possibility, the analysis can be restricted to those first employed after 1930, at which time there is a reduction in risk for nonmalignant respiratory disease.

However, for this subcohort and for the exposures associated with it, there still is no excess observed in lung cancer mortality (28 observed vs. 30.9 expected).

It should be noted that U.S. lung cancer mortality rates, which were used to generate the expected number of deaths in this study, have been higher than those of South Dakota, the state where the majority of the study population has resided. According to the National Cancer Institute,²⁰ for the years 1950 to 1969, which represents an approximate mid-point of the study, the age adjusted lung cancer mortality rates for white males in the U.S. were 37.98 deaths/100,000, whereas, the corresponding South Dakota rates were 23.71 deaths/100,000. Therefore, if South Dakota rates had been used to generate expected deaths, the SMR's could have been elevated by as much as 38%. It should also be noted that smoking was prohibited in the mine from the early 1900's until 1952. This rule was strictly enforced due to the fire hazard. Any interaction between smoking and occupational exposure would be minimized during this period. However, the findings related to lung cancer should be viewed as truly showing no association with exposures in the mine for three reasons: 1) The patterns regarding latency and dose are inconsistent with an etiology related to exposures experienced at the mine. 2) The number of expected deaths in this cohort allows for a very powerful analysis; we had a 90% probability of detecting a true relative risk of 1.5 for lung cancer, based on a one-sided

test. When the cohort was restricted to those with at least 15 years latency there was still an 38% probability of detecting the same risk and when restricted to at least 15 years latency and at least 5 years employment underground, there was still a 76% probability of detecting the risk. 3) Arsenic exposure may have been high enough especially in the years prior to 1952, to produce slight excesses in lung cancer mortality. These exposures may confound the analysis of effects from either amphibole fibers or silica.

The overall risk for nonmalignant respiratory disease coded as either RTB or NMRD is significantly elevated. Both of these categories reflect dust-related pneumoconiotic diseases, and in this case, reflect silicosis-related disease; where the excess risk in RTB is due to excess mortality in silicotuberculosis and the excess risk in NMRD is due to excess mortality in silicosis. Based on pathological reports obtained from hospitals, at least two-thirds of the individuals who died from NMRD had a confirmed diagnosis of silicosis.

An analysis of RTB and NMRD by length of employment in underground jobs, by dose (dust-days), and by latency indicate that both of these causes of death are associated with exposures experienced at the mine. For both causes a positive dose-response gradient is observed whether the dose is measured by length of employment or by dust-days; and the significant excess in both causes occurred after

a latent period predictable for an occupationally-related chronic disease. The dose-response relationship for NMRD is not strictly linear a finding which may be due to two factors. First, the category, NMRD, includes diseases that are not specifically related to silica dust exposure, for example, emphysema. Second, the calculations of length of employment and dose in terms of dust-days for the cohort is underestimated because jobs other than those classified as "full time underground" were not counted in these calculations and some of these jobs had dust exposure.

The excess in RTB occurred almost entirely among miners first employed prior to 1930 with only one death due to RTB occurring after 1969. In part, this is a reflection of a general decline in death due to tuberculosis, because of better standards of living and improvements in medical care, and because of improvements in the diagnosis of silicosis. Similarly, most of the excess risk due to NMRD is among those first employed prior to 1930. However, the risk remains above normal for miners first employed in subsequent time periods as well. For the subcohort first employed after 1951 when dust levels were substantially reduced, the risk for NMRD was still high with an SMR of 308 (4 observed vs. 1.4 expected). It is not known whether or not this excess is due to silicosis. For the only two deaths where pathological information was available, there was no mention of silicosis and in none of these four cases was silicosis mentioned on the death certificate. The number of deaths in this subcohort is small, and continuing follow-up will evaluate the possibility of silicosis among miners exposed after 1951.

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U.S. DHEW, NCI, Publication No. (NIH) 74-615.

Table 1

TIME WEIGHTED AVERAGE AIRBORNE EXPOSURE (Optical Microscopy)
TO FIBERS GREATER THAN 5 MICRONS AMONG HOMESTAKE WORKERS

Job Category	Range (fibers/cm ³)	Geometric Mean
Miners	0.08-4.01	0.44
Underground Jobs Excluding Miners	0.02-2.57	0.24
Crushing Department Workers (Surface)	0.12-5.34	1.16

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Table 2

AVERAGE YEARLY DUST CONCENTRATION
(in millions of particles per cubic foot)
BY YEAR BY JOB GROUPING AT THE HOMESTAKE GOLD MINE
(1937-1975)

Year	JOB CATEGORY				
	1	2	4	6	7
1937	8.1	6.6	12.8	7.4	95.1
1938	9.3	34.7	13.8	22.0	61.1
1939	12.0	21.0	15.7	16.5	27.1
1940	9.1	13.0	12.5	11.0	11.1
1941	12.4	10.2	14.2	11.3	11.1
1942	12.8	49.8	16.9	31.3	11.1
*1943-1945	—	—	—	—	—
1946	12.3	26.9	9.6	19.6	23.6
1947	12.3	26.9	9.6	19.6	23.6
1948	11.3	11.6	12.2	11.4	23.6
1949	22.1	12.3	15.5	17.2	20.0
1950	4.1	14.0	7.1	9.0	16.5
1951	6.3	6.7	10.7	6.6	13.0

Table 2
(Continued)

Year	JOB CATEGORY				
	1	2	4	6	7
1952	3.6	7.1	6.1	5.3	9.5
1953	1.5	5.5	7.3	3.5	0.7
1954	2.0	8.1	7.0	5.0	0.8
1955	3.2	5.4	5.5	4.3	0.9
1956	1.4	5.4	5.9	3.4	1.0
1957	2.6	5.0	4.4	3.8	1.1
1958	2.2	4.7	3.8	3.4	1.2
1959	2.6	4.3	4.2	3.5	1.3
1960	2.4	4.2	4.1	3.3	1.4
1961	2.1	4.0	3.8	3.2	1.5
1962	1.7	3.8	3.6	2.6	1.6
1963	1.3	3.6	3.4	3.0	1.7
1964	0.9	3.4	3.2	2.9	1.8
1965	0.9	4.5	3.2	3.6	2.8

Table 2
(Continued)

Year	JOB CATEGORY				
	1	2	4	6	7
1966	2.0	3.3	4.1	3.5	3.8
1967	1.7	3.1	3.6	2.8	7.9
1968	1.5	3.2	4.0	3.8	12.0
1969	1.5	2.9	3.6	2.9	9.8
1970	1.4	3.0	3.6	3.0	4.8
1971	0.7	2.5	3.6	3.4	4.1
1972	0.8	2.9	3.6	3.6	3.4
1973	0.8	2.9	3.7	3.8	2.7
1974	0.9	2.6	3.9	4.0	2.1
1975	0.9	2.6	3.9	4.0	2.1

* Except for limited activity, the mine was closed from October 16, 1942 to July 1, 1945.

FROM: May 27, 1976, memo from J. K. Waterland to D. T. Delicate -
Homestake Mining Company, Lead, South Dakota.

Table 3

INDEX OF DOSE BY JOB CATEGORY AND YEAR

Year	Job Category				
	1	2	4	6	7
Pre-1920	100.0	100.0	100.0	100.0	100.0
1920-32	74.0	74.0	74.0	74.0	74.0
1933	73.0	73.0	73.0	73.0	73.0
1934	68.0	68.0	68.0	68.0	68.0
1935	65.7	65.7	65.7	65.7	65.7
1936	65.7	65.7	65.7	65.7	65.7
1937	53.5	43.6	84.5	48.8	627.7
1938	61.4	229.0	91.1	145.2	403.3
1939	78.0	136.5	102.1	107.3	176.2
1940	58.2	83.2	80.0	70.4	71.0
1941	70.7	58.1	80.9	64.4	63.3
1942	76.8	298.8	101.4	187.8	66.6
*1943	10.0	10.0	10.0	10.0	10.0
*1944	10.0	10.0	10.0	10.0	10.0
*1945	10.0	10.0	10.0	10.0	10.0
1946	66.4	145.3	51.8	105.8	127.4
1947	66.4	145.3	51.8	105.8	127.4
1948	59.9	61.5	64.7	60.4	125.1
1949	117.1	65.2	82.2	91.2	106.0
1950	23.0	78.4	39.8	50.4	92.4
1951	38.4	40.9	65.3	40.3	79.3
1952	20.2	39.8	34.2	29.7	53.2
1953	9.8	35.8	47.5	22.8	4.6
1954	13.0	52.7	45.5	32.5	5.2
1955	20.5	34.6	35.2	27.5	5.8
1956	9.1	35.1	38.4	22.1	6.5
1957	16.9	32.5	28.6	24.7	7.2
1958	14.5	30.6	24.7	22.1	7.8
1959	16.9	28.0	27.3	22.8	8.5
1960	15.6	27.3	26.7	21.5	9.1
1961	13.7	26.0	24.7	20.8	9.8
1962	11.1	24.7	23.4	16.9	10.4
1963	8.5	23.4	22.1	19.5	11.1
1964	5.9	22.1	20.8	18.9	11.7
1965	5.9	29.3	20.8	23.4	18.2
1966	12.8	21.1	26.2	22.4	24.3
1967	10.5	19.2	22.3	17.4	49.0
1968	9.0	19.2	24.0	22.8	72.0
1969	9.3	18.0	22.3	18.0	60.8
1970	8.8	18.9	22.7	18.9	30.2
1971	4.6	16.3	23.4	22.1	26.7
1972	4.5	16.2	20.2	20.2	19.0
1973	4.4	16.0	20.4	20.9	14.9
1974	5.0	14.3	21.5	22.0	11.6
1975	5.0	14.3	21.5	22.0	11.6

For 1943-1945, the mine was closed but there was limited activity. An index of dust exposure equal to 10.0 was assigned for these years for all jobs. Prior to 1937, no dust counts were available, therefore, they were estimated by extrapolating from 1937-1952 data and from personal (Homestake) knowledge of conditions in mines with similar work practices.

Table 4

VITAL STATUS ASCERTAINMENT FOR THE HOMESTAKE COHORT

Year First Employed	Alive (%)	Dead (%)	Unknown (%)	Total
<1930	106 (23)	335 (74)	11 (3)	452
1930 - < 1935	121 (45)	134 (50)	13 (5)	268
1935 - < 1940	173 (62)	96 (34)	12 (4)	281
1940 - < 1945	140 (67)	55 (26)	15 (7)	210
1945 - < 1950	547 (76)	113 (16)	55 (8)	715
1950 - < 1955	499 (82)	78 (13)	32 (5)	609
1955 - < 1960	379 (86)	29 (7)	32 (7)	440
1960 - < 1965	317 (90)	21 (6)	15 (4)	353
Total	2282 (68.5)	861 (25.9)	185 (5.6)	3328

Table 5

DISTRIBUTION OF WORKERS BY DATE FIRST EMPLOYED AND LENGTH OF
EMPLOYMENT IN UNDERGROUND JOBS AMONG THE HOMESTAKE COHORT

Date First Employed	Length of Underground Employment											Total (%)
	1 < 2	2 < 3	3 < 4	4 < 5	5 - 9	10 - 14	15 - 19	20 - 24	25 - 29	> 30		
< 1920	1	0	0	2	2	4	10	19	27	23	88 (3)	
> 1920 - 1924	0	1	0	1	15	18	45	19	12	9	120 (4)	
> 1925 - 1929	0	3	2	1	38	111	47	22	9	11	244 (7)	
> 1930 - 1934	2	3	2	2	125	80	19	11	6	18	268 (8)	
> 1935 - 1939	13	27	57	57	82	15	6	7	8	9	281 (8)	
> 1940 - 1944	96	24	13	9	30	10	5	12	8	3	210 (6)	
> 1945 - 1949	283	126	51	30	78	30	25	34	48	10	715 (22)	
> 1950 - 1954	160	76	39	41	98	70	37	71	17	0	609 (18)	
> 1955 - 1959	93	60	44	26	94	41	46	36	0	0	440 (13)	
> 1960 - 1964	94	61	56	35	58	34	15	0	0	0	353 (11)	
Total	742	381	264	204	620	413	255	231	135	83	3328	
Percent	(22)	(11)	(8)	(6)	(19)	(12)	(8)	(7)	(4)	(3)		

Table 6

CAUSE-SPECIFIC MORTALITY OF THE HOMESTAKE COHORT

<u>Cause</u>	<u>ICD Codes (7th Revision)</u>	<u>Observed Deaths</u>	<u>Expected Deaths</u> ¹	<u>SMR</u>	<u>95% Confidence Intervals for SMR</u> ²
Respiratory Tuberculosis	001-008	36	9.9	364	(270 ----)
Other Tuberculosis	010-019	3	0.6	500	- - - - -
All Malignant Neoplasms	140-205	137	141.0	97	(82 - 115)
MN of Buccal Cavity and Pharynx	140-148	6	4.8	125	(46 - 272)
MN of Esophagus	150	1	3.3	30	- - - - -
MN of Stomach	151	5	8.8	57	(18 - 133)
MN of Intestine	152,153	10	12.6	79	(38 - 146)
MN of Rectum	154	6	5.0	120	(44 - 261)
MN of Biliary Passages and Liver	155	3	2.5	120	- - - - -
MN of Pancreas	157	8	7.8	103	(44 - 202)
MN of Peritoneum and Unspecified Digestive Organs	158,159	2	0.7	286	- - - - -
MN of Larynx	161	2	2.2	91	- - - - -
MN of Trachea, Bronchus, and Lung	162-163	43	42.9	100	(73 - 135)
MN of Other Parts of Respiratory System	160,164	3	0.6	500	- - - - -
MN of Prostate	177	11	7.7	143	(72 - 259)
MN of Other Male Genital Organs	178-179	2	1.1	182	- - - - -
MN of Urinary Organs	180-181	2	7.7	26	- - - - -
MN of Other and Unspecified Major Sites	156B,165,190-199	14	17.7	79	(43 - 133)
Lymphosarcoma and Reticulosarcoma	200	6	4.0	150	(55 - 326)
Hodgkin's Disease	201	2	2.1	95	- - - - -
Leukemia and Aleukemia	204	10	5.9	169	(81 - 312)
Other Neoplasms of Lymphatic and Hematopoietic Tissues	202,203,205	1	2.0	50	- - - - -
Neoplasms of Brain	223,237	3	1.6	188	- - - - -
Diabetes Mellitus	260	8	10.9	73	(37 - 146)

(continued)

<u>Cause</u>	<u>ICD Codes (7th Revision)</u>	<u>Observed Deaths</u>	<u>Expected Deaths 1</u>	<u>SMR</u>	<u>95% Confidence Intervals for SMR2</u>
Diseases of Blood and Blood-Forming Organs	290-299	4	2.0	200	- - - - -
Mental, Psychoneurotic and Personality Disorders	300-326	4	4.4	91	- - - - -
Diseases of Nervous System	330-334, 345	41	50.5	81	(58 - 110)
Diseases of Circulatory System	400-468	285	337.3	84	(75 - 95)
Influenza	480-483	2	1.6	125	- - - - -
Pneumonia (Except Newborn)	490-493	16	16.7	96	(55 - 156)
Bronchitis	500-502	2	2.5	80	- - - - -
Other Respiratory Diseases	510-527	53	19.0	279	(219 - ----)
Diseases of Stomach and Duodenum	540, 541, 543	7	6.9	101	(41 - 209)
Hernia and Intestinal Obstruction	560, 561, 570	6	3.0	200	(73 - 435)
Cirrhosis of Liver	581	15	21.0	71	(40 - 118)
Chronic and Unspecified Nephritis; Other Renal Sclerosis	592-594	8	5.0	160	(69 - 315)
Diseases of Skin	690-716	2	0.6	333	- - - - -
Diseases of Bones and Organs of Movement	720-730	2	0.7	286	- - - - -
Unknown Causes (Blank)	780-793, 795, blank	63	--	--	- - - - -
Transportation Accidents	800-866	35	33.7	104	(72 - 144)
Accidental Poisonings	870-895	2	2.4	83	- - - - -
Accidental Falls	900-904	11	6.7	164	(82 - 294)
Other Accidents	910-936, 960-962	58	17.5	331	(263 - ----)
Violence	963-964, 970-985	18	25.9	69	(41 - 110)
All Others		40	35.8	--	- - - - -
All Deaths		861	765.8	112	(105- 120)

Table 7

LUNG CANCER MORTALITY BY LATENCY AND LENGTH OF EMPLOYMENT UNDERGROUND FOR HOMESTAKE WORKERS

Latency (Years)	Length of Employment (Years)							Total
	1 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25 - 29	> 30	
< 15								
O/E	0/ 1.62	1/ 0.78	1/0.51					2/ 2.91
SMR	—	128	196					69
15 - 19								
O/E	1/ 1.92	1/ 0.79	0/0.61	1/0.67				3/ 3.99
SMR	52	127	—	149				75
20 - 24								
O/E	5/ 3.11	0/ 1.18	0/0.69	2/0.62	0/0.70			7/ 6.30
SMR	161	—	—	323	—			111
25 - 29								
O/E	1/ 3.33	1/ 1.47	0/0.85	0/0.44	0/0.65	0/0.40		2/ 7.14
SMR	30	68	—	—	—	—		28
30 - 34								
O/E	3/ 2.28	1/ 1.78	3/1.20	1/0.50	1/0.46	0/0.50	0/0.15	9/ 6.87
SMR	132	56	250	200	217	—	—	131
35 - 39								
O/E	1/ 1.43	4/ 2.00	3/1.52	2/0.66	0/0.41	1/0.37	0/0.42	11/ 6.81
SMR	70	200	197	303	—	270	—	162
> 40								
O/E	1/ 0.26	1/ 2.07	4/2.66	2/1.57	0/0.85	0/0.57	1/0.88	9/ 8.86
SMR	385	48	150	127	—	—	115	102
Total								
O/E	12/13.95	9/10.07	11/8.04	8/4.46	1/3.07	1/1.84	1/1.45	43/42.88
SMR	86	89	137	179	33	54	69	100

Table 8

LUNG CANCER MORTALITY BY LATENCY AND DOSE FOR HOMESTAKE WORKERS

Latency (Years)	Dose (Dust-Days)							
	20000	20000-40000	40000-80000	80000-160000	160000-320000	320000-640000	≥ 640000	Total
< 15								
O/E	0/0.47	0/0.53	1/0.80	1/0.67	0/0.32	0/0.11	0/0	2/2.91
SMR	--	--	125	149	--	--	--	69
15-19								
O/E	0/0.46	0/0.58	2/0.93	0/0.96	1/0.68	0/0.40	0/0	3/4.01
SMR	--	--	215	--	147	--	--	75
20-24								
O/E	1/0.51	1/0.87	3/1.38	0/1.24	1/1.35	1/0.91	0/0.07	7/6.33
SMR	196	108	217	--	74	110	--	111
25-29								
O/E	0/0.11	0/0.81	1/1.39	1/1.39	0/1.68	0/1.59	0/0.25	2/7.22
SMR	--	--	72	72	--	--	--	28
30-34								
O/E	0/0.01	0/0.34	0/0.79	2/1.14	2/1.82	4/2.30	1/0.51	9/6.91
SMR	--	--	--	175	110	174	--	131
35-39								
O/E	0/0.01	0/0.08	1/0.45	0/0.85	2/1.85	8/2.87	0/0.72	11/6.83
SMR	--	--	222	--	108	279	--	161
≥ 40								
O/E		0/0.01	1/0.06	0/0.29	1/1.68	6/5.07	1/1.77	9/8.88
SMR	--	--	1667	--	60	118	56	101
Total								
O/E	1/1.57	2/3.22	9/5.80	4/6.54	7/9.38	19/13.25	2/3.32	43/43.10
SMR	63	62	155	61	75	143	60	100

Table 9

NONMALIGNANT RESPIRATORY DISEASE MORTALITY BY LATENCY AND
LENGTH OF EMPLOYMENT UNDERGROUND FOR HOMESTAKE WORKERS

Latency (Years)	Length of Employment (Years)							Total
	1 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25 - 29	≥ 30	
< 15								
O/E	1/ 0.63	1/ 0.30	0/0.18					2/ 1.11
SMR	159	333	—					180
15 - 19								
O/E	0/ 0.54	0/ 0.23	0/0.19	0/0.20				0/ 1.16
SMR	—	—	—	—				—
20 - 24								
O/E	1/ 0.94	1/ 0.36	1/0.21	0/0.19	0/0.22			3/ 1.92
SMR	106	278	476	—	—			156
25 - 29								
O/E	2/ 1.20	1/ 0.55	0/0.28	4/0.14	1/0.22	1/0.14		9/ 2.53
SMR	167	182	—	2857	455	714		356
30 - 34								
O/E	1/ 1.02	0/ 0.83	2/0.50	1/0.19	2/0.19	3/0.20	0/0.05	9/ 2.98
SMR	98	—	400	526	1053	1500	—	302
35 - 39								
O/E	1/ 0.75	2/ 1.08	2/0.79	2/0.32	1/0.22	1/0.18	1/0.18	10/ 3.52
SMR	133	185	253	625	455	556	556	284
> 40								
O/E	0/ 0.16	3/ 1.28	4/1.71	4/1.05	1/0.63	5/0.38	3/0.57	20/ 5.78
SMR	—	234	234	381	159	1316	526	346
Total								
O/E	6/ 5.24	8/ 4.63	9/3.86	11/2.09	5/1.48	10/0.90	4/0.80	53/19.00
SMR	115	173	233	526	338	1111	500	279

Table 10

NONMALIGNANT RESPIRATORY DISEASE MORTALITY BY LATENCY AND DOSE FOR HOMESTAKE WORKERS

Latency (Years)	Dose (Dust-Days)									
	< 20000	20000-40000	40000-80000	80000-160000	160000-320000	320000-640000	> 640000	Total		
< 15										
O/E	0/0.17	0/0.20	2/0.30	0/0.24	0/0.14	0/0.06	0/0	2/1.11		
SMR	---	---	667	---	---	---	---	180		
15-19										
O/E	0/0.12	0/0.16	0/0.26	0/0.27	0/0.20	0/0.15	0/0	0/1.16		
SMR	---	---	---	---	---	---	---	---		
20-24										
O/E	0/0.14	0/0.25	1/0.41	2/0.39	0/0.42	0/0.29	0/0.02	3/1.92		
SMR	---	---	---	513	---	---	---	156		
25-29										
O/E	0/0.03	0/0.26	1/0.46	2/0.54	1/0.62	3/0.55	2/0.08	9/2.52		
SMR	---	---	---	370	161	545	2500	357		
30-34										
O/E	0/0	0/0.13	0/0.32	1/0.54	0/0.84	5/0.96	3/0.18	9/2.97		
SMR	---	---	---	185	---	521	1667	303		
35-39										
O/E	0/0	0/0.03	0/0.23	2/0.45	2/1.00	4/1.47	2/0.32	10/3.50		
SMR	---	---	---	444	200	272	625	286		
>40										
O/E	0/0	0/0	0/0.04	0/0.18	2/1.04	9/3.28	9/1.21	20/5.76		
SMR	---	---	---	---	192	274	744	347		
Total										
O/E	0/0.46	0/1.03	4/2.01	7/2.61	5/4.26	21/6.76	16/1.82	53/18.95		
SMR	---	---	199	268	117	311	879	280		

Table 11

RESPIRATORY TUBERCULOSIS MORTALITY BY LATENCY AND LENGTH OF EMPLOYMENT UNDERGROUND
FOR HOMESTAKE WORKERS

Latency (Years)	Length of Employment (Years)							Total
	1 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25 - 29	≥ 30	
< 15								
O/E	0/ 1.98	0/ 1.10	0/0.49					0/ 3.57
SMR	—	—	—					—
15 - 19								
O/E	0/ 0.34	1/ 0.43	2/0.63	0/0.32				3/ 1.72
SMR	—	233	317	—				174
20 - 24								
O/E	0/ 0.26	0/ 0.31	0/0.39	3/0.39	1/0.20			4/ 1.55
SMR	—	—	—	769	500			258
25 - 29								
O/E	1/ 0.17	0/ 0.22	0/0.25	1/0.20	4/0.22	0/0.12		6/ 1.18
SMR	588	—	—	500	1818	—		508
30 - 34								
O/E	0/ 0.08	0/ 0.15	1/0.18	2/0.13	1/0.13	2/0.14	0/0.07	6/ 0.88
SMR	—	—	556	1538	769	1429	—	682
35 - 39								
O/E	0/ 0.04	1/ 0.09	2/0.11	1/0.08	4/0.07	0/0.08	3/0.08	11/ 0.55
SMR	—	1111	1818	1250	5714	—	3750	2000
≥ 40								
O/E	0/ 0.01	0/ 0.06	0/0.09	3/0.08	0/0.07	3/0.06	0/0.09	6/ 0.46
SMR	—	—	—	3750	—	5000	—	1304
Total								
O/E	1/ 2.88	2/ 2.36	5/2.14	10/1.20	10/0.69	5/0.40	3/0.24	36/ 9.91
SMR	35	85	234	833	1449	1250	1250	363

Table 12

RESPIRATORY TUBERCULOSIS MORTALITY BY LATENCY AND DOSE FOR HOMESTAKE WORKERS

Latency (Years)	Dose (Dust-Days)									
	< 20000	20000-40000	40000-80000	80000-160000	160000-320000	320000-640000	≥ 640000	Total		
< 15										
O/E	0/0.15	0/0.42	0/0.73	0/0.82	0/0.92	0/0.56	0/0	0/3.60		
SMR	---	---	---	---	---	---	---	---		
15-19										
O/E	0/0.03	0/0.06	0/0.12	0/0.19	1/0.39	2/0.94	0/0.01	3/1.72		
SMR	---	---	---	---	256	213	---	174		
20-24										
O/E	0/0.01	0/0.04	0/0.09	0/0.14	0/0.28	3/0.85	1/0.12	4/1.54		
SMR	---	---	---	---	---	353	833	260		
25-29										
O/E	0/0	0/0.02	1/0.06	0/0.09	0/0.20	4/0.57	1/0.23	6/1.18		
SMR	---	---	1667	---	---	702	435	508		
30-34										
O/E	0/0	0/0.01	0/0.02	0/0.05	0/0.13	4/0.38	2/0.28	6/0.88		
SMR	---	---	---	---	---	1053	714	682		
35-39										
O/E	0/0	0/0	0/0.01	0/0.02	1/0.08	4/0.25	6/0.18	11/0.55		
SMR	---	---	---	---	1250	1600	3333	2000		
≥ 40										
O/E	0/0	0/0	0/0	0/0.01	0/0.05	3/0.22	3/0.18	6/0.46		
SMR	---	---	---	---	---	1364	1667	1304		
Total										
O/E	0/0.19	0/0.55	1/1.03	0/1.32	2/2.05	20/3.77	13/1.00	36/9.93		
SMR	---	---	96	---	98	531	1300	362		

Table 13

NONMALIGNANT RESPIRATORY DISEASE AND RESPIRATORY TUBERCULOSIS MORTALITY BY LATENCY
AND LENGTH OF EMPLOYMENT UNDERGROUND FOR HOMESTAKE WORKERS

Latency (Years)	Length of Employment (Years)							
	1 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25 - 29	≥ 30	Total
< 15								
O/E	1/ 2.61	1/ 1.40	0/0.67					2/ 4.68
SMR	38	71	—					43
15 - 19								
O/E	0/ 0.88	1/ 0.66	2/0.82	0/0.52				3/ 2.88
SMR	—	152	244	—				104
20 - 24								
O/E	1/ 1.20	1/ 0.67	1/0.60	3/0.58	1/0.42			7/ 3.47
SMR	83	149	167	517	238			202
25 - 29								
O/E	3/ 1.37	1/ 0.77	0/0.53	5/0.34	5/0.44	1/0.26		15/ 3.71
SMR	219	130	—	1471	1136	385		404
30 - 34								
O/E	1/ 1.10	0/ 0.98	3/0.68	3/0.32	3/0.32	5/0.34	0/0.12	15/ 3.86
SMR	91	—	441	938	938	1471	—	389
35 - 39								
O/E	1/ 0.79	3/ 1.17	4/0.90	3/0.40	5/0.29	1/0.26	4/0.26	21/ 4.07
SMR	127	256	444	750	1724	385	1538	516
> 40								
O/E	0/ 0.17	3/ 1.34	4/1.80	7/1.13	1/0.70	8/0.44	3/0.66	26/ 6.24
SMR	—	224	222	619	143	1818	455	417
Total								
O/E	7/ 8.12	10/ 6.99	14/6.00	21/3.29	15/2.17	15/1.30	7/1.04	89/28.91
SMR	86	143	233	638	691	1154	673	308

Table 14

**NONMALIGNANT RESPIRATORY DISEASE AND RESPIRATORY TUBERCULOSIS MORTALITY
BY LATENCY AND DOSE FOR HOMESTAKE WORKERS**

Latency (Years)	Dose (Dust-Days)							
	< 20000	20000-40000	40000-80000	80000-160000	160000-320000	320000-640000	> 640000	Total
< 15								
O/E	0/0.32	0/0.62	2/1.03	0/1.06	0/1.06	0/0.62	0/0	2/4.71
SMR	---	---	194	---	---	---	---	42
15-19								
O/E	0/0.15	0/0.22	0/0.38	0/0.46	1/0.59	2/1.09	0/0	3/2.88
SMR	---	---	---	---	169	183	---	104
20-24								
O/E	0/0.15	0/0.29	1/0.50	2/0.53	0/0.70	3/1.14	1/0.14	7/3.46
SMR	---	---	200	377	---	263	714	202
25-29								
O/E	0/0.03	0/0.28	2/0.52	2/0.63	1/0.82	7/1.12	3/0.31	15/3.70
SMR	---	---	385	317	122	625	968	405
30-34								
O/E	0/0	0/0.14	0/0.34	1/0.59	0/0.97	9/1.34	5/6.46	15/3.85
SMR	---	---	---	169	---	672	1087	390
35-39								
O/E	0/0	0/0.03	0/0.24	2/0.47	3/1.08	8/1.72	8/0.50	21/4.05
SMR	---	---	---	426	278	465	1600	519
> 40								
O/E	0/0	0/0.02	0/0.04	0/0.19	2/1.09	12/3.50	12/1.39	26/6.22
SMR	---	---	---	---	184	343	863	418
Total								
O/E	0/0.65	0/1.60	5/3.05	7/3.93	7/6.31	41/10.53	29/2.80	89/28.87
SMR	---	---	164	178	111	389	1036	308

Table 13

MORTALITY ACCORDING TO SUBCORTS BY YEAR FIRST EMPLOYED
IN UNDERGROUND JOBS AT HONESTAKE

Cause of Death	Year First Employed Underground								After 1951
	Before 1930	1930-34	1935-39	1940-44	1945-49	1950-54	1955-59	1960-64	
Respiratory Tuberculosis (RTB) O/E SHR	32/ 4.04 792	2/ 1.99 101	0/ 0.10 ---	0/ 0.05 ---	2/ 0.92 217	0/ 0.36 ---	0/ 0.11 ---	0/ 0.03 ---	0/ 0. ---
"Other" Non-Malignant Respiratory Diseases (NMRD) O/E SHR	33/ 6.47 510	5/ 3.89 129	6/ 3.40 176	3/ 1.46 205	2/ 2.11 95	3/ 1.12 268	1/ 0.40 250	0/ 0.17 ---	4/ 1. 286
Lung Cancer O/E SHR	15/ 12.02 125	8/ 7.72 104	6/ 7.15 84	4/ 3.64 110	6/ 6.66 90	3/ 3.84 78	1/ 1.45 69	0/ 0.41 ---	3/ 4. 63
Diseases of the Circulatory System O/E SHR	117/118.90 98	50/ 60.85 82	30/ 53.21 56	20/24.37 82	38/ 42.16 90	21/24.16 87	6/ 9.24 65	3/ 4.16 72	26/31. 63
"Other" Accidents O/E SHR	9/ 3.21 280	5/ 2.12 236	2/ 2.26 88	3/ 1.43 210	15/ 3.72 403	12/ 2.53 474	9/ 1.44 625	3/ 0.83 361	21/ 4. 496
All Malignant Neoplasms O/E SHR	51/ 44.90 114	25/ 24.92 100	18/ 22.37 80	11/10.83 102	17/ 19.77 86	12/11.61 103	1/ 4.73 21	2/ 1.85 108	14/15.8 91
All Deaths O/E SHR	335/247.89 135	134/130.27 103	96/116.65 82	55/56.10 98	113/106.46 106	78/64.50 121	29/28.86 100	21/14.67 143	109/92.6 118

Table 16

MORTALITY FROM CHRONIC RENAL DISEASE BY
LENGTH OF UNDERGROUND EMPLOYMENT AMONG HOMESTAKE WORKERS

Length of Underground Employment (Years)	Observed Deaths	Expected Deaths	SMR
1 - 4	1	1.72	58
5 - 9	1	1.09	92
10 - 14	2	0.92	217
15 - 19	1	0.55	182
20 - 24	1	0.35	286
25 - 29	1	0.23	435
<u>></u> 30	1	0.18	556
Total	8	5.04	159

Table 17

MORTALITY FROM CHRONIC RENAL DISEASE
BY DOSE AMONG HOMESTAKE WORKERS

Dose (Total Dust-Days)	Observed Deaths	Expected Deaths	SMR
< 20000	0	0.28	—
20000 ~ 40000	0	0.42	—
40000 ~ 80000	1	0.66	152
80000 ~ 160000	0	0.68	—
160000 ~ 320000	1	0.91	110
320000 ~ 640000	4	1.56	256
<u>≥</u> 640000	2	0.54	370
Total	8	5.04	159

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Mortality and Industrial Hygiene Study
of Styrene-Butadiene Rubber Workers

Theodore J. Meinhardt
Richard A. Lemen
Michael S. Crandall
Ronald J. Young

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MORTALITY AND INDUSTRIAL HYGIENE STUDY OF
STYRENE-BUTADIENE RUBBER WORKERS

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The results of a retrospective cohort mortality study and an industrial hygiene assessment conducted at two adjacent styrene-butadiene rubber producing facilities in Eastern Texas will be reviewed. This study was initiated based on the company and union request following the deaths, due to leukemia in January, 1976, of two men who worked at these plants. Personnel records were available from 1943 at plant A and from 1950 at plant B to the study cut-off date of March 31, 1976. Although not statistically significant, excesses in several cause-specific categories of hematopoietic and lymphatic tissue malignancies were observed in plant A. The SMR for the leukemia and aleukemia cause-specific category was 203. When the analysis of the worker population was restricted to individuals employed 6 months or longer prior to 1950, a time period of process development and crisis operation, a marginally significant SMR for leukemia and aleukemia, 278, was observed. Other cause-specific categories of death were assessed to assure no unrecognized hazard was operating in this population.

Recent relevant findings from epidemiologic investigations of styrene-butadiene rubber worker populations and from toxicological studies on 1,3-butadiene will be discussed. Plans for and progress on updating the mortality analysis and the possibility of utilization of specific job assignments will be outlined.

Acknowledgement: This research is supported by The National Cancer Institute.

Introduction

Early in 1976, the National Institute for Occupational Safety and Health (NIOSH) was requested to evaluate the potential risk of leukemia associated with employment in the styrene-butadiene rubber (SBR) production industry. This request was made by a synthetic rubber producing corporation and the International Oil, Chemical and Atomic Workers Union; prompted partially by the fact that two men who had been employed at adjacent SBR production facilities in Port Neches, Texas, died of leukemia during January 1976, and partially by the reported association between leukemia and benzene exposure occurring in other rubber processes. Simultaneous with NIOSH receiving this request, an article⁹ was published demonstrating an association between leukemia in a large rubber manufacturing plant in Ohio and employment in the synthetic rubber production facility.

Based on preliminary observations, we decided to conduct detailed environmental and epidemiological studies at these two facilities to test the hypothesis that employment in the SBR production industry was associated, specifically, with an increased risk of leukemia and, more generally, with an increased risk of other malignancies of hematopoietic and lymphatic tissues. In the course of this investigation, mortality information on malignancies of hematopoietic and lymphatic tissues, as well as on other causes of death was collected. Information on general mortality was used to explore the possible existence of other previously unrecognized unusual mortality patterns and is presented along with the information on leukemia.

SBR is the most widely used synthetic rubber in the world. The production of this synthetic was not actively pursued in the United States or elsewhere in the world until the supply of natural rubber had been curtailed by war activities in the Pacific during the late 1930s and early 1940s. In 1941, the Rubber Reserve Company of the U.S. Reconstruction Finance Corporation initiated a program to construct 15 plants which would permit the production of synthetic rubber.¹⁸

Process Description and Environmental Concentrations

SBR is a copolymer of styrene and 1,3-butadiene made through an emulsion polymerization process. A typical emulsion system contains water, monomers, initiators, and an emulsifier (soap).¹⁴ A typical recipe, as described by one of the plant managers, is shown in Table 1.

The two plants included in this study began operations in 1943. Plant A has been operated continuously. Personnel employment records and occupational histories were available for all personnel since 1943. According to best estimates, a total of 3,494 persons have worked at this plant. Of this total population, 1,662 were white males who had at least six months of employment. In this latter group of employees, the average length of employment was 9.48 years, and the average annual turnover rate was 7%. An estimated 700 people were employed at this plant when the study was initiated.

Plant B was operated from 1943 to 1947 and then shut down. The plant was reactivated in 1950 and has been in continuous operation from that date. Personnel employment records documenting the employment of 2,015 workers were available from 1950 on. Attempts to identify employees at this plant prior to 1950, through the Social Security Administration, have been unsuccessful. Of the total identified worker population, 1,094 were white males having at least six months of employment. In this latter group of employees, the average length of employment was 10.78 years, and the average annual turnover rate was 5%. An estimated 485 people were employed at this plant when the study was initiated.

Processes and environmental conditions of an industry change with time. To illustrate the changes in this particular industrial process, some of the important process changes which have occurred at Plant A since 1943 are briefly described. Originally, this plant produced rubber by a hot temperature, batch polymerization process which was then converted to a continuous feed operating system in 1946. In 1949, a cold temperature, continuous polymerization process was developed to go along with the hot process. Extender oils were added to the chemical process in 1951 to insure that the rubber product was not too hard for future processing. A pilot polyethylene operation was constructed at this plant in 1960, but was discontinued in 1965. A general modernization program of the production area was begun in 1967. Undoubtedly, such changes influence environmental conditions within the plant. Similar process evolution has taken place at Plant B.

Styrene and butadiene were considered as the target chemicals for this study because they are the two major components of SBR, and benzene exposure was evaluated due to its known leukemogenic activity. Measurements were taken in all areas of both SBR production facilities with an approved method.³

The results of the environmental samples are presented in Table 2. All of the samples were well within existing standards of the Occupational Safety and Health Administration and threshold limit values recommended by the American Conference of Governmental Industrial Hygienists (100 ppm of styrene, 1,000 ppm of butadiene and 10 ppm of benzene for an 8-hour time-weighted average). No historical monitoring data were available.

Methods

The cohorts of workers to be evaluated in this study were defined as all white male workers who had been employed at least six months since January 1, 1943 at Plant A or since January 1, 1950 at Plant B. The other sex-ethnic subgroups were not large enough for meaningful evaluation. However, the mortality experience of black males was evaluated and no excesses were obvious. It was felt that an employment period shorter than six months would be difficult to correlate with chronic health conditions attributable to this industry.

The vital status of as many of the former employees from these two facilities was determined as possible because the death information on all individuals, regardless whether they qualified for the study cohorts, could provide useful insight. Vital status determinations were made through March 31, 1976 for all cohort members with the help of several follow-up sources, including the Social Security Administration, the U.S. Post Office, the Texas Bureau of Motor Vehicles, and a commercial case location service. Death certificates were obtained for any study members known to be deceased. The underlying causes of death taken from the certificate were coded by one nosologist in accordance with the revision of the International Lists of Disease and Causes of Death in effect at the time of death and then converted into the 7th revision code for the purposes of comparison. Persons of unknown vital status were considered alive in these evaluations. Deaths which occurred after the study cutoff date (March 31, 1976) are not included in the analyses. Any individual who was known to be deceased but for whom no death certificate had been obtained was included in the unknown cause of death category.

A modified life-table^{4,5,17} technique was used to obtain the person-years at risk of dying by 5-year age groups, by 5-year calendar time periods, by duration of work experience (exposure), and by time from initial employment (latency) in the S3R production industry. Person-years at risk of death began accumulating at six months after initial employment. The age, race, sex, calendar time and cause-specific mortality rates of the U.S. population were then applied to the appropriate stratum of person-years at

risk to generate the expected number of cause-specific deaths in the study population. This expected number of deaths was then compared to the observed number of deaths in the study population so that any statistically significant differences and the standardized mortality ratio (SMR) could be determined [$SMR = \text{Observed/Expected} \times 100$]. The magnitude of the differences for subcategories of the hematopoietic and lymphatic tissue malignancies was evaluated with a test statistic based on the Poisson distribution.

Results

The determination of vital status for the workers in Plants A and B are shown in Table 3. At Plant A, 1,356 individuals were identified as alive, 252 individuals were found to be deceased (with death certificates received for 246 individuals), and for 54 individuals (3.25%) vital status was unknown. In the data analyses, the latter 54 were all considered alive, and they thus contributed to the person-years at risk and made the cause-specific death results conservative. A total of 34,187 person-years at risk of dying had accumulated for the entire Plant A study group. At Plant B, 980 individuals were identified as alive, 80 individuals were found to be deceased (with death certificates received for 78 individuals) and for 34 individuals (3.11%) vital status was unknown. As with their counterparts at Plant A, this last group was considered alive. A total of 19,742 person-years at risk of dying had accumulated for the entire Plant B study group.

Table 4 shows the mortality patterns for malignancies of the lymphatic and hematopoietic tissues and for subcategories of these malignancies from Plants A and B. Nonsignificant excesses were observed in the overall category and in three of the subcategories based on the mortality experience of Plant A employees. The major increase was an approximate doubling of the number of expected deaths due to leukemia. No appreciable excesses were observed in these categories based on the mortality experience of Plant B employees.

The general mortality pattern of the Plant A and Plant B populations are presented in Table 5. None of the major categories considered were in excess based on the mortality experience of the worker population from either plant. Many of these categories, including total mortality, appear to have substantial deficits in observed deaths compared to the expected number of deaths. Such observations have been explained by the selection of healthy individuals when initially employed in this industry, and other physically demanding industries.^{6,8} In other words, this effect results from the differences in the mortality experience of a selected healthy worker population and in the mortality experience of a general reference group like the U.S. population which is comprised of people in a spectrum of health status.

When the records of the individuals identified with leukemia (for those who qualified and did not qualify for inclusion in the study analyses) at the Plant A were reviewed (presented in Table 6), it was observed that most of

these employees had started work before the end of December, 1945. This date corresponds to the time when the batch process was converted to a continuous feed operation and the war time production conditions were discontinued. Because of these process changes, it was decided to evaluate the mortality experience of Plant A white male employees with at least six months of employment between the beginning of January, 1943, and the end of December, 1945. This subgroup of the Plant A population consisted of 600 workers. For this group of employees, the average length of employment was 11.89 years and the average annual turnover rate was 7%. Table 7 presents the vital status: 365 individuals were identified as alive, 201 individuals were found to be deceased, and for 34 individuals (5.6%) the vital status was unknown. The experience of this subgroup resulted in the accumulation of 17,086 person-years at risk of death.

The mortality patterns for malignancies of the hemotopoietic and lymphatic tissues and for general mortality of the subgroup defined above, are presented in Tables 8 and 9, respectively. Of course the SMRs for the subcategories of lymphatic and hematopoietic tissue malignancies in this restricted subgroup are greater since this subgroup contained not only all qualifying leukemia deaths, but also all of the remaining lymphatic and hematopoietic tissue deaths which qualified for inclusion in the analysis. Since the evaluation of this subgroup's mortality experience was a posteriori, the use of statistical tests may not appropriately assess chance occurrence. However, if a one-sided test statistic was used to evaluate the excesses observed in the subcategories of the lymphatic and hematopoietic tissue malignancies, then the excess in the leukemia and aleukemia category

was significant with $p < 0.05$. Similar to what was observed in the overall populations from both plants, none of the general mortality categories were in excess for the restricted subgroup compared to the expected U.S. mortality experience.

Discussion

At the same time the National Institute for Occupational Safety and Health became aware of the deaths from leukemia at the SBR facilities in Port Neches, Texas, an article by McMichael et al.,⁹ reported on the mortality experience of 6,678 male rubber workers observed between 1964 and 1973. The study examined total mortality as well as various cause-specific categories in the overall rubber worker population and specific worker subgroups. McMichael et al., examined age-standardized risk ratios for cause-specific deaths in the specific worker subgroups. The risk ratio for lymphatic and hematopoietic malignancies was 6.2 in the synthetic plant. The synthetic plant would be similar to the two Port Neches facilities as far as the styrene-butadiene production operations were concerned. However, this plant also produced other types of rubber. Later, other researchers in the Occupational Health Study Group of the University of North Carolina (where the research effort of the McMichael et al., study was based) expressed reservations about the publicity and the significance of the relative risks since they were based on small numbers.¹⁵

The findings by McMichael et al., prompted a more detailed case-referent study of these cancer deaths by Spirtas et al.¹⁶ This study revealed an estimated 2.4 relative risk for deaths due to lymphatic and hematopoietic malignancies associated with employment in the synthetic rubber production plant.

Andjelkovich et al.,¹ reported the mortality experience of a population from another rubber manufacturing plant observed between 1964 and 1973. The population studied was defined as "any persons who, as of January 1, 1964 were 40 or more years of age and an active or living retired hourly worker from the plant". The SMR for neoplasms of the lymphatic and hematopoietic tissues for white male cohort members in the 65 to 84 year-old age group and the 40 to 85 year-old age group demonstrated a statistically significant excess in the monocytic leukemia and other neoplasms of the lymphatic and hematopoietic tissues subcategories.

In a second paper, Andjelkovich et al.,² reported SMRs for persons employed in specific work areas at the same rubber manufacturing plant studied earlier; they used the entire cohort's mortality experience as a reference for comparison. A statistically significant excess of deaths due to leukemia [SMR = 246] was reported for persons whose most representative work area was general services, which would not necessarily involve contact with SBR production. A statistically significant excess of deaths for malignant neoplasms of lymphatic and hematopoietic tissues [SMR = 426] was observed in the industrial products subgroup of the general services department.

In a paper by Monson et al.,¹⁰ mortality experience of all members of the same local union who worked for one rubber company in Akron, Ohio was examined. The study included 13,571 men who had worked during or after 1935 for at least five years. Excesses of deaths due to leukemia were observed for those men working in the tire and the processing divisions with SMRs of 150 and 240, respectively. The processing department would include the activities which produce the synthetic rubber. The results of the Monson et al. study reinforce the possibility of an association between exposure to SBR production and the development of lymphatic and hematopoietic malignancies.

A final study, as yet not published, by Matanoski et al.,⁷ has looked at the mortality experience of 13,920 male synthetic rubber production workers with at least 12 months of employment from eight different plants in the U.S. and Canada. The dates of plant operations and the dates from which complete records are available vary between plants. These researchers achieved a 93.4% follow-up in cohort members. They did not observe any excess risk in the overall study population for malignancies of the hematopoietic and lymphatic tissues or for leukemia specifically. However, since the majority of the cohort studied by Matanoski et al., began employment after 1950, only a portion of this population's experience provides insight into the early years of operation in this industry during which rapid changes occurred.

Currently, we are proceeding with analyses based on detailed work histories of the study population at Plant A and on updating the vital status experience of the study population from both Plant A and Plant B. The first of these efforts has been dependent on obtaining company and

corporate input about the probability of exposure by department. Developing an exposure scheme has been quite difficult and since little historic environmental data were available and we have had to rely on the personal experience of long-term industry employees. In the past several months, we have reached agreement on a simplified stratification of exposure potential. This scheme identifies high exposure departments consisting of the laboratory, tank farm, reactor and recovery departments; a medium or an intermittent exposure department, the maintenance department; and low exposure departments consisting of all other departments including process, warehousing, utilities, and administration.

Exposure data gathered in the 1976 industrial hygiene survey was used to validate this department exposure stratification scheme. The scheme seems reasonable for butadiene exposures at the time of the 1976 survey. The scheme did not appear to reflect styrene exposure as well. In the absence of other more extensive data, we have decided to assign as relative weights the actual arithmetic mean butadiene exposure values (ppm) measured during the 1976 survey in an attempt to assess the impact of that chemical. We have assumed that the relative exposure relationship between departments has remained similar and recognize this as a very limited method to assess exposure within the rubber industry. These relative weighting factors are shown in Table 10 and are 1.41 for the high exposure departments, 0.77 for the medium or intermittent exposure department and 0.22 for the low exposure departments. Lifetable analyses incorporating dose for the Plant A study population is underway.

Efforts to update the vital status and mortality information on the study populations from both plants are proceeding. The original study populations from both plants have been sent to SSA which will update vital status on all individuals from March 31, 1976 to December 31, 1981. This vital status update has identified approximately 210 additional deaths occurring to former employees at these two plants. Records on all live or unknown vital status study members have been submitted to the National Death Index Registry. Of the individuals newly identified through SSA as being deceased, 206 or 98% were denoted formerly as alive and 4 or 2% were denoted as unknown vital status.

The two major monomers used in this industry have each independently stimulated occupational health concern. NIOSH initiated a mortality study of styrene exposed workers based on initial reports of increased leukemogenic risk in the synthetic rubber and other styrene-based industries and based on the large number of workers potentially exposed to styrene. This research will be summarized later by Jay Beaumont and Mike Crandall. Recently, two animal bioassay^{12,13} have shown butadiene to be carcinogenic in rats and mice, in both sexes at numerous sites. The major cancer excesses reported in these two studies are summarized in Table 11. No epidemiological data has been found which specifically addresses the effects of butadiene. Due to the limited number of workers with exposure solely to butadiene at any particular site, traditional epidemiologic techniques may not be capable of evaluating this potential health exposure relationship. However, we are currently considering the feasibility of conducting a traditional epidemiological study or alternately an evaluation of the genotoxicity effects in exposed workers associated with butadiene.

Conclusion

The continued analysis of the mortality occurring to styrene-butadiene rubber production workers will probably never provide definitive answers about the health effects associated with either styrene or butadiene because of the concomitant exposure to many other chemicals and because of the defused and poorly defined nature of exposure in this industry. At the present time it is not clear whether a real leukemogenic risk is associated with SBR production or whether the reported excesses in lymphatic and hematopoietic malignancies are artifacts in the populations studied. However, since NIOSH continues to receive reports of additional occupational cancers occurring to individuals exposed to this industrial process and since the evaluation of a mixed chemical exposure environment with very low levels of exposure may provide evidence of health risks not detected in single agent evaluation, the continued evaluation of workers in this industry seems warranted.

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Table 1
Typical Recipe for Production
of Styrene-Butadiene Rubber

Ingredients	Parts	Percentage
Butadiene	75.0	26.2
Styrene	25.0	8.8
n-Dodecyl Mercaptan	0.5	0.2
Paramenthane Hydroperoxide	0.1	0.1
Sodium Formaldehyde Sulfoxyde	0.1	0.1
Iron EDTA	0.01	0.01
Soap Flakes	5.0	1.8
Water	180	63.0

Source: Correspondence from D.T. Boumans, Plant Manager,
B.F. Goodrich, December 29, 1976.

Table 2

Detectable Range and Mean
TWA Environmental Sampling Results

Plant A

Contaminant	Number of Samples	Range (ppm)	Concentration Mean (ppm)	S.D.+
Styrene	55	0.03-6.46	0.94	1.23
Butadiene	41	0.11-4.17	1.24	1.20
Benzene	3	0.08-0.14	0.10	0.04

+ Standard Deviation

Plant B

Contaminant	Number of Samples	Range (ppm)	Concentration Mean (ppm)	S.D.+
Styrene	35	0.05- 12.30	1.99	3.00
Butadiene	47	0.34-174.00	13.50	29.90
Benzene	0	--	--	--

+ Standard Deviation

Table 3

Vital Status of White Males with at Least Six
Months of Employment at Plant A and Plant B

Vital Status	Plant A		Plant B	
	Number of Study Members	Percentage	Number of Study Members	Percentage
Alive	1356	81.59	980	89.58
Deceased	252	15.16	80	7.31
Unknown	54	3.25	34	3.11
Total	1662	100.00	1094	100.00

Person-years at risk from Plant A study population was 34,187.
Person-years at risk from Plant B study population was 19,742.

Table 4

Mortality Due to Malignancies of the Lymphatic and Hematopoietic
Tissues in White Males with at Least Six Months of Employment

Cause-Specific Death Categories	ICD Codes	Plant A			Plant B		
		Number of Deaths		Standard Mortality Ratio	Number of Deaths		Standard Mortality Ratio
		Observed	Expected		Observed	Expected	
Lymphatic & Hematopoietic Tissues	200-205	9	5.79	155	2	2.55	78
Lymphosarcoma & Reticulosarcoma	200	3	1.66	181	1	0.76	132
Hodgkin's Disease	201	1	0.87	115	0	0.51	0
Leukemia and Aleukemia	204	5	2.47	203	1	0.99	101
Other Neoplasms of the Lymphatic & Hematopoietic Tissues	202,203 205	0	0.79	0	0	0.29	0

Table 5

General Mortality in White Males with at Least Six Months of Employment

Cause-Specific Death Categories	ICD Codes	Plant A			Plant B		
		Number of Deaths		Standard Mortality Ratio	Number of Deaths		Standard Mortality Ratio
		Observed	Expected		Observed	Expected	
Tuberculosis	001-019	1	3.66	27	0	0.77	0
Malignant Neoplasms	140-205	45	57.33	78	11	20.78	53
Diseases of the Nervous System	330-339 345	17	20.90	81	3	5.69	53
Diseases of the Circulatory System	400-468	123	139.84	88	39	46.32	84
Diseases of the Respiratory System	470-527	8	16.29	49	5	5.13	97
Diseases of the Digestive System	540-581	10	12.44	80	2	5.43	37
Accidents	800-962	19	26.33	72	11	14.07	78
All Other Causes		29	38.32	76	9	16.80	54
Total		252	315.11	80	80	114.99	66

Table 6

Description of Cases Involving Leukemia
That Have Occurred Among Plant A

Plant A Qualifying Cases

Age	Beginning Dates of Employment	Time From Date of Death	Initial Employment to Death	ICD Codes	Cause of Death or Disease Condition
55	1944	11/14/47	3 yrs.	204.1	1a) Circulatory Failure 1b) Due to General Arterial Venous Thrombosis 1c) Chronic Myeloid Leukemia
77	1943	9/29/72	29 yrs.	204.3	1a) Leukemia 1b) Acute Lymphoblastic Leukemia 1c) Osteomyelitis Transverse Myelitis, Paraplegia
32	1943	5/12/46	3 yrs.	204.1 340.3	1a) Myelogenous Leukemia Other) Psychosis with Syphilitic Meningo Encephalitis
46	1943	1/22/67	23 yrs.	204.3	1a) Intracerebral Hemorrhage 1b) Thrombocytopenia 1c) Acute Leukemia Myeloblastic
64	1943	12/06/71	28 yrs.	204.1	1a) Chronic Granulocytic Leukemia

Plant A Non-Qualifying Cases

54	1944	5/18/72	28 yrs.	204.3	1a) Respiratory Arrest 1b) Acute Leukemia and Pseudomonas
69	1943	1/13/64	20 yrs.	593 204.1	1a) Medullary Failure 1b) Cardiac Arrest 1c) Renal and Hepatic Failure 2a) Myelogenous Leukemia
42	1951	2/14/78	27 yrs.	204.3	1a) Acute Myelogenous Leukemia 1b) Sepsis
58	1951	1/05/76	25 yrs.	204.3	1a) Gastrointestinal Hemorrhage 1b) Thrombocytopenia 1c) Acute Myelocytic Leukemia
66	1942	Alive			Chronic Lymphatic Leukemia
51	1956	Alive			Chronic Leukemia

Table 7

The Vital Status of the Restricted Plant A
White Males With at Least 6 Months
of Employment Between the Beginning of
January 1943 Through the End of December 1945

Status	Number of Study Members	Percentage
Alive	365	60.83
Deceased	201	33.50
Unknown	34	5.67
Total	600	100.00

Person-Years at Risk From This Study Population: 17,085.53.

Table 8

Mortality Due to Malignancies of the Lymphatic and Hematopoietic Tissues
in White Males with at Least Six Months of Employment at Plant A Between
the Beginning of January 1943 and the End of December 1945

Cause-Specific Death Categories	ICD Codes	Number of Deaths		Standard Mortality Ratio
		Observed	Expected	
Lymphatic and Hematopoietic Tissues	200-205	9	4.25	212
Lymphosarcoma and Reticulosarcoma	200	3	1.34	224
Hodgkin's Disease	201	1	0.47	213
Leukemia and Aleukemia	204	5	1.80	278
Other Neoplasms of the Lymphatic and Hematopoietic Tissues	202, 203 205	0	0.64	0

Table 9

General Mortality in White Males with at Least Six Months of Employment at Plant A Between the Beginning of January 1943 and the End of December 1945

Cause-Specific Death Categories	ICD Codes	Number of Deaths		Standard Mortality Ratio
		Observed	Expected	
Tuberculosis	001-019	1	3.13	32
Malignant Neoplasms	140-205	39	45.14	86
Diseases of the Nervous System	330-339 345	15	17.85	84
Diseases of the Circulatory System	400-468	102	113.69	90
Diseases of the Respiratory System	470-527	8	13.43	60
Diseases of the Digestive System	540-581	7	8.88	79
Accidents	800-962	7	14.05	50
All Other Causes		22	25.92	85
Total		201	242.09	83

Table 10
Dose Analysis

Exposure Categories	Exposure Weighting
High Exposure Departments	1.41
Intermittent Exposure Department	0.77
Low Exposure Departments	0.22

Table 11

Animal Bioassay Significant Results of Butadiene Exposure Studies

Hazleton Laboratory Study(12) of Sprague/Dawely Rats Doses 0, 1000 and 8000 ppm		National Toxicology Program Study (13) of B6C3F Mice Doses 0, 625 and 1250 ppm	
Male	Female	Male	Female
Pancreatic Tumors	Mammary Tumors	Hemangiosarcoma	Hepatocellular Adenomas and Carcinomas
Leydig Cell Tumors (In Testes)	Thyroid-Follicle Tumors		Granuloma of the Ovary
	Uterus/Vaginal Stromal Sarcomas		Acinar Carcinoma of the Mammary Gland
			Papillomas and Carcinoma of Forestomach
Zymbol Gland Tumors		Epithelial Hyperplasia of the Forestomach	
		Alveolar/Bronchiolar Adenomas and Carcinomas	

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

A Retrospective Cohort Mortality Study of a
Phosphate Fertilizer Production Facility

Leslie T. Stayner
Theodore Meinhardt
Richard Lemen
David Bayliss
Robert Herrick
Gordon R. Reeve
Alexander B. Smith
William Halperin

National Institute for Occupational Safety and Health

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A RETROSPECTIVE COHORT MORTALITY STUDY
OF A PHOSPHATE FERTILIZER PRODUCTION FACILITY

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ABSTRACT

A retrospective cohort mortality study of phosphate fertilizer production workers was undertaken to determine whether this group is at increased risk of dying from any cause, and particularly from lung cancer. Altogether 3199 workers who had ever been employed at one facility were included in this investigation. These workers were followed for vital status ascertainment from their first date of employment up to December 1, 1977, or the date of death, which ever occurred first. Overall, no statistically significant elevations in cause specific mortality were observed for the entire study population. However, when the analysis was stratified by duration of employment, and length of followup, a statistically significant ($p < .05$) excess in lung cancer mortality was observed among workers with greater than 10 years of employment and followup ($SMR=411$). Because of the small number of deaths involved, and because we had prior knowledge of a lung cancer cluster at this plant, we believe that these findings should be viewed as suggestive, and that other investigations in plants with similiar exposures are needed to clarify whether an occupationally related lung cancer excess truly exists.

Introduction

Phosphate ore contains uranium as a natural constituent. The mining and processing of this ore may result in human exposures to uranium, and to radon daughters and thorium which are decay products of uranium. Concern has been raised about these exposures due to the well known association between radon daughter exposure and the development of respiratory cancer in uranium miners.¹

There is only limited epidemiologic data on the issue of whether the mining and processing of phosphate ore is associated with an increased risk for lung cancer. Lung cancer rates in Polk County, Florida an area which has a large phosphate mining and processing industry, are known to be significantly higher than the U.S. population overall.² In a recent study a significant ($p < .001$) association was observed between counties in the U.S. with high rates of lung cancer and counties containing phosphate deposits, mines or processing plants; however, when an adjustment was made for urban-rural differences this association was no longer statistically significant.³

Study Background

The National Institute for Occupational Safety and Health (NIOSH) received a report of several cases of lung cancer that occurred among workers at a

phosphate fertilizer plant. It was learned subsequently that three black men, each fifty years of age or less, who were reportedly nonsmokers, had developed lung cancer. According to a supervisor at the plant, two of these workers smoked cigars, while the third was a moderate cigarette smoker. All 3 of these men were also reported to have been involved in the cleaning of a phosphoric acid vessel; however, plant records indicate that only one of these men was definitely involved in this activity, another may have been occasionally, while the third was not involved at all.

The plant where the individuals had worked is a phosphate fertilizer production facility. This facility began operations in 1953 and was purchased by a second company in 1969. The plant has operated continuously since 1953 with very little employment turnover when management changed.

The principal products of this facility are diammonium and dicalcium phosphates, which are predominantly used for fertilizers. A pilot project was operated between 1953 and 1958 which recovered uranium from the phosphate ore.

In 1976 NIOSH conducted an industrial hygiene survey at this facility ⁴ to evaluate in particular occupational exposures in the cleaning of a reaction vessel, a task in which all three of the lung cancer cases had reportedly performed. Crushed phosphate ore is mixed in these vessels with sulfuric acid to produce dicalcium phosphate and gypsum as a byproduct. Workers enter the vessel once a year to chip away gypsum deposits from the agitator. Since 1975, these deposits have been removed from outside the

vessel using a high pressure water gun. Environmental samples were taken for uranium, radon daughters, fluoride, sulfuric acid, phosphoric acid, cadmium, chromium, vanadium and arsenic. A summary of the results from this survey is presented in Table I. All samples were found to be below all applicable standards except for two fluoride samples.

To investigate further the relationship between work in the phosphate industry and the risk of developing lung cancer, NIOSH initiated a study to evaluate the mortality experience of workers in the phosphate fertilizers industry. A number of other companies were visited by NIOSH in an attempt to identify a suitable location for this study. After completing this survey, NIOSH selected the facility described above. The other companies were rejected because either they had operated for a briefer period of time, employed fewer production workers, or lacked critical personnel information.

Methods

All personnel records were microfilmed at both the company that originally owned the facility, and the company that purchased it in 1969. From these records we were able to obtain the following information: employee's name, social security number, birth date, and race. The personnel files often did not contain detailed occupational histories, but did have the date first and last employed at the facility, and the job titles held on these dates. Demographic information, the date first and last employed at the facility and the corresponding job titles were coded and entered into a computer file.

The vital status of study subjects was ascertained using a number of sources of information. All study subjects social security numbers were sent to the Social Security Administration (SSA) for linkage with their files. SSA provided us with vital status information that was current as of December 31, 1977. Inquiries were sent for individuals who were not identified by SSA to the Internal Revenue Service (IRS) and to the Florida Department of Motor Vehicles, Division of Driver Licenses. The IRS only provides addresses, and does not provide a date of last contact. The addresses identified through the IRS search were sent to the local post office for confirmation that the individuals still resided at that address. For the limited number of subjects not successfully traced through these methods, a private locator service was used. Using these methods, subjects were followed for vital status ascertainment from their first date of employment up to December 31, 1977, or the date of death, whichever came first.

Death certificates were obtained from state vital statistics departments. Cause of death was coded by a nosologist using the revision of the International Classification of Diseases in effect at the time of death. Deceased workers for whom no death certificate have yet been located were assumed dead on the date specified by the reporting agency and the cause of death was coded unknown.

The analysis was performed using a modified life table analysis program developed by NIOSH.⁵ Person-years at risk of dying (PYARs) were calculated from the first date of employment at the plant through the end of

study date (12/31/77), the date of death, or the date when the subject was lost to follow-up, whichever occurred first. PYARs were obtained for each sex, race, five-year age and calendar period groups. The expected deaths were calculated by multiplying the appropriate PYARs by the U.S. death rates specific for five year age groups, five year calendar periods, sex, race and cause of death. The results were summed over the specific categories to obtain the total expected deaths for each cause. The findings were also stratified by duration of employment and length of followup. Length of followup was intended to act as a surrogate measure for latency, and duration of employment as a surrogate measure for exposure in this study.

Standardized Mortality Ratios (SMRs) were calculated for each race and sex category by dividing the number of observed deaths by the number expected and multiplying by 100. An exact test based on the Poisson distribution was used for testing statistical significance. Ninety percent confidence limits were calculated using Fisher exact limits. For death categories with less than two observed deaths, no statistical tests, SMRs or confidence limits were computed. A one tailed p value of 0.05 was used as the minimum criteria for statistical significance. Confidence limits and the statistical tests were computed with the aid of the Hewlett Packard Programs described in Rothman and Boice.⁶

RESULTS

Altogether 3199 workers were identified as having worked at this plant from 1953 to 1976. Dates of employment were not available for 39 workers (1.2 percent). These subjects could not be included in the life table analysis because person years at risk and duration of employment could not be calculated. None of these individuals had died from respiratory cancer.

The vital status of this cohort at the end of the study date (12/31/77) is presented in Table II. Over 96 percent of the study population were determined to be either alive or deceased. For the known deceased, approximately 93 percent of the death certificates have been obtained. The remaining 7 percent of the death certificates were not obtainable even after exhaustive efforts.

Table III presents a frequency distribution of the cohort for sex, race, age at hire and length of employment. Overall, this population was predominantly white and male. The majority of the workers were in their 20's when hired. Fifty four percent of the workforce had worked less than six months.

The cause specific mortality experience for all study subjects is presented in Table IV. None of the cause specific SMRs were significantly ($p < .05$) elevated. There was a significant ($p < .001$) excess of death in the unknown category. This excess was attributable to the 7 percent of death

certificates that were not obtainable (Table II). There was a deficit for all causes of death combined (SMR = 84), which was primarily due to a large deficit for diseases of the circulatory system (SMR = 62).

Sex and race specific mortality was examined, although the detailed results are not presented in this paper. Again, no cause specific SMRs were significantly ($p < .05$) elevated for any of the sex-race groups. White males had a SMR that was greater than one hundred for malignancies of the genitals (SMR = 253), urinary organs (SMR = 247), diabetes mellitus (SMR = 208) and diseases of the respiratory system (SMR = 119). Black males had SMRs that were greater than one hundred for tuberculosis (SMR = 178) and for malignancies of the trachea, bronchus and lung (SMR = 181). White females had an elevated risk for diseases of the circulatory system (SMR = 246). No deaths were observed among black females.

In Tables V and VI, the relationship between lung cancer, duration of employment and length of followup is presented. For white males (Table VIII) no trends were evident for either duration of employment or length of follow-up. Extremely small excesses were evident for the 0-10 year and 10-20 year length of follow-up groups which had SMRs of 118 and 105 respectively. For black males (Table VI) the SMRs appeared to increase with both duration of employment and length of followup, although there were only a few deaths in each of these categories. Workers with greater than 20 years of employment had a SMR of 1250 which was statistically significant ($p < .05$). The length of followup categories of 10-20 years and 20+ years had SMRs of 173 and 357 respectively.

Hospital records were obtained for the five black males workers who died from lung cancer, which confirmed the death certificate diagnosis of lung cancer. Four were confirmed histologically to be squamous cell carcinomas, and for one individual no histologic information was provided. One was described as a non-smoker, two were described as smokers and the records for the other two did not divulge their smoking habits.

Finally, in an attempt to determine whether the five black workers who died from lung cancer shared a common exposure, their job titles were reviewed. Three of the five workers held the position of cleanup man when they first started working at the plant. There was no clustering of job titles for the position held on the last day of work. There was also no indication in the employees' personnel records that any of them had worked in the uranium recovery operation. In order to determine whether the observation that 3 of these individuals were first employed as cleanup men represented a significant excess, a life table analysis was performed for black males that were first employed as cleanup men. This analysis revealed that 2.36 deaths were expected versus 3 observed, yielding a SMR of 127, which was not statistically significant.

Discussion

Overall, no statistically significant increases in cause specific mortality were observed for the entire study population, or for any of the individual sex-race groups. However, when lung cancer mortality was stratified by duration of employment and length of followup there was an apparent excess among black males with greater than 10 years of employment and followup. This pattern is consistent with the effect of a respiratory carcinogen, which is expected to increase with duration of exposure, and often only appears after a latency period of about 20 years. If the analyses were restricted to black males with at least 10 years of employment and followup, this would yield a SMR of 411 which is statistically significant (p one tail < .05).

Two of the five black male lung cancer victims were among the original 3 reported to NIOSH. The third individual is still alive. Thus, this study confirms an excess of lung cancer originally identified by a cluster. It would have been valuable to look for an excess of lung cancer at a similar plant where there was no a priori knowledge that a cluster existed.

Inferences in regard to causality must await observations by other investigations in this industry.

A possible explanation for the fact that the excess risk of lung cancer was only apparent in black males is that blacks may have been hired into less hygienic positions, resulting in higher exposures to respiratory

carcinogens. Other studies have noted that lung cancer excesses in blacks, not seen in whites may be related to certain jobs being assigned preferentially to nonwhites.⁷

Sixty percent (3/5) of the lung cancer cases among black males first worked as cleanup men at the plant, which paralleled the fact that of 690 blacks in this study 404 (59 percent) had worked as cleanup men, whereas, of 2498 whites in this study only 26 (1 percent) had held this job. This position involved cleaning the phosphoric acid reaction vessel, a task which (Table 1) may result in exposures to several potential respiratory carcinogens including chromium, arsenic, uranium and radon daughters. There was a slight lung cancer excess for this job (SMR = 127) which was not statistically significant. This excess was based upon a difference of 0.7 between the observed and expected number of deaths and may easily be a result of chance variation.

This plant is located in Polk County, which has significantly higher lung cancer mortality rates than the United States as a whole (4). However lung cancer rates in Polk County are only elevated for white males; nonwhite males during the period of 1950 to 1969 in Polk County had lung cancer rates that were 18% lower than U.S. rates (6). Therefore, the observed excess of lung cancers among black males at this plant is not a result of elevated background rates.

Although only 93 percent of the death certificates were obtained, the net effect of not ascertaining the remaining 7 percent of deaths would be to reduce the probability of detecting an effect. These deaths contributed person-time to the study without contributing disease specific deaths and would thereby inflate the expected number of deaths in the cause specific categories. We believe this effect to be minimal, since the amount of person-time contributed by these 13 subjects was small relative to the entire study population.

The majority of expected deaths in Tables V and VI had less than 20 years of followup, since about 93 percent of the person years in this study were in the less than 20 years of followup category, and many of the study subjects are still relatively young. The relatively short length of followup limits the ability of this study to detect an association between employment at this plant, and the risk of dying from diseases with a long latency period (e.g. lung cancer). As this population matures, the number of subjects with adequate length of follow-up (e.g. 10-20 years) will increase. Therefore, this cohort will be followed and reported on in the future.

In summary, a statistically significant excess in lung cancer mortality was observed among black males in the longest duration of employment and followup categories. Although the small number of deaths involved preclude any conclusion, this finding is suggestive of a possible association with

occupational exposure. A slight excess was found for black males that first started as cleanup men. However, this excess was not statistically significant, and may very well have been due to chance variations. The followup of this study population will be continued, since it is still fairly young. Inferences of causality are undermined by our prior knowledge of the lung cancer cluster and the small number of deaths involved. Other investigations in plants with similar exposures are needed to clarify whether an occupationally related lung cancer excess really exists.

TABLE I
Environmental Data - Personal and Area Samples
mg/m³

	Number of Samples	Range	Average
Fluoride	7	0.02 - 13.24	3.39
Sulfuric Acid	8	0.013 - 0.22	0.11
Phosphoric Acid	8	0.03 - 0.52	0.25
Cadmium	11	0.002	0.002
Chromium (total)	11	0.001 - 0.02	0.002
Uranium	2	0.005 - 0.014	0.010
Vanadium	11	0.01	0.01
Arsenic	4	0.001	0.001

Radon Daughter Sample Results

Number of Samples	Counts per minute (range)	Working Level* (range)
7	0 - 25	0.00 - 0.02

*Working Level is defined as any combination of short-lived radon-222 daughters, polonium-218, lead-214, bismuth-214, and polonium-214, in one liter of air, without regard to the degree of equilibrium, that will result in the ultimate emission of 1.3×10^5 MeV of alpha particle energy.

TABLE II
VITAL STATUS
(As of 12-31-77)

	NUMBER	PERCENT
Known to be alive	2,910	91.0
Known to be deceased	176	5.5
Death certificates obtained	163	
Death certificates outstanding	13	
Not known to be alive or deceased	113	3.5
	3,199	100

TABLE III
Sex, Race, Age and Length of Employment
Distribution of Study

	NUMBER	PERCENT
Sex		
Male	2981	93.2
Female	212	6.6
Unknown	6	.2
Race		
White	2498	78.1
Black	690	21.6
Other	11	.3
Age at Hire*		
18 to 28	1973	61.7
28 to 43	1022	31.9
43 to 58	159	5.0
58+	4	.1
Unknown	41	1.3
Length of Employment		
0 to 1 year	1733	54.2
1 to 10 years	1028	32.1
10 to 20 years	312	9.8
20+ years	87	2.7
Unknown	39	1.2

*Age when subjects began work at the facility

TABLE IV
Cause Specific Mortality For All Study Members

CAUSE OF DEATH	OBSERVED	EXPECTED	SMR**	90 PERCENT
				CONFIDENCE*** INTERVAL
Tuberculosis	2	1.63	123	22,386
All malignant neoplasms	22	28.99	76	51,108
Buccal cavity & pharynx	0	1.08	--	--
Digestive Organs & Peritoneum	3	7.23	41	11,107
Larynx	0	.43	--	--
Trachea, Bronchus & Lung	10	8.85	113	61,192
Other parts of the Respiratory system	0	.15	--	--
Breast	1	.55	--	--
Female Genital Organs	0	.36	--	--
Male Genital Organs	2	1.09	183	33,578
Urinary Organs	2	1.10	182	32,572
Lymphatic & Hematopoietic	2	3.78	53	09,167
Other & Unspecified Sites	2	4.40	98	08,143
Benign & Unspecified Neoplasms of the brain	0	.43	--	--
Diabetes Mellitus	4	2.58	155	53,355
Diseases of the Blood & Blood forming organs	0	.74	--	--
Psychoneurotic, Personality & Mental Disorders	0	2.92	--	--
Diseases of the Nervous System	3	8.73	34	09,89
Diseases of the Circulatory System	36	54.56	66	49,87
Diseases of the Respiratory System	5	7.93	63	25,133
Diseases of the Digestive System	2	9.66	21	04,65
Diseases of the Genito-urinary system	2	2.27	88	16,277
Diseases of the Skin	0	.23	--	--
Diseases of the Bone & Organs of Movement	0	.13	--	--
Accidents & Violence	54	53.94	100	79,126
Unknown	16	4.30	372*	233,565
All others	9	9.66	93	49,163
All deaths	155	189.51	82	71,93

*p (one tail) < .001

** SMRs were not calculated for death categories with only one death.

*** Fischer exact limits were used.

TABLE V
Length of Followup & Duration of Employment at the Plant for
Malignancies of the Trachea, Bronchus & Lung Among White Males

Duration of Employment (Years)				
Length of Followup (Years)	0 - 10	10 - 20	20 +	Total
0 - 10				
Observed	2	0	0	2
Expected	1.69	0	0	1.69
SMR*	118	-	-	118
10 - 20				
Observed	2	1	0	3
Expected	1.91	.95	0	2.86
SMR*	105	-	-	105
20 +				
Observed	0	0	0	0
Expected	.97	.17	.22	1.36
SMR*	-	-	-	-
Total				
Observed	4	1	0	5
Expected	4.57	1.12	.22	5.91
SMR*	88	-	-	85

*SMRs were not computed for categories with only one death.

TABLE VI
Length of Followup & Duration of Employment at the Plant
for Malignancies of the Trachea, Bronchus & Lung Among Black Males

Duration of Employment (Years)				
Length of Followup (Years)	0 - 10	10 - 20	20 +	Total
0 - 10				
Observed	0	0	0	0
Expected	0.45	0	0	.45
SMR*	-	-	-	-
10 - 20				
Observed	2	1	0	3
Expected	1.21	0.52	0	1.73
SMR*	165	-	-	173
20 +				
Observed	0	0	2	2
Expected	0.35	0.05	0.16	0.56
SMR*	-	-	1250 **	357
Total				
Observed	2	1	2	5
Expected	2.01	0.57	0.16	2.74
SMR*	100	-	1250 **	182

*SMRs were not calculated for categories with only one death.

**p (one tail) < .05

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Mortality Study of Workers Exposed to Styrene in the
Reinforced Plastic Boat Building Industry

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MORTALITY STUDY OF WORKERS EXPOSED TO STYRENE IN THE
REINFORCED PLASTIC BOAT BUILDING INDUSTRY

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ABSTRACT

Cause of death patterns among workers exposed to styrene were studied at two boat building plants in order to test the hypothesis that leukemia and lymphoma may be associated with exposure to styrene. 5,021 workers met the criterion of having worked at the two plants between 1959 and 1978, of whom 2,060 worked in departments that were classified as having high exposure to styrene. This exposure classification was based on industrial hygiene surveys conducted at the two plants in 1978. It was found that 176 workers were deceased as of the study end date (December 31, 1978), in comparison to 195.3 deaths expected on the basis of U.S. death rates. No deaths were observed from leukemia (1.7 were expected) or lymphoma (2.1 were expected). Accidental deaths were in excess in the high exposure group (18 observed versus 12.4 expected), but they did not appear to be due to styrene's acute narcotic effect on the central nervous system since none of the individuals were employed at the boat building facilities at the time of their death. However, the possibility of a chronic effect from styrene exposure could not be dismissed. While cancer did not appear to be related to styrene exposure, it was concluded that the study had little ability to demonstrate carcinogenic activity. The study's low sensitivity was due primarily to insufficient length of observation and the young age of the cohort members. It was also concluded that this population should provide increasingly useful information on chronic styrene toxicity as it ages and the observation period increases.

INTRODUCTION

Statement of the Problem

There is some, although limited, evidence that styrene may cause cancer in both laboratory animals and humans. The purpose of the present study was to investigate the existence of suggested excess mortality risks, especially from leukemia and lymphoma, among workers occupationally exposed to styrene. This cohort mortality study, using a group of workers not previously examined, was designed to provide additional information for the evaluation of the potential carcinogenicity of styrene exposure in humans.

Styrene Production and Usage

Styrene, also known as vinyl benzene, is an unsaturated, aromatic monomer. It is a volatile, colorless liquid with a molecular formula of $C_6H_5CH=CH_2$ and a molecular weight of 104.14. Unlike other aromatic hydrocarbons such as toluene, the xylenes, and ethyl benzene which occur naturally, styrene and its oxide result only from synthetic production. The most widely used method of manufacturing styrene is by alkylating benzene with ethylene in the presence of a catalyst to form ethylbenzene. The ethylbenzene is then dehydrogenated to form styrene (1).

Styrene was first discovered in the nineteenth century, although it was not until the late 1930's that a high purity styrene monomer was manufactured commercially in the United States (2). Today styrene is used extensively in

the manufacturing of plastics and synthetic rubber. The production of styrene in the United States has grown from 2.4 million pounds in 1940 to 7.5 billion pounds in 1979. U.S. production of styrene is expected to grow to 3.4 billion pounds by 1984, with 62% being consumed in polystyrene production, 22% in copolymers, 7% in styrene butadiene rubber, 7% in unsaturated polyester resins, and 2% in miscellaneous uses (3). Based on the National Occupational Hazard Survey, NIOSH estimates that 30,000 workers in the U.S. are potentially exposed to styrene on a full-time basis and that an additional 300,000 individuals work at facilities which use compounds containing styrene and may be periodically exposed (4). Occupations with potential styrene exposure are shown in Table 1 (5).

Exposure Standards

The present OSHA standard for styrene includes a time weighted average (TWA) of 100 ppm, a ceiling concentration of 200 ppm, and a maximum peak concentration of 600 ppm for five minutes in any three hour period. NIOSH recommends that exposure to styrene be controlled so that workers are not exposed to concentrations greater than 50 ppm, determined as a TWA concentration for up to a 10-hour workshift, 40-hour workweek. A ceiling concentration of 100 ppm, as determined during any 15-minute sampling period, is also recommended (6).

HEALTH EFFECTS

Styrene is an irritant of the skin and mucous membranes and at high levels acts as a central nervous system narcotic. Styrene may be absorbed into the blood stream by all routes, including ingestion, inhalation, or absorption through the skin. In industrial settings, inhalation of vapors and mists and skin contact with the liquid are the most common modes of exposure (7). Styrene's odor threshold is about 1 ppm and concentrations greater than 100 ppm are regarded as having a very unpleasant odor. Transient irritations of the eyes, nose, and oral cavity may develop when concentrations exceed 100-200 ppm and coughing may occur around 500 ppm (8,9).

Neurotoxic Effects

Styrene is a known neurotoxic agent. The neurotoxic effects are well recognized and have been demonstrated in laboratory animals, such as rats (10), mice (11), and guinea pigs (10); and in humans, in both experimental (12,13) and occupational settings (14,15). Central nervous system (CNS) effects have been observed with both acute and chronic styrene exposures. When rats and guinea pigs were continuously exposed to styrene concentrations ranging from 1,300 to 10,000 ppm, the animals exposed to the higher concentrations experienced immediate weakness, loss of equilibrium, convulsions, and death within three hours. When exposed to 1,300 ppm, some rats (but no guinea pigs) survived more than 40 hours of continuous exposure (10). Humans experimentally exposed to 800 ppm styrene showed immediate CNS effects of listlessness, drowsiness, and impaired balance. Slight muscular

weakness and unsteadiness continued after the termination of exposure (12). At styrene concentrations of 350-375 ppm, overt CNS effects have been observed in humans including inability to perform a modified Romberg test (13), decreased manual dexterity and coordination (13), and increased reaction time (9). Subjective CNS effects such as headache, unusual fatigue, difficulty concentrating, and a feeling of tension were reported during styrene exposure (13). Workers exposed to styrene in the manufacturing of reinforced plastics had increased reaction times (14), reduced radial and peroneal nerve conduction velocities (15), abnormal electroencephalograms (16,17), and subjective symptoms such as headaches, fatigue, tension, and dizziness (15,16). Reaction times and digit symbols (WAIS subtest) have been shown to be related to both intensity of exposure and duration of exposure when comparing workers exposed to styrene to unexposed controls (18). Cases have also been reported where workers exposed to styrene have experienced chronic neurologic problems such as reflex differences in the upper and lower extremities, and in a few individuals, cerebral lesions have been reported (19,20).

Hepatotoxic Effects

Styrene has also been shown to be an hepatotoxic agent. The hepatotoxic effects have been hypothesized to be due to a metabolite of styrene binding covalently to liver macromolecules (protein and DNA) (21). Rats exposed to a single dose of 1,300 ppm for 30 hours had apparent damage to the liver parenchyma, although at similar exposure levels, no liver damage was found for either rabbits or monkeys (10). Rats intubated with 400 mg

Carcenogenic Potential

The evidence as to the carcinogenicity of styrene is unclear. Both male and female 020 mice treated with 1,350 mg/kg styrene for 16 weeks had a significant excess of lung tumors when compared to olive oil treated animals. However, when compared to untreated mice, the difference was only statistically significant for the females (33). When Fischer 344 rats and B6C3F1 mice were given styrene by stomach tube five times a week for 78 weeks, excess carcinomas and adenomas of the lungs were observed for the male mice, but no difference in tumor incidence was observed in the female mice or rats of either sex (34). A chronic inhalation study of Sprague-Dawley rats exposed to styrene for 18-20 months did not reveal any statistically significant excess of tumors although the authors commented that the incidence of lymphatic and hematopoietic cancers appeared to be higher than expected based on their experience with that strain of rat (35).

Part of the concern over the potential carcinogenicity of styrene is based upon the fact that styrene oxide may be an intermediate in the metabolism of styrene, and styrene oxide has been shown to be mutagenic in tests using microorganisms (36-38). The principal evidence in favor of this hypothesis is the observation that microsomes from livers of rabbits pretreated with phenobarbital converted styrene to styrene oxide (39). Based upon this evidence a scheme for the metabolism of styrene to mandelic acid through styrene oxide and phenylethylene glycol was proposed (40). However, when styrene oxide was fed to rabbits, phenylethylene glycol was not found (41) and there have been no investigations which have shown the presence of styrene oxide as a product of styrene metabolism in vivo.

styrene/kg/day, 5 days a week for 26 weeks showed slight increases in liver weights, while at 667 mg styrene/kg/day, moderate increases in liver weights were observed (22). After a single dose of styrene to rats (2.5 g/kg), a 50% decrease in hepatic glycogen was found (23). In humans, investigators have reported possible damage to liver function due to styrene exposure based upon a variety of clinical tests such as increased serum levels of beta and gamma globulins (24) and elevated serum levels of aspartate-amino-transferase and alanine-amino transferase (25). Increased gamma-glutamyl transpeptidase (GGTP) activity has been demonstrated in polystyrene workers in Germany (26) and the United States (27).

Mutagenic Potential

Styrene has shown little and poorly reproducible potential for mutagenicity in microorganisms (28,29), although occupational exposure to humans has indicated that styrene may cause changes in genetic material. Workers who manufactured fiberglass reinforced plastics products have shown more frequent abnormal chromosomes in their lymphocytes than do unexposed controls (30,31). Pero et al. (32) have suggested that styrene may act as a promoter of genotoxic activity since the carcinogen n-acetoxy-2-acetylaminofluorene was shown to induce higher levels of unscheduled DNA synthesis in the lymphocytes of workers occupationally exposed to styrene when compared to the lymphocytes of unexposed controls.

Epidemiologic Studies

Several epidemiologic studies have been conducted on workers employed in the synthetic rubber industry (including styrene-butadiene rubber) and in the production of styrene and styrene-based products. Studies of rubber industry workers by McMichael et al. (42), Monson and Nakano (43), and Andjelkovick et al. (44) have all shown excess deaths due to malignant neoplasms of the lymphatic and hemotopoietic system. Excess deaths due to cancer of the stomach, prostate, and bladder were also found in at least two of the three studies. No one chemical can be implicated in these patterns of excesses mortality since the rubber industry has used a wide variety of chemicals, including styrene and butadiene, as well as other known or suspect carcinogens such as benzene and beta-naphthylamine. However, in another study Meinhardt et al. (45) examined the mortality experience of workers at two styrene-butadiene rubber producing plants where the most prevalent chemicals used were styrene and butadiene. The investigators found excess mortality due to malignant neoplasms of the lymphatic and hemotopoietic system for the workers at one of the two facilities, with the excess approaching significance for the group of workers employed before 1946.

Studies of workers employed in the production of styrene and styrene-based products have shown inconsistent results for excess mortality (Table 2). Frentzel-Beyme et al. (46) and Nicholson et al. (47) reported negative results in mortality studies of styrene-polystyrene polymerization workers. In the Nicholson report, the criteria for inclusion in the study cohort was

employment for five or more years as of May 1, 1960. The authors indicated that when randomly collected death certificates of individuals who did not have five years of employment as of 5/1/1960 were also considered, the data was suggestive of an excess risk for leukemia and lymphoma deaths. Ott et al. (48) examined the mortality experience of employees engaged in the development or production of styrene-based products. Deaths due to malignant neoplasms were fewer than expected (282 obs. vs 358 exp.) for the total cohort, although an increase in lymphatic leukemia (6 obs. vs 2.9 exp.) was observed among a subgroup of employees who had exposure to polymer extrusion fumes, solvents, and colorants. Ahlmark (49) conducted a survey of Swedish workers who had been employed at facilities using styrene between 1950 and 1975. When the names of these individuals were matched against a national death registry and a national cancer incidence registry, no excesses of death or disease were observed. Werner (50) reported a statistically significant excess of lymphoma deaths among men who worked at least one year in the production, polymerization and processing of styrene. The author suggested caution when interpreting the results, due to small numbers (3 lymphoma deaths) and lack of association with duration of exposure.

METHODS

Cohort Selection

To further investigate the health effects of occupational exposure to styrene, NIOSH conducted a cohort mortality study of two reinforced plastic boat building facilities. The reinforced plastic industry was chosen since it had the highest levels of styrene exposure, with few confounding exposures, and the greatest number of potentially exposed workers. The selection of an appropriate cohort was a difficult task since the use of styrene in industry is relatively new and for most of the workers the number of years since initiation of styrene exposure is short. NIOSH investigated the feasibility of conducting a study at 14 various reinforced plastic companies located throughout the United States. The two facilities selected were chosen based on the number of years they had been involved in the production of reinforced plastic products and on the availability of adequate personnel records necessary to conduct a retrospective cohort mortality study.

Company A, located in Washington State, began production of fibrous glass reinforced plastic boats in 1957. At the time of the feasibility study in 1978, there were 485 employees of whom 14% were females. Company B, also located in Washington State, changed its operations from the production of wood boats to fibrous glass boats in 1957. The company moved to its present location in 1959. At the time of the feasibility study in 1978, Company B employed 292 individuals of whom 32% were females. The rate of turnover was

high for both companies, approximately 40% annually, with even higher rates for jobs involving high styrene exposure. The individuals at both plants were members of local unions.

Criteria for Inclusion in the Cohort

Individuals were eligible for inclusion in the cohort if they worked for Company A or Company B after December 31, 1958. Individuals employed at either company who discontinued their employment before 1959 were not included in the cohort since adequate personnel records were not maintained at either company before this date.

Industrial Hygiene Data

In-depth industrial hygiene surveys were conducted at the two facilities to determine the employees' full-shift time weighted average exposure to styrene and to classify the jobs and departments within the plants according to levels of styrene exposure. The surveys were also used to identify other exposures present at the two facilities which could potentially produce adverse health effects. The survey information was used in the mortality study to define the low/high styrene exposure groups.

Data Collection and Coding

The personnel records from the companies and the local union offices were microfilmed during October and November 1978. From these records,

demographic data and work history information were abstracted and coded into a masterfile for each company. The demographic data included name, social security number, sex, race, date of birth, place of birth, last known address, and spouse's or next of kin's address. The work history information included the general first and last dates of employment and a detailed work history comprised of the dates of employment along with a code for each specific job held by an individual. The coding scheme for the detailed work histories was consistent with job groupings used in the industrial hygiene surveys at the two companies. All the jobs within the companies were grouped into exposure categories based on the extent of styrene exposure (Appendix A and B). No smoking data was available for any individuals in the study cohort.

Cohort Validation

In order to assess the completeness of the cohort derived from the personnel and union records, the companies were asked to request the Quarterly Earnings Reports (QER), form 941, from the Social Security Administration. These reports listed all the employees of a company for every quarter, each year in which they received wages. A computerized listing of the names and social security numbers of the individuals in the cohort masterfiles was matched against those in the QER for each company (51). Lists were prepared of those individuals who appeared in the QER but not in the cohort masterfiles. These lists were sent to the companies for help in resolving the discrepancies. Individuals who appeared in the cohort masterfiles but not in the QER were not identified for verification since personnel records were considered the primary source of information.

Missing Information

Demographic or work history information missing from company and union records was obtained through various sources. Information on date of birth, sex, and race was provided by the Social Security Administration through an interagency agreement. Missing beginning or ending dates of employment were assigned using information from the QER. For individuals appearing in more than one quarter, the missing date was assigned the 15th day of the mid month for the quarter in which an individual first or last appeared in the QER. For individuals appearing in only one quarter, the assigned date was approximately one-half the maximum time possible for that individual.

Vital Status & Death Certificate Ascertainment

NIOSH attempted to identify all the deaths that occurred among the cohort through December 31, 1978. The sources used included the Social Security Administration, Internal Revenue Service, Washington State Department of Motor Vehicles, and for difficult to trace individuals, a case location service.

For all the individuals identified as deceased, NIOSH attempted to obtain a death certificate. For those certificates obtained, the underlying cause of death was coded by a qualified nosologist according to the revision of the International Classification of Diseases in effect at the time of death.

Mortality Analysis

The cohort was analyzed using a modified life table technique (52). The life table analyses took into account the potentially confounding variables of age, race, sex, and calendar year. The effects of both latency (time since first employment) and duration of employment were examined in five year intervals. The statistics generated from the life table analyses were standardized mortality ratios (SMR's). The SMR's were ratios of the number of observed deaths with the number of expected deaths generated from comparison death rates using the number of person-years at risk for the cohort. The comparison death rates were the rates for the United States general population.

Life table analyses were conducted for the whole cohort and separately for the high and low exposure groups as determined by the job categories. Individuals were included in the high exposure group if they worked for any length of time in a job included in the fibrous glass or plastician exposure category. Plastician is to an industry term for individuals working with fibrous glass reinforced plastics. To avoid calculation of "immortal" person years at risk of dying, person years were not calculated prior to January 1, 1959 due to incomplete records at the study facilities before that date. The analysis for the high exposure group included the person-years at risk for each individual from their date of first employment in a high exposure job (or January 1, 1959, which ever was later) until their date of death or the study end date (December 31, 1978). The analysis for the low exposure group was conducted as follows: 1) for individuals who

never worked in a high exposure job; the person years at risk from their date of employment (or January 1, 1959, which ever was later) until their date of death or the study end date and, 2) for individuals who began work in a low exposure job and then transferred to a high exposure job; the person years at risk from their date of employment in a low exposure job (or January 1, 1959, which ever was later) until their date of transfer to a high exposure job.

RESULTS

Industrial Hygiene

Styrene Exposure - The exposure to styrene in the two study facilities resulted from the use of thermoset polyester resins. The type of resin used in these facilities contained approximately forty percent by weight styrene monomer as a reactive diluent. During manual laminating and spraying operations, as much as ten percent of the styrene could potentially be volatilized into the workplace air with the remainder being consumed in the chemical reaction (53).

The amount of styrene to which the study individuals were exposed was dependent upon the particular job being performed. Individuals who worked in jobs titled fiberglass or plastician, where the employees worked directly with the polyester resins, had the highest exposures. The extent of styrene exposure within these high exposure jobs varied according to specific factors within each job such as the size and configuration of the part being made and the method of resin application. Hull lamination exemplifies the

influence of both size and part configuration on styrene exposure since this job required the largest volume of resin, and the configuration of the hull is such that styrene vapors can become entrapped. Tables 3 and 4 show the mean styrene concentrations for particular jobs within the high exposure category for Plant A and Plant B. The composite mean concentration of airborne styrene for all high exposure jobs was 42.5 ppm at Company A and 71.7 ppm at Company B (54). All the other jobs within the two facilities had either low or minimal exposure to styrene .

Other Exposures - Significant exposure to acetone was present at both facilities. Acetone was used extensively as a cleaning solvent. The range of TWA exposure levels of acetone found at the two study facilities was 17.0 ppm to 252 ppm at Company A and 8.2 ppm to 396 ppm at Company B. The OSHA standard for acetone is 1000 ppm for an 8-hour TWA. It is believed to be a relatively innocuous compound, as indicated by its high exposure limit.

Exposures to fibrous glass and organic peroxides were present in the lamination areas at both facilities. Fibrous glass in the form of both woven and chopped strand roving was used as a binder for the catalyzed polyester resin. The most commonly used catalyst in the polyester resin was methyl ethyl ketone peroxide. In boat assembly areas, potential exposures included wood dusts, noise, ergonomic stress, and a variety of solvent exposures including toluene, the xylenes, and naphthas. The exposure to these solvents was in the form of a mixture of substances rather than a single solvent exposure. In addition, during the use of urethane paints and varnishes, exposure to isocyanates (TDI, MDI, HMDI) was also possible.

Exposure Control Techniques

At the time of the industrial hygiene survey in 1978, the control techniques available at both facilities to reduce potential exposures included dilution ventilation and personal protective equipment. The dilution ventilation techniques at Plant A included an exhaust fan in the gel coat booth, two 4 feet diameter exhaust fans in the lamination building, a number of high speed circulating fans, and natural ventilation. The personal protective equipment available included rubber gloves and respirators. Rubber gloves were used by some of the employees in the fibrous glass jobs while over half the individuals wore respirators. At Company B, the dilution ventilation procedures included an exhaust fan in the lamination area, a number of high speed circulating fans, and natural ventilation. Almost all the employees in the fibrous glass jobs used rubber gloves and, although respirators were available, they were not used by most of the employees.

There was limited historical industrial hygiene data available for either of the two facility. The Washington State Department of Labor and Industries conducted industrial hygiene sampling at the two facilities starting in 1974 for Company A (55) and in 1975 at Company B (56). It appeared from the data that exposure levels of styrene may have decreased over time but, due to the limitations of the data, the extent of the possible decrease could not be determined.

Cohort Description

The completed cohort consisted of 5,201 individuals, 3,503 who worked for Company A and 1,705 who worked for Company B. Seven individuals worked at both facilities. The cohort included 4,269 white males, 640 white females, 250 nonwhite males, and 42 nonwhite females. As of December 31, 1978, 4924 cohort members were alive (94.7%), 176 were dead (3.4%) and vital status was unknown for 101 individuals (1.9%) (Table 5). Death certificates were obtained for 165 (94%) of the 176 deceased individuals. The largest percentage of workers were first employed during the late 1960's with the second largest group first employed during the early 1970's (Table 6). Individuals with dates of employment before 1957 were considered to be first employed as of January 1, 1957 since this was the beginning of the use of styrene at the study facilities. The distribution of the person-years at risk by calendar year and age for the total cohort is shown in Table 7. It can be seen that the majority of the person-years were accumulated during the later time periods and in the younger age groups. 78% of the person-years were accumulated since 1970 and 81% of the person-years were accumulated for individuals under age 45.

There were 2,060 individuals, 39.6% of the total cohort, who worked at least one day in any of the high styrene exposure jobs. This group included 1502 white males, 411 white females, 117 nonwhite males, and 30 nonwhite females. As of December 31, 1978, 1966 of the high exposure group individuals were alive (95.4%), 47 were dead (2.3%) and vital status was unknown for 47 individuals (2.3%). Death certificates were obtained for 44 (94%) of the 47 deceased individuals.

There were 3102 individuals, 59.6% of the total cohort who never worked in any high styrene exposure job. This low exposure group included 2735 white males, 222 white females, 133 nonwhite males, and 12 nonwhite females. As of December 31, 1978, 2921 individuals were alive (94.2%), 127 were deceased (4.1%) and vital status was unknown for 54 individuals (1.7%). Death certificates were obtained for 122 (96%) of the deceased individuals.

The cohort included 39 individuals without sufficient detailed work history information to allow for their classification into either the high or low exposure group. This group included 32 white males and 7 white females. As of December 31, 1978, 2 individuals were deceased and death certificates were obtained for both individuals.

Duration of Employment in a High Styrene Exposure Job - Duration of

employment for the majority of the 2,060 individuals who worked at least one day in any of the high styrene exposure jobs was relatively short (Table 8). Nine individuals (0.4%) worked for only 1 day, 125 individuals (6.1%) worked between 2 and 7 days, and 381 individuals (18.5%) worked between 8 days and 1 month. Therefore, a total of twenty-five percent of the individuals worked for one month or less in jobs classified as having high styrene exposure. Forty eight percent of the individuals worked from one month to one year, 21 percent worked from one year to five years, and only seven percent of the high exposure employees worked for 5 or more years in high styrene exposure jobs (Figure 1).

Time Since First Employment in a High Styrene Exposure Job - Using time since first employment in any high styrene exposure job as a surrogate measure for latency, it was possible to evaluate the length of time that has passed since initiation of exposure to styrene to allow for the manifestation of disease. Since styrene's use in industry is relatively new, the latency potential for most individuals in the high exposure group was short (Table 8). Only 39% of the 2060 individuals were observed for more than 10 years from the date of their first employment in a high exposure job. Four percent of the individuals were observed for more than 15 years and less than one percent of the high exposure individuals were observed for more than 20 years since their date of first employment in a high styrene exposure job (Figure 2).

Validation of the Cohort

There were 764 nonmatches between individuals who appeared in the QER file but not in the master file for Company A. Most of the nonmatches were due to individuals being included in the QER who worked at a second boat building facility started by Company A in North Carolina in 1972. There were 348 individuals in the QER file who began work after 1972 and had North Carolina assigned social security numbers. These individuals were excluded from the group of nonmatches since they were assumed to have worked at the North Carolina facility. A list of the names and social security numbers of 263 nonmatches who began work after 1972 and did not have North Carolina assigned social security numbers was sent to the North Carolina facility for clarification. Only 18 of the 263 individuals could not be identified as

having worked at the North Carolina plant. The remaining 153 nonmatches were individuals who began work before 1972 and therefore before the start of the North Carolina plant. These nonmatches could not be resolved since a fire at the Washington facility in 1980 destroyed all the personnel records for that plant. A total of 171 nonmatches, 5% of Company A's cohort, could not be resolved.

There were 93 nonmatches between the QER file and the masterfile for Company B. Sixteen of these individuals had their entire work history before 1959 and therefore did not meet the cohort inclusion criteria. An additional 40 individuals were identified by the company as having worked at a different facility. The remaining 37 individuals could not be identified, yielding 2% nonmatches for Company B.

The unresolved nonmatches from both plants were not added to the masterfile since there was no demographic or work history information for these individuals which would be needed in the life table analyses. The numbers of nonmatches are presented to show that the cohort was verified to be relatively complete (96%).

Observed and Expected Deaths

Table 9 shows the observed and expected deaths with the corresponding SMR's for the total cohort along with those for the high and low exposure groups. Most of the SMR's were unstable (had considerable variability) since they were based on very small numbers. The table includes information for those

disease categories where there was at least one death in either the high or low exposure groups. Even though there were no leukemia or lymphoma deaths, these categories were included because of their relevance to the study hypothesis. The total cohort included two deaths not found in either the high or low exposure groups since the appropriate exposure category for these individuals could not be determined. One death was due to coronary heart disease and the second was due to an accident.

There were no statistically significant excesses of deaths for any of the disease categories when all four race-sex categories were combined. This was true for the total cohort and for both the high and low exposure groups. Although there were no significant excesses, the all causes SMR was elevated for the high exposure group and there was no deficit of deaths due to diseases of the circulatory system as would normally be expected from a group of healthy workers. Since all 47 deaths in the high exposure group were among white males, the excesses became more apparent when white males were considered separately (Table 10). The all cause SMR was elevated with the majority of this excess contributed by deaths due to accidents (18 observed vs. 11.14 expected).

Since styrene exposure can cause narcosis, the possible relationship between styrene narcosis in the high exposure group individuals and the excess risk of death due to accidents was examined by comparing the date of death with the last date of employment for each of the accidental death victims. It was hypothesised that if an acute narcotic effect from styrene exposure was causing excess accidental deaths, the individuals would still be employed at

the facilities at the time of their deaths. The comparison revealed that not one of the 18 accidental deaths occurred while the individual was still employed at either of the two study facilities and that two months was the shortest time period between the end of employment and the date of death (Figure 3). In addition, none of the 18 death certificates listed a usual occupation with any apparent relationship to boat building which could have indicated a continued exposure to styrene after discontinuing employment at the study facilities. There was, however, one individual who could not be assigned to either the high or low exposure group who died in an accident while still employed at one of the study facilities.

Other disease categories having elevated SMR's and more than three deaths included all malignant neoplasms (32 obs. vs 28.8 exp.) for the low exposure group, and the main contributor to that excess, cancer of the trachea, bronchus, and lung (15 obs. vs 9.7 exp.). The excess of lung cancer in this group did not appear to be associated with styrene exposure since there was no excess of lung cancer deaths in the high exposure group and there was no relationship with duration of employment. Eight of the 15 deaths had less than six months of employment and 13 of the 15 deaths had less than 5 years of employment at either of the study facilities. There did, however, appear to be an increase in the risk of lung cancer by time since first employment for the less than 5, 5-10 and 10-15 year latency categories (Table 11). The death certificates for the 15 lung cancer cases were reviewed for an indication of a consistent cell type. No assessment was possible since 8 (53%) of the certificates had no indication of cell type. The remaining 7 certificates included four squamous cell, two oat cell, and one large cell carcinoma.

Statistical Power

Statistical power calculations were performed to estimate the study's probability of detecting an excess risk of death from leukemia or lymphoma. The formula used was that of Breslow (57), with the expected number of deaths obtained from the comparison rates (U.S. population) used in the life table analyses. Power calculations were performed for 1) the high exposure group with no minimum latency (time since first employment in a high exposure job) or exposure (length of employment in a high exposure job) specified, 2) the high exposure group with individuals having 10 or more years latency and no minimum exposure, and most importantly, 3) the high exposure group with individuals having 10 or more years latency and a minimum of one year exposure in a high exposure job.

Even when considering large relative risks, the power of the study to detect a significant excess mortality for leukemia and lymphoma was small (Table 12). When no minimum latency or exposure was specified, the power of the study to detect a significant excess at a relative risk of five was 49% for leukemia and 54% for lymphoma. At a relative risk of 10, the power was 89% for leukemia and 94% for lymphoma. When a 10 year minimum latency was required, the power of the study to detect significant excess mortality at a relative risk of 10 was 32% for leukemia and 36% for lymphoma. When a one year minimum exposure was required in addition to the 10 year minimum latency, the power of the study to detect a significant excess mortality at a relative risk of 10 was further reduced to 20% for leukemia and 22% for lymphoma.

DISCUSSION

There were no disease categories which showed significant excess mortality risks for the total cohort or for either the high or low exposure groups. A nonsignificant increase risk of accidental deaths (18 obs. vs. 12.3 exp.) for the high exposure group did not appear to be related to an acute narcotic effect from styrene exposure since none of the individuals were employed at the boat building facilities at the time of their death. However, the possibility of a chronic effect from styrene exposure could not be dismissed. An increased risk of cancer of the trachea, bronchus, and lung (15 obs. vs 9.7 exp.) for the low exposure group also did not appear to be associated with styrene exposure since there was no excess of lung cancer deaths in the high exposure group and there was no relationship with duration of employment. The increasing risk of lung cancer for the time since first employment categories of less than 5, 5-10, 10-15 years did not appear to be related to styrene exposure at these facilities, since occupational lung cancer usually has a minimum of 15-20 years latency.

The a priori diseases of interest were leukemia and lymphoma. Through December 31, 1978, no deaths due to any malignant neoplasm of the lymphatic and hematopoietic system were found for individuals in either the high or low exposure groups. Additional follow-up of the cohort revealed one lymphoma death (in 1981) occurring in an individual employed as a shipwright (low exposure group) from September 17, 1958 until November 13, 1968. This additional follow-up is very incomplete and no conclusions can be based upon it at this time.

It will be a number of years before this cohort will be able to provide information as to the carcinogenic potential of styrene exposure in humans. With vital status follow-up through 1978, only a small portion (39%) of the individuals in the high exposure group have been observed for more than 10 years since their initial employment and, therefore, little time has passed since initiation of styrene exposure to allow for manifestation of disease (15-20+ years usually for occupational cancers). In addition, the cohort is very young. As of December 31, 1978, only 3% of the total cohort and 2% of the individuals in the high exposure group, were deceased.

Even though no statistically significant excess mortality risks were found, the study should not be interpreted as negative, but rather as nonpositive or inconclusive. For a study to be truly negative, it must have sufficient power to detect excess risks if they exist. Power calculations performed for the leukemia and lymphoma deaths for the high exposure group revealed that when a 10 year minimum latency (time since first employment in a high exposure job) was required, the power of the study to detect a significant excess at a relative risk of 10 was 32% for leukemia and 36% for lymphoma. The power of the study was further reduced to 20% for leukemia and 22% for lymphoma when a one year minimum exposure was required in addition to a 10 year minimum latency. Therefore, this study could only have detected very large relative risks.

It was known at the time of planning this study that it would need to have a prospective component since styrene's widespread use in industry was relatively recent. Therefore, it is recommended that the cohort be updated

through 1983 when the death data become available through the National Death Index. With this additional five years of follow-up, 71% of the individuals in the high exposure group will have been observed for more than 10 years since their date of first employment and 39% will have been observed for more than 15 years. Even with this additional follow-up, more time may still be required before the study will have sufficient power to evaluate the carcinogenic potential of styrene exposure in humans.

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TABLE 1
POTENTIAL OCCUPATIONAL EXPOSURES

Adhesive makers	Polystyrene makers
Boat makers	Potting compound workers
Emulsifier agent makers	Protective coating makers
Insulator makers	Rubber makers
Organic chemical synthesizers	Sports car body makers
Petroleum refinery workers	Styrene workers
Plastic luggage makers	Swimming pool makers
Polyester resin laminators	Varnish makers

TABLE 2

EPIDEMIOLOGIC STUDIES OF WORKERS EXPOSED TO STYRENE

PRIMARY AUTHOR	YEAR PUBLISHED	STUDY POPULATION	CRITERIA FOR STUDY POPULATION	TYPE OF PLANT OR EXPOSURE	# OF DEATHS	RESULTS
Nicholson, W.	1978	560	employed for 5 years as of 5/1/1960	production of styrene and polystyrene	83	no significant excess deaths*
Frentzel- Beyme, R.	1978	1960	one month employment since 1931	production of styrene and polystyrene	74	no significant excess deaths
Ott, M.	1980	2904	> = 1 years employment 1937-1970	development and production of styrene-based products	303	lymphatic leukemia among a subgroup of polymerization, coloring, and extrusion workers
Ahlmark, A.	unpublished Sweden	1205	employed between 1950-1975	various styrene consumers but mainly reinforced plastic facilities	14	no significant excess cancer cases
Werner, J.	unpublished England	622	> = 1 years employment 1945-1974	production of styrene and polystyrene; manufacturing of finished products	34	significant excess of lymphoma deaths

* Authors note that data is suggestive of an excess risk for leukemia and lymphoma deaths when the cohort definition is expanded to include individuals with less than 5 years of employment as of 5/1/1960.

TABLE 3

COMPANY A - RANGE AND MEAN TWA STYRENE EXPOSURES
BY JOB WITHIN THE HIGH STYRENE EXPOSURE CATEGORY

Job Performed	Number Sampled	Styrene Concentrations (ppm)		
		Range	Mean	Std. Error
Hull Lamination	18	12.0 - 82.7	51.1	4.2
Deck Lamination	11	12.3 - 66.2	37.2	4.9
Small Parts Lamination	15	20.1 - 84.7	45.8	4.5
Gel Coat	4	22.5 - 44.9	34.2	4.8
Mold Repair & Patch	4	7.3 - 19.1	11.8	2.7
Model Development	1		53.7	
Composite	53	12.0 - 84.7	42.5	2.6

TABLE 4

PLANT B - RANGE AND MEAN TWA STYRENE EXPOSURES
BY JOBS WITHIN THE HIGH STYRENE EXPOSURE DEPARTMENTS

Job Performed	Number Sampled	Styrene Concentrations (ppm)			
		Range	Mean	Std. Error	
Hull Lamination	27	33.6 - 183.0	96.1	7.9	
Deck Lamination	12	31.4 - 58.3	44.7	3.0	
Small Parts Lamination	8	23.7 - 46.8	33.7	2.8	
Gel Coat	2	28.7 - 29.4	29.0	0.4	
Stringer Installation	8	33.6 - 156.0	106.0	16.2	
Overlay & Patch	6	10.4 - 61.3	31.1	7.7	
Model Development	4	63.1 - 88.5	76.6	6.8	
Composite	67	10.4 - 183	71.7	5.2	

TABLE 5

VITAL STATUS FOLLOW-UP AS OF DECEMBER 31, 1978

VITAL STATUS	ALIVE		DECEASED		UNKNOWN		TOTAL	
	#	%	#	%	#	%	#	%
WHOLE COHORT								
White Males	4025	77.4	167	3.2	77	1.5	4269	82.1
White Females	622	12.0	2	0.1	16	0.3	640	12.3
Nonwhite Males	235	4.5	7	0.1	8	0.2	250	4.8
Nonwhite Females	42	0.8	0	0.0	0	0.0	42	0.8
TOTAL	4924	94.7	176	3.4	101	1.9	5201	100.0
HIGH EXPOSURE								
White Males	1425	69.2	47	2.3	30	1.5	1502	72.9
White Females	399	19.4	0	0	12	0.6	411	20.0
Nonwhite Males	112	5.4	0	0	5	0.2	117	5.7
Nonwhite Females	30	1.5	0	0	0	0.0	30	1.5
TOTAL	1966	95.4	47	2.3	47	2.3	2060	100.0
LOW EXPOSURE								
White Males	2570	82.8	118	3.8	47	1.5	2735	88.2
White Females	216	7.0	2	0.1	4	0.1	222	7.2
Nonwhite Males	123	4.0	7	0.2	3	0.1	133	4.3
Nonwhite Females	12	0.4	0	0.0	0	0.0	12	0.4
TOTAL	2921	94.2	127	4.1	54	1.7	3102	100.0
UNKNOWN EXPOSURE								
White Males	30	76.9	2	5.1	0	0	32	82.1
White Females	7	17.9	0	0	0	0	7	17.9
Nonwhite Males	0	0	0	0	0	0	0	0
Nonwhite Females	0	0	0	0	0	0	0	0
TOTAL	37	94.9	2	5.1	0	0	39	100.0

TABLE 6
DATE OF FIRST EMPLOYMENT

YEARS	NUMBERS EMPLOYED	PERCENTAGE
1957* - 1959	121	2.33
1960 - 1964	394	7.58
1965 - 1969	1988	38.22
1970 - 1974	1643	31.59
1975 - 1978	1055	20.28

* Individuals employed prior to January 1, 1957 were considered first employed as of January 1, 1957

TABLE 7

DISTRIBUTION OF PERSON YEARS BY CALENDAR YEAR AND AGE

AGE	<u>CALENDAR YEAR</u>				TOTAL
	1959-64	1965-69	1970-74	1975-78	
15-24	285.3	2658.3	4052.5	2557.7	9553.6
25-34	508.6	2272.0	6347.6	8600.7	17728.9
35-44	451.4	1240.7	2373.5	3280.3	7346.0
45-54	264.5	996.6	1724.2	1894.4	4880.2
55-64	76.9	404.8	1010.4	1152.8	2644.9
65+	18.2	75.7	226.8	406.0	726.5
TOTAL	1604.8	7648.0	15735.4	17891.9	42880.2

TABLE 8

DISTRIBUTION OF INDIVIDUALS BY DURATION OF EMPLOYMENT AND
TIME SINCE FIRST EMPLOYMENT AS OF 12/31/78 IN HIGH EXPOSURE JOBS

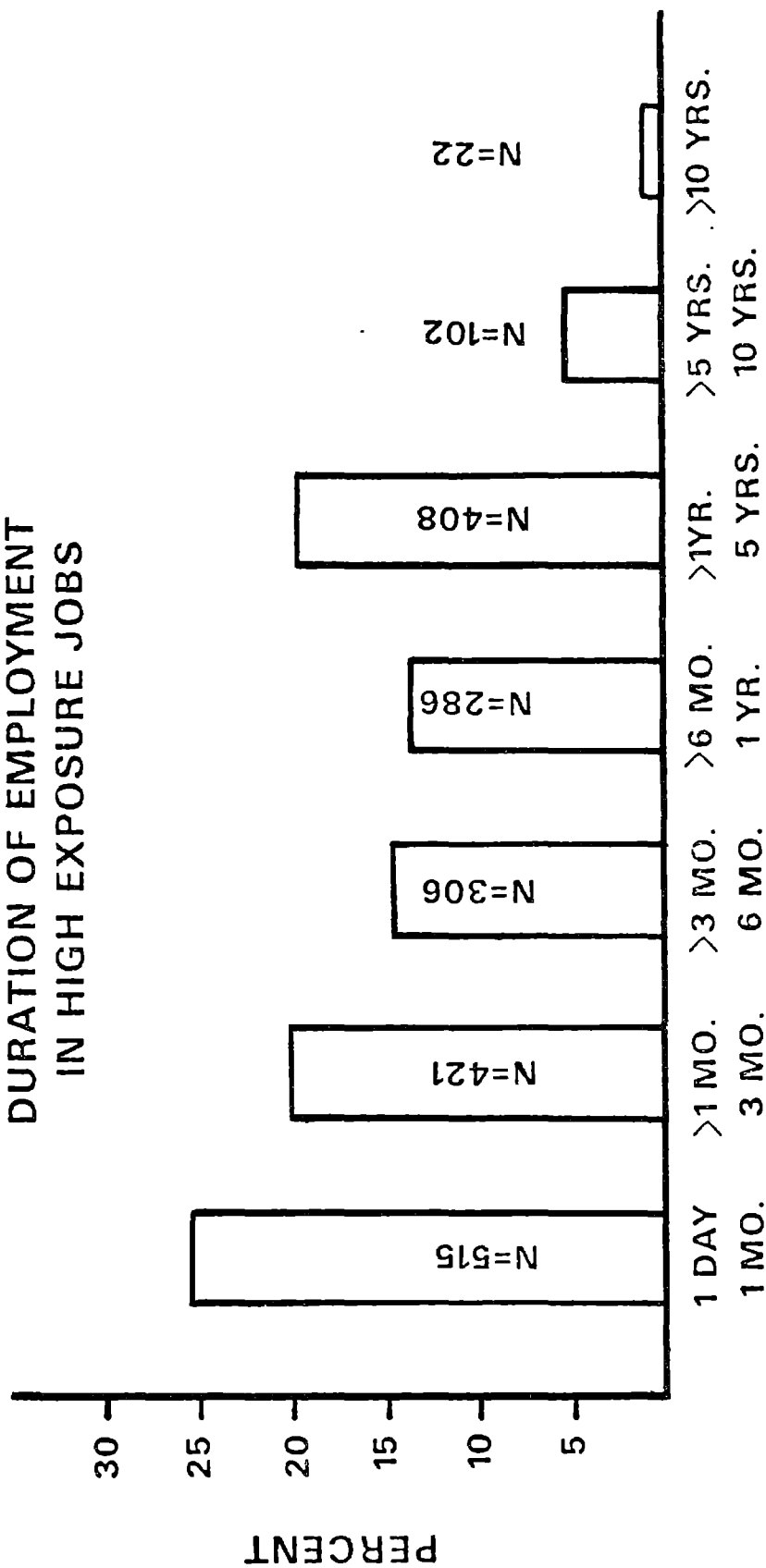
DURATION* FREQUENCY PERCENT	TIME SINCE FIRST EMPLOYMENT AS OF DECEMBER 31, 1978 (Years**)					Total
	0 - 5	5 - 10	10 - 15	15 - 20	20+	
1 day	2 0.10	4 0.19	3 0.15	0 0.00	0 0.00	9 0.44
2 - 7 days	34 1.65	40 1.94	47 2.28	4 0.19	0 0.00	125 6.07
8 days - 1 month	89 4.32	103 5.00	184 8.93	5 0.24	0 0.00	381 18.50
1 month - 1 year	318 15.44	326 15.83	343 16.65	26 1.26	0 0.00	1013 49.18
1 year - 5 yrs.	145 7.04	138 6.70	107 5.19	17 0.83	1 0.05	408 19.81
5-10 yrs.	0 0.00	53 2.57	33 1.60	10 0.49	6 0.29	102 4.95
10-15 yrs.	0 0.00	0 0.00	14 0.68	3 0.15	2 0.10	19 0.92
15-20 yrs.	0 0.00	0 0.00	0 0.00	1 0.05	0 0.00	1 0.05
20+ yrs.	0 0.00	0 0.00	0 0.00	0 0.00	2 0.10	2 0.10
TOTAL	588 28.54	664 32.23	731 35.49	66 3.20	11 0.53	2060 100.00

* Duration of employment

**Time Since First Employment (High Exposure Job) - surrogate measure for latency.

FIGURE I

DURATION OF EMPLOYMENT IN HIGH EXPOSURE JOBS



DURATION OF EMPLOYMENT

FIGURE II

TIME SINCE FIRST EMPLOYMENT HIGH EXPOSURE JOBS

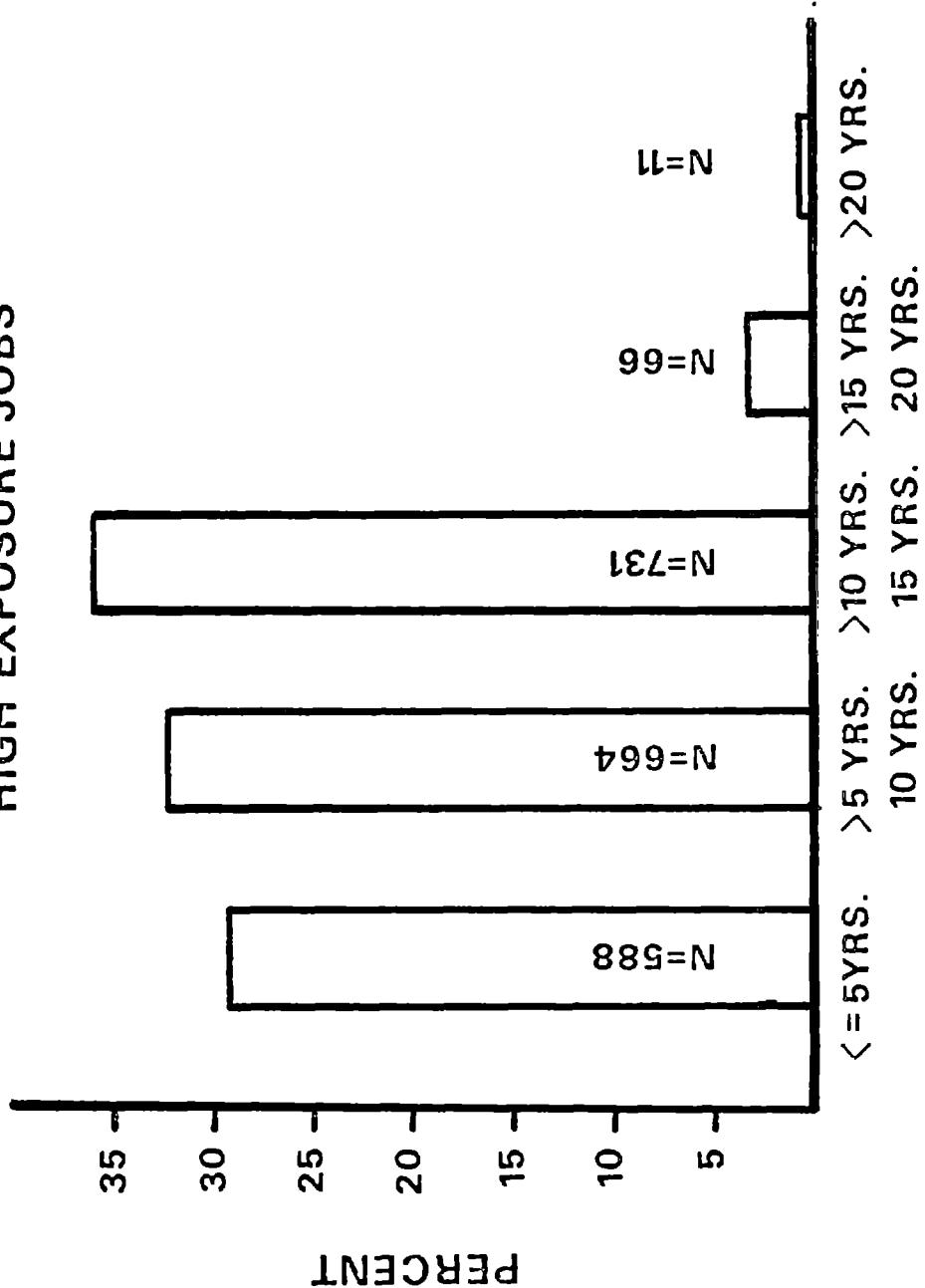


TABLE 9

COMPARISON OF OBSERVED AND EXPECTED DEATHS

CAUSE OF DEATH (ICD Code)	TOTAL COHORT			HIGH EXPOSURE			LOW EXPOSURE		
	OBS.	EXP.	SMR	OBS.	EXP.	SMR	OBS.	EXP.	SMR
All Causes	176 ⁺	195.3	90	47	41.5	113	127	148.8	85
Tuberculosis	1	0.5	188	1	0.1	1111	0	0.5	—
All Malignant Neoplasms	36	35.0	103	4	5.9	67	32	28.8	111
Cancer of digestive organs	8	8.4	95	1	1.1	87	7	7.2	84
Cancer of trachea, bronchus, and lung	16	11.2	143	1	1.4	71	15	9.7	155
Cancer of male genital organs	4	1.8	224	1	0.3	352	3	1.5	201
Cancer of female genital organs	1	0.4	268	0	0.2	—	1	0.2	557
Cancer of urinary organs	3	1.6	190	1	0.2	517	2	1.4	146
Cancer of other and unspecified	4	5.1	79	0	1.1	—	4	3.9	101
Leukemia	0	1.7	—	0	0.4	—	0	1.3	—
Lymphomas*	0	2.1	—	0	0.5	—	0	1.5	—
Other lymphatic & hematopoietic	0	0.4	—	0	**	—	0	0.4	—
Diabetes Mellitus	4	2.5	162	1	0.5	220	3	2.1	150
Psychoneurotic, Personality, and Mental Disorders	1	1.8	56	0	0.5	—	1	1.2	80

+Two deaths not included in either the high or low exposure groups

* Lymphosarcoma, reticulosarcoma, and hodgkins Disease

** < 0.1

TABLE 9 (continued)

COMPARISON OF OBSERVED AND EXPECTED DEATHS

CAUSE OF DEATH (ICD Code)	TOTAL COHORT			HIGH EXPOSURE			LOW EXPOSURE		
	OBS.	EXP.	SMR	OBS.	EXP.	SMR	OBS.	EXP.	SMR
Diseases of the Nervous System	7	8.5	82	2	1.2	162	5	7.2	69
Diseases of the Circulatory System	46*	64.3	72	9	8.3	108	36	55.1	65
Diseases of the Respiratory System	7	8.5	82	2	1.3	156	5	7.2	67
Diseases of the Digestive System	6	7.6	79	0	1.5	--	6	6.0	99
Accidents	38*	32.4	117	18	12.4	146	19	19.9	96
Violence	17	15.8	108	6	6.0	102	11	9.8	111
Unknown	8	3.1	260	3	0.8	368	5	2.2	223
All others	5	9.2	54	1	2.5	39	4	6.6	61

* One death not included in the high or low exposure groups.

TABLE 10
COMPARISON OF OBSERVED AND EXPECTED DEATHS
WHITE MALES HIGH EXPOSURE CATEGORY

CAUSE OF DEATH (ICD Code)	OBSERVED	EXPECTED	SMR	95% C. I.	
				Lower	Upper
All Causes	47	34.77	135	0.99	1.80
Tuberculosis	1	0.09	1111	0.15	61.82
All Malignant Neoplasms	4	4.63	86	0.23	2.21
MN digestive organs	1	0.93	107	0.01	5.98
MN lung	1	1.23	81	0.01	4.52
MN male genital	1	0.28	358	0.05	19.91
MN urinary organs	1	0.17	590	0.08	32.81
Diabetes	1	0.36	278	0.04	15.46
Diseases of the Nervous System	2	0.93	215	0.24	7.76
Diseases of the Circulatory System	9	7.35	123	0.56	2.33
Arteriosclerotic heart disease	8	5.96	134	0.58	2.64
Hypertension with heart disease	1	0.08	1250	0.16	69.55
Diseases of the Respiratory System	2	1.05	190	0.21	6.88
Accidents	18	11.14	162	0.96	2.55
Violence	6	4.64	129	0.47	2.81
Other Causes	1	2.00	50	0.01	2.78
Unknown Causes	3	0.81	370	0.74	10.82

TABLE 11
OBSERVED AND EXPECTED LUNG CANCER DEATHS
FOR THE LOW EXPOSURE GROUP

Latency† (Years)	DURATION OF EMPLOYMENT											
	6 mo		6 Mo		1 yr		5 yrs		10 yrs		15 yrs	
	obs	exp	obs	exp	obs	exp	obs	exp	obs	exp	obs	exp
5	2	1.4		0.3	1	1.1						
5-10	4	1.5		0.3	1	0.9	1	0.8				
10-15	2	0.8	1	0.2	2	0.5		0.3		0.5		
15-20		0.2		*	0	0.1		0.2	1	0.1		0.3
20+		*				*		*		*		*
TOTAL	8	3.8	1	0.9	4	2.6	1	1.4	1	0.6		0.3
											15	9.4

† Latency = Time since first employment

* < 0.1

TABLE 12

POWER TO DETECT EXCESS RISK OF DEATH: HIGH EXPOSURE GROUP

DEATH CATEGORY	NO MINIMUM LATENCY NO MINIMUM EXPOSURE		10 YR. MINIMUM LATENCY NO MINIMUM EXPOSURE		10 YR. MINIMUM LATENCY 1 YR. MINIMUM EXPOSURE	
	Expected		Expected		Expected	
	Deaths	Power	Deaths	Power	Deaths	Power
Leukemia						
RR=2	0.43	14%	0.07	08%	0.03	07%
RR=5	0.43	49%	0.07	16%	0.03	12%
RR=10	0.43	89%	0.07	32%	0.03	20%
Lymphoma*						
RR=2	0.54	15%	0.09	08%	0.04	07%
RR=5	0.54	57%	0.09	18%	0.04	12%
RR=10	0.54	94%	0.09	36%	0.04	22%

Latency = Time Since First Employment - High Exposure Job.

Exposure = Length of Employment - High Exposure Job.

* Lymphosarcoma, Reticulosarcoma, and Hodgkins Disease

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION I - EPIDEMIOLOGY
Chairman: Dr. Carl M. Shy
University of North Carolina

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. SHY: Could I ask the speakers to come up to the front so that the questions can be directed to them as they are visible to the audience? We have about 15 or 20 minutes for questions and we will take those now.

QUESTION: I have a question for Dr. Brown. The excess mortality from accidents in the Gold Mine Study: how many of those were on-the-job accidents, or was the excess in the finding on-the-job accidents?

DR. BROWN: We really haven't looked at them in that much detail. But just glancing at their certificates, there are a lot of obvious mine accidents--crushing, things like that. But we really haven't looked at that in detail.

There is another part of the accident group: falls. Falls are also at excess and I think some of those are also due to mining accidents.

It may not have been at Home State Mine where they had these mining accidents, but miners tend to migrate from mine to mine, so they could have occurred in other mines.

DR. SHY: Dr. Bresnick?

DR. BRESNICK: Two questions. One for the group that deals with the styrene business and the other for Dr. Brown. The first question is: what attempt was made to assess the quality of the styrene? For example, how much styrene oxide or what other types of contaminants are present in either the styrene or the butadiene?

MR. CRANDALL: We monitored for styrene and acetone. We attempted to sample for styrene oxide and the method that we used was not good. The results just didn't turn out. We didn't sample for fibrous glass or other potential exposures. Our main interest was in styrene, so we pretty much stuck to that.

DR. BRESNICK: The second question to the mining business again. As you know, with the asbestos group there is a tremendous synergistic effect upon bronchogenic carcinoma with smoking. Have you been able to sort out any type of a smoking plus the asbestos group that you are dealing with?

ANSWER: Unfortunately we don't have information on smoking for all these cohort members. There is some data on smoking from a silicosis study that was done back in 1960, but it is only a small part of the cohort that we looked at, that were included in this survey. So we don't really have that kind of data to look at any kind of synergistic effect. I would have to review it, but I think that among those in 1960 the smoking habits were about what you see with the U.S. in general. But we weren't able to look at any synergism.

DR. LANDRIGAN: Philip Landrigan, from NIOSH. I might be able to add something relevant to that point. We recently did a cross-sectional morbidity study at a small foundry up in Pennsylvania where there was heavy exposure to silica. The overall prevalence of silicosis in past and present workers was approximately 10 percent, with an 80 percent prevalence in workers with more than 20 years' employment exposure.

The company had an active medical screening program for the workers, and they had collected smoking history data on roughly three-fourths of the work force. We were able, therefore, to examine the prevalence of radiologically visible silicosis according to smoking history; smoking history was expressed both categorically (as yes/no) and also in terms of cumulative pack-years. We found that there was no relationship between the occurrence of silicosis and history of smoking. Furthermore, there was no correlation between severity of silicosis and history of smoking.

On the other hand, when we examined prevalence of chronic cough, phlegm production, and shortness of breath, there was clearly an interactive relationship, in which both smoking and silicosis contributed to the severity and frequency of these symptoms.

QUESTION: Well, what about with fibers?

DR. LANDRIGAN: There were no fibers involved in that study.

QUESTION: I know. This was a silicosis. But what might you expect with a fiber?

DR. LANDRIGAN: With classic asbestos fibers you would see interaction.

QUESTIONER: Right. Yes.

QUESTION: I would like to ask this question about the convention in use for reporting ratios, particularly specific mortality ratios in some of these studies. It has to do with the concept of significant figures.

In some of these studies the number of cases is really very low. For example, one case of leukemia was identified in one of these plants against an expected incidence of something like 0.11, and a specific mortality ratio of 11.11 for significant figures was presented. A ratio of that sort cannot possibly be as precise as is suggested by those tables.

I wonder if any of the speakers would care to comment on the convention of carrying nonintegral numbers out far beyond the number of figures that are justified by the data. Is this convention a general one and do you think that it suggests a greater validity than is justified by the findings?

DR. BEAUMONT: Your point is well taken. The reason we have confidence limits and P values is to associate some confidence with these numbers. I am not sure that there is a clear distinction between such ratios based upon small numbers and those based upon large numbers.

COMMENT: It seems to me there is a distinction to be made between something being not due to chance but still being chancy. If you have an observed of 2 over an expected of, say, 0.15, that may be statistically significant; but a change of 1 in the observed, say, from 2 to 1, would remove that significance. That is what I am calling chancy.

Some people feel that when an observed falls below, say, 4 or 5, and the expected is less than that, that one should not even report statistical significance -- maybe give the ratio, but asterisk and say that significance testing was not performed.

We frequently get into this problem of small numbers when we divide cohorts by length of employment and length of follow-up. The only way to handle the problem is to follow cohorts for a longer period of time.

DR. SHY: Further questions, please? Yes?

DR. WEISBURGER: I wasn't quite certain what you did about these fibers in Dr. Brown's study. Did you call them asbestos and they were not? Or, what was the situation?

ANSWER: We called them asbestos because they do meet the NIOSH definition for an asbestos fiber.

DR. WEISBURGER: Were they mineralogically and chemically asbestos? That is the question.

ANSWER: If you ask the U.S. Bureau of Mines, no. Or, if you ask IARC or some other agencies, no. But NIOSH bases it on health effects or potential health effects, not on the mineralogical definition.

DR. WEISBURGER: But isn't this confusing then?

ANSWER: Very.

DR. WEISBURGER: But why introduce a source of confusion?

ANSWER: It is a controversial subject as to what the definition of asbestos should be, and various groups have their own definitions. OSHA, I think, doesn't even consider cummingtonite-grunerite asbestos as fibrous. I am not sure about that. But it is a problem. That is why I described what it is physically. I guess it doesn't really matter what the definition is. What matters is what it is causing in terms of health effects.

COMMENT: I think there is reasonable compromise here, but I don't think you can say they are asbestos. I think you can call them "asbestos-like" or something else. But certainly I think the definition does have a meaning and you ought to stick to it. I mean, you don't call butadiene not butadiene. It's butadiene. If they are chemically defined or mineralogically defined, I think you have to call them that. I do agree with Elizabeth that you are introducing something here that is confusing. If you want to call them "asbestos-like" or call them fibers, I think that is fine.

QUESTION: Is there time for one more question?

DR. SHY: Yes, sir. There are ten more minutes.

QUESTION: Again, this is hammered at Dave Brown's data, I guess. You dismissed the radon contribution because it is roughly a third of what, I guess, the OSHA standard is. From what I saw, it appears to be rather alarming that since 1974 the contribution of radon was increasing, and increasing in some cases almost logarithmically from .01 to 0.1, for example. Well, albeit it's less, but it's going up.

DR. BROWN: It isn't really going up. It is a matter of how the surveys were conducted. The earlier surveys did not take into consideration where they took the sample. In the later surveys they took that into consideration and they took it nearer the ventilation intake, or the outtake.

It makes a big difference as to where you take the samples, and the earlier surveys did not take that into consideration. I don't think it is fair to say that the radon daughter levels are going up.

QUESTION: Well, from the values you showed me, it is fair to say that. But your qualification is in order.

DR. BROWN: Yes. And I do discuss that in the paper.

DR. SHY: Yes?

DR. WEISBURGER: I have a question about the boat building industry. I noticed that several of those people in the pictures were not wearing their respirators. They had them hanging down around their chests. What can be done about that sort of situation?

DR. LANDRIGAN: The proper way to protect those workers against styrene vapors is to ventilate their factories. Respirators should be used only as an ancillary means of protection.

QUESTION: How can you ventilate them if that's inside the hull?

DR. LANDRIGAN: You have to use a flexible local ventilation system. That is a problem throughout the industry, how to devise adequate local ventilation. Most of the plants use some sort of dilution ventilation, but in general it is not very effective for the workers who are working right at the mold. They are always within an arm's length of the mold's surface and that is where the highest exposures are found.

NIOSH has conducted a controlled technology study to assess the problem, and results are available from Mr. William Todd of NIOSH's Division of Physical Sciences and Engineering in Cincinnati.

MR. MEINHARDT: Mike, do you want to mention the dermal contact, the fact that many of these people do not only wear gloves --

MR. CRANDALL: Well, I will mention that styrene is absorbed by the skin and they do have a lot of skin contact with it. Protective equipment use was -- well, let me say that it was provided, respirators were provided, and for the most part protective clothing was provided -- aprons and gloves and that sort of thing.

But the workers either did not care to use it or they felt that they could do their job better without it. They felt they had a good feel for the surface, since there was a lot of smoothing out and making sure everything was good with the mold and all that. So they felt that they couldn't feel it if they had gloves on. So they did not wear them.

Protective equipment is a problem in the industry and, as Phil says, I don't know if it is the solution.

DR. SILVERSTEIN: Mike Silverstein, with the United Auto Workers. Since the question with respect to the boat building plant is relative, first, let me just comment that there are techniques of local exhaust ventilation that could have been applied fairly effectively to some of the work situations shown in the slides. I am interested in knowing whether or not there were epoxy resins used in those operations?

MR. CRANDALL: No. One of the problems with local ventilation is that the molds they use are movable. They start the process in one place and then they move it to another place and then they move it to another place -- two or three moves within the process -- and it would require a lot of, well, more than one system of local ventilation. If it were a stationary situation, it would be a lot easier to install local ventilation. But that is not to say they should not do that.

QUESTION: I have one question that perhaps Leslie Stayner could address and maybe Phil Landrigan might follow up on it. A common deficiency in these cohort studies was relatively small numbers of person-years of follow-up and, therefore, low power to detect effects which may not appear until perhaps ten more years of follow-up. And we may have ten such weak studies for every one that gives a strongly positive result.

The question is: what kind of priorities can we apply to select from among those ten studies those which we should definitely follow up? Or, are you saying that you are going to follow up every one of them in ten years?

MR. STAYNER: In the case of the phosphate study, I certainly see us following it up in ten years. I think in general that any study where the length of follow-up has been inadequate to give confidence in the findings should be updated periodically.

COMMENT: I want to point out that the one exception was the gold miners' study. We had an excellent chance of detecting a risk of excess lung cancer if it had really occurred in this population, even with restricting it to people with long employment and adequate latency.

DR. LANDRIGAN: The beauty of doing five-year or ten-year follow-up evaluations of occupational cohorts is that once you have defined the cohort membership and obtained Social Security numbers and other identifiers, it is relatively inexpensive and highly cost effective to simply put the numbers to sleep in the computer for a decade and then, like Rip Van Winkle, wake them up and see if they are still alive.

The cohorts that I suspect we will wish to re-examine will be those where the results are not already firm. I don't think, for example, that we would examine the gold miners again, but we would almost certainly take a second look at the styrene-butadiene workers.

We are continuing at the moment to follow up beryllium workers and benzene workers. Biologic plausibility, regulatory interest and the quality of the data all influence the decisions.

DR. SHY: Any further questions? If not, we thank the speakers for staying on their time and all of you for being a good audience.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Thursday Morning, March 22

SESSION II - EXPERIMENTAL STUDIES
Chairman: Dr. Frode Ulvedal
Environmental Protection Agency

SESSION II - EXPERIMENTAL STUDIES
Chairman: Dr. Frode Ulvedal, EPA

DR. ULVEDAL: We are ready to start the Second Session, which is on Experimental Studies.

The first speaker for the Second Session is Dr. Mike Waters, from EPA, and he will give us some information on interaction between normal human diploid cells and chemical carcinogens and mutagenicity in vitro.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Interaction Between Normal Human Diploid Cells and
Chemical Carcinogens/Mutagens In Vitro

M.D. Waters
Environmental Protection Agency

S.L. Huang
Environmental Health Research and Testing, Inc.
Research Triangle Park, N.C.

INTERACTION BETWEEN NORMAL HUMAN DIPLOID CELLS AND CHEMICAL CARCINOGENS/MUTAGENS IN VITRO

M. D. Waters

Genetic Toxicology Division, Health Effects Research Laboratory
U. S. Environmental Protection Agency, Research Triangle Park, NC

S. L. Huang

Environmental Health Research and Testing, Inc.
Research Triangle Park, NC

INTRODUCTION

The objectives of the present studies were to develop sensitive, reproducible methods for detecting mutations in normal human fibroblast cells and to demonstrate dose-related mutagenesis by known and potential carcinogens. We have modified conventional test procedures for detecting mutations in human fibroblasts (Jacobs and DeMars, 1977) to enhance the system's sensitivity. These modifications include the use of cell synchronization (Huang et al., 1981), multiple treatments (Huang et al., 1982), and exogenous metabolic activation (Huang and Waters, 1983).

In bacteria and viruses, mutagens act primarily on replicating DNA and preferentially at the replication point. They increase the mutation frequency of a given gene most effectively if administered while that gene is being replicated (Cerda-Olmedo et al., 1968; Baker and Tessman, 1968; Zampieri et al., 1968; Orkin and Littlefield, 1971; Burke and Fangman, 1975; Kee and Haber, 1975; Suzuki and Okada, 1975). Experiments with synchronized mammalian cells have indicated that DNA synthesis is required for mutation. Mutants resistant to 6-thioguanine (6TG) are induced by 5-bromodeoxyuridine only during early S phase (Aebersold and Burki, 1976; Rasko et al., 1976; Tsutsui et al., 1978). The occurrence of mutations appears to be related to the state of S phase, and the mutation frequencies to the number of S-phase cells in a mutagen-treated cell population. One objective of the present studies was to provide information about conditions for inducing mutations in synchronized cultures of human fibroblasts.

Metabolic activation is necessary to elicit dose-dependent responses in genetic bioassays of most mutagenic and carcinogenic chemicals (Miller, 1970; Heidelberger, 1975). Cell-mediated activation has been used in a few studies with human fibroblasts (Aust et al., 1980; Michalopoulos et al., 1981). Our studies with human fibroblasts have employed rat-liver S-9 microsomal activation (Huang and Waters, 1983). We assayed the activities of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 4-aminobiphenyl (4AB), with and without S-9 activation, by two different protocols: single treatment of synchronized cells and multiple treatment of nonsynchronized cells. We also assayed the suspected human carcinogens *N*-ethyl-*N*-nitrosourea, *N,N*-bis(2-chloroethyl)-*N*-nitrosourea, cyclophosphamide, and melphalan in nonsynchronized normal human fibroblasts, using multiple treatments.

In addition, we have used prolonged treatment with MMS to induce mutations in nonsynchronized human fibroblasts, and we have used the Luria-Delbrück fluctuation test (Luria and Delbrück, 1943) to determine the

mutation rate (Huang et al., 1982). The Luria-Delbrück fluctuation test has previously been used to detect the effects of weak mutagens or mutagens at low concentrations (Voogd et al., 1974; Cole et al., 1976; Green et al., 1976).

MATERIALS AND METHODS

Cell Lines, Cultures, Media, and Staining

Human fibroblast lines were derived from neonatal foreskins. Cells were grown in 85 vol of Ham's F10 medium (without hypoxanthine) and 15 vol of fetal bovine serum (FBS) with penicillin (100 IU/ml) and kanomycin (0.1 mg/ml) at 37°C in a 5% carbon dioxide atmosphere. The 6TG selective medium consisted of F10 and FBS with 7.5×10^{-5} M 6TG. Cloning efficiency was measured by inoculating 100 cells into a 60-mm petri dish, renewing the medium after one week, and, at two weeks, rinsing the cells with saline, fixing them with 10% formaldehyde, and staining them with 2% crystal violet.

Metabolic Activation

The S-9 fraction was prepared using 8- to 10-week-old rats (Sprague-Dawley, Madison, WI) primed with Aroclor 1254. Each 10 g of liver was homogenized in 20 ml of 0.25 M sucrose solution and centrifuged at 9000 g. The supernatant fraction (S-9) was collected and stored at -70°C. S-9 batches were prescreened for toxicity; the least toxic batches were used in the experiments. For single treatments, 10% S-9 was used, and for multiple treatments, 0.25% S-9. In methodological control experiments, cell death at these concentration averaged 60%. (The salts and cofactors caused no cell death at the concentrations used; see below.)

Single Treatment of Synchronized Cells (with Metabolic Activation)

Salts and cofactors (23.6 mg of potassium chloride, 22.6 mg of glucose-6-phosphate, 11.6 mg of NADP, and 16 mg of magnesium chloride hexahydrate) were dissolved in 0.4 ml of water. The treatment solution was prepared by mixing 0.4 ml of salts and cofactors, 1 ml of S-9, test chemical dissolved in 0.1 ml of dimethylsulfoxide, and 8.5 ml of F10 medium. Human fibroblasts were synchronized by the method of Milo and DiPaolo (1978). Logarithmically growing cells (approximately 1.5×10^6) were transferred to Dulbecco's modified Eagle's medium (MEM; Biolab, Chicago) lacking arginine and glutamine and supplemented with 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate. After 24 h, the cells were grown for 16 h in MEM supplemented with 10% FBS plus insulin (0.5 IU/ml). The cells progressed into S phase (Huang et al., 1981) and were exposed to the treatment solution for 4 h in incubators at 37°C in a 5% carbon dioxide atmosphere.

Determination of DNA Synthesis in Synchronized Cell Cultures

For determination of DNA synthesis, [^3H]thymidine was added to the above medium to a final concentration of 0.2 $\mu\text{Ci/ml}$. At intervals of 1, 2, 3, 4, 5, 6, 7, 8, 16, 17, 18, 20, and 22 h following the release of nutrient stress, cultures received [^3H]thymidine and were labelled for 1 h. The cells were then washed twice with saline and dissolved in 0.1 N NaOH. DNA in the

lysate was precipitated with 5% cold tetrachloric acid, and the lysate was filtered through a Whatman GF/C filter paper. The trapped DNA was washed twice with 5% cold tetrachloric acid and then with 95% ethanol. The filter paper was dried and then transferred to a vial containing toluene-based scintillation fluid. The counts for each culture were obtained using a Packard Tri-Carb scintillation counter.

Induction of 6-Thioguanine-Resistance Mutation in Synchronized Cell Cultures During Different Periods of the G₁ and S Phases

Approximately 1.5×10^6 cells were plated on 100-mm culture dishes, synchronized, and released from DNA-synthesis blockage. After intervals of 7, 8, 16, 17, 18, 19, 20, and 22 h, the cultures were treated with NA-AAF (4.2 $\mu\text{g/ml}$) for 1 h.

Mutagenesis with MMS in Cells Encompassing Different Cell Phases

After release from DNA synthesis blockage, the synchronized cells in 100-mm culture dishes were treated with MMS for 16 h. Treatment encompassed the G₁ and S phases (8 to 24 h after release), S and G₂ phases (16 to 32 h after release), and G₂ and G₁ phases (24 to 40 h after release).

Multiple Treatment of Nonsynchronized Cells (with Metabolic Activation)

The chemicals to be tested were dissolved in dimethylsulfoxide or acetone at a concentration of 1 mg/ml and diluted with F10 medium to give the final concentrations (ranging from 0.0015 to 80 $\mu\text{g/ml}$). Salts and cofactors (147.5 mg of potassium chloride, 141.5 mg of glucose-6-phosphate, 72.5 mg of NADP, and 100 mg of magnesium chloride) and 63 μl of S-9 (or more, depending on its toxicity) were added to each 25 ml of solution. Approximately 2×10^5 cells/dish were grown in FBS-F10 medium for 24 h and then exposed to the treatment mixture (5 ml/dish) for 4 h in an incubator at 37°C in 100% air. The cultures were given this 4-h treatment on each of four consecutive days. After each treatment, the cells were rinsed once with F10 medium and then cultured in regular medium.

Prolonged Treatment of Nonsynchronized Cells with MMS

Approximately 1.5×10^6 cells were plated on 100-mm dishes for 24 h and then treated daily with MMS in fresh culture medium, for a total treatment time of 24, 48, or 72 h. For longer treatment, a small number of cells were inoculated into culture dishes and exposed to MMS for five days.

Phenotypic Expression and Estimation of Mutation Frequency

After treatment, cells were cultured in FBS-F10 medium for seven days, with two passages. After expression, cells were pooled (five dishes per concentration) for determination of mutation frequencies. Approximately 10^4 cells were plated per 60-mm dish; 120 dishes were prepared for each dose. All dishes received 6TG at a final concentration of 7.5×10^{-5} M in 3 h. The 6TG selective medium was renewed once per week. At three weeks, the cells were rinsed with saline, fixed with 10% formaldehyde, and stained with 2% crystal violet. Mutant colonies were counted, and the mutation frequencies determined according to the Poisson expectation (Albertini and

DeMars, 1973; Maher and Wessel, 1975; Jacobs and DeMars, 1977, 1978; Huang and Lieberman, 1978). The mean number of mutant colonies per dish (x) was obtained according to $P_0 = e^{-x}$, where P_0 was the fraction of dishes bearing no mutants. The number of surviving cells per dish was determined by multiplying the initial number of cells per dish (10^4) by the cloning efficiency. Mutation frequency was calculated by dividing the mean number of mutant colonies per dish by the number of surviving cells per dish. Induced mutation frequency was obtained by subtracting the background mutation frequency from the treatment mutation frequency.

Estimation of Mutation Rates Using Luria-Delbrück Fluctuation Analysis

Cells were inoculated in 40 dishes (3000 cells/dish) and allowed to grow in the presence of MMS for five days, with daily renewal of MMS. The number of cells (N_1) was determined for each dish 7 to 9 days after inoculation, and 10^4 cells from each dish were transferred to new dishes. All the new dishes received 6TG at a final concentration of 7.5×10^{-5} M on the same day. The selective medium was renewed once per week. Dishes for estimation of cell cloning efficiency were fixed and stained after two weeks, and those for selection of mutants after three weeks.

Mutation events were assumed to be distributed among the culture dishes randomly (according to a Poisson distribution). The mean number of mutants per dish, x , was estimated as the negative log of P_0 . The average number of cell generations per dish, N , was obtained according to the Luria and Delbrück formula $N = (N_1 - N_0)/\ln 2$, where N_0 is the number of cells inoculated before MMS treatment and N_1 is the number of cells at the onset of selection. N_0 and N_1 were corrected for cloning efficiency before mutation rates were calculated. The mutation rate (a) was calculated using the Luria and Delbrück formula $a = x(\ln 2)/(N_1 - N_0)$.

RESULTS

Mutagenic Activities of 7,12-Dimethylbenz(a)anthracene and 4-Aminobiphenyl

Both DMBA and 4AB induced dose-dependent mutations in normal human fibroblasts in the presence of rat S-9 microsomes when administered by either single or multiple treatment (Table 1). The effective chemical concentrations differed between the two methods of administration. Multiple treatment of nonsynchronized cells caused more cell death than did single treatment of synchronized cells, and the effective doses were much lower. Thus, multiple treatment of nonsynchronized cells appeared to be the more sensitive method.

Prolonged Treatment of Nonsynchronized Cultures with MMS

Following repeated treatment with MMS, mutation frequencies in nonsynchronized human fibroblast cultures gradually increased (Table 2). Exposure to MMS for 24 h did not induce mutations in nonsynchronized cultures. However, after a three-day exposure, the increase in mutation frequency was statistically detectable, and a dose-response relationship was demonstrated (Figure 1). The Luria and Delbrück formula was used to calculate mutation rates for cultures exposed to MMS for five days.

TABLE 1. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY
7,12-DIMETHYLBENZ(a)ANTHRACENE AND 4-AMINOBIIPHENYL^a

Conc. (µg/ml)	Single treatment of synchronized cells			Multiple treatment of nonsynchronized cells		
	Relative cloning effic. (%)	Mean mutants per dish	Induced mutation freq. (x 10 ⁻⁶)	Relative cloning effic. (%)	Mean mutants per dish	Induced mutation freq. (x 10 ⁻⁶)
7,12-DIMETHYLBENZ(a)ANTHRACENE						
0	100	0.009	--	100	0.124	--
0.20				108	0.192	21.30
0.39				94	0.288	73.50**
0.78				105	0.341	79.80**
1.56				89	0.325	97.30**
3.13	95	0.017	2.47	61	0.452	248.80**
6.25	90	0.027	5.68			
12.50	90	0.073	19.43*			
25.00	80	0.077	23.58**			
50.00	63	0.146	59.85**			
4-AMINOBIIPHENYL						
0	100	0.008	--	100	0	--
3.13				85	0.025	11.5
6.25				99	0.051	20.1*
12.50				98	0.087	34.4*
25.00				84	0.118	54.5**
40.00	95	0.033	4.41			
60.00	65	0.042	15.54*			
80.00	80	0.060	18.58*			

^aLinear regressions: DMBA: single treatment, $\ln(y) = -0.27 + 1.13 \ln(x)$
multiple treatment, $\ln(y) = 4.55 + 0.75 \ln(x)$
4AB: single treatment, $\ln(y) = -6.34 + 2.15 \ln(x)$
multiple treatment, $\ln(y) = 1.61 + 0.75 \ln(x)$

*Significantly different from control, $p < 0.05$.

**Significantly different from control, $p < 0.01$.

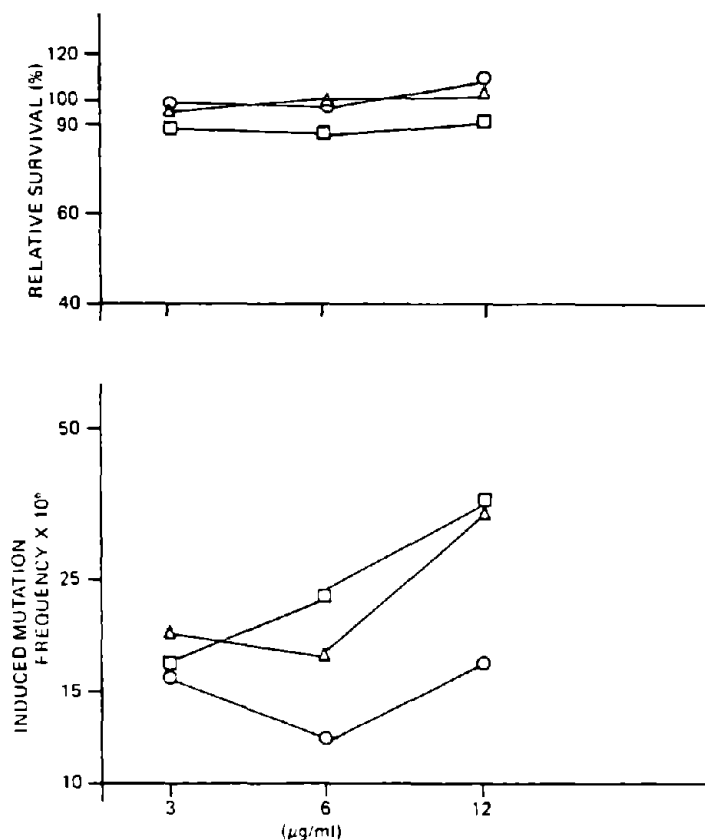


Figure 1. Mutagenesis and cytotoxicity in nonsynchronized human fibroblasts treated with MMS for 24 h (O), 48 h (Δ), or 72 h ($\square$). The linear regression for mutation frequency after 72-h treatment is $\ln(y) = 2.29 + 0.55 \ln(x)$.

continued DNA synthesis. MEM supplemented with 10% FCS plus insulin (0.5 IU/ml) released cells from G_1/S interphase blockage. New DNA synthesis began at 16 h after release and peaked at 20 h. As shown in Figure 3, the increase in DNA synthesis did not result from an increase in numbers of cells.

One-hour exposure of cells to NA-AAF at different stages of the cell cycle allowed observation of the temporal pattern of mutation. Exposure of cells during the G_1 phase produced no obvious mutations (Table 5); mutations were induced only during S phase. Mutations frequencies increased as S phase progressed, were highest when DNA synthesis reached a maximum, and declined after the midpoint of S phase, even though DNA synthesis continued in some portion of the cell population. MMS treatment starting during G_1 and including the entire S phase induced mutations in a dose-dependent fashion, as did treatment including S phase and G_2 phase (Table 6). However, treatment including G_2 and G_1 phases did not induce mutations. Induction of 6TG resistance was linear with increasing dose, and mutation induction

TABLE 2. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY REPEATED TREATMENT WITH MMS

Treatment period (h)	Conc. (µg/ml)	Cloning effic. (%)	P ₀	No. of mutants per dish	No. of viable cells per dish	Mutation freq. (x 10 ⁻⁶)	Induced mutation (x 10 ⁻⁶)
24	0	33.4	0.962	0.038	3674	10.47	--
	3	34.6	0.904	0.101	3806	26.56	16.09
	6	34.4	0.918	0.085	3784	22.50	12.03
	12	37.6	0.892	0.114	4136	27.59	17.12
48	0	33.8	0.973	0.028	3718	8.18	--
	3	34.0	0.900	0.105	3740	30.98	22.80
	6	35.2	0.909	0.095	3872	27.08	18.90
	12	38.8	0.836	0.179	4268	46.05	37.87
72	0	33.8	0.973	0.028	3718	8.18	--
	3	28.8	0.927	0.076	3168	26.22	18.04
	6	27.8	0.911	0.094	3058	33.64	25.46
	12	31.6	0.862	0.148	3476	46.85	38.67

Spontaneous mutation rates were estimated to be 4 to 20 x 10⁻⁷/cell per generation. Mutation rates were significantly higher in treated cultures than in controls, and induced mutation rates increased linearly with increasing concentrations of MMS (Figure 2). Prolonged treatment increased the fraction of dishes with mutants.

Multiple Treatment of Nonsynchronized Cultures with Four Suspected Human Carcinogens

All four agents caused dose-dependent mutations when administered in multiple treatments to nonsynchronized human fibroblast cultures (Tables 3 and 4). N-ethyl-N-nitrosourea was found to be a potent direct-acting mutagen, and N,N-bis(2-chloroethyl)-N-nitrosourea to be a weaker direct-acting mutagen. Both melphalan and cyclophosphamide required metabolic activation, and melphalan appeared to be the more potent mutagen. Neither melphalan nor cyclophosphamide caused dose-dependent mutation when administered to synchronized cells in a single treatment. The results obtained with the multiple treatment method confirm the mutagenicity observed with these agents in other genetic bioassays. Multiple treatment of nonsynchronized human fibroblast cultures appears to be a relatively sensitive method for testing mutagenicity in human cells.

Cell Synchronization

Dulbecco's MEM lacking arginine and glutamine and supplemented with 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate stopped most cell growth and decreased DNA synthesis; the cells were blocked in G₁/S phase within 24 h. However, as shown in Figure 3, some cells escaped blockage and

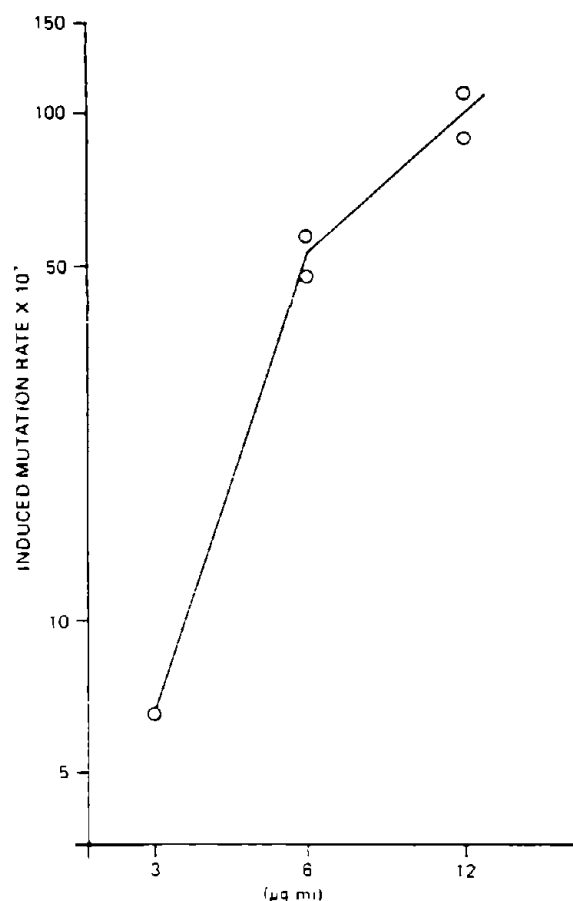


Figure 2. Mutation rates for human fibroblasts treated with MMS for five consecutive days, calculated by the Luria and Delbrück formula. The linear regression for mutation frequency after five-day treatment is $\ln(y) = 0.11 + 1.91 \ln(x)$.

followed one-hit kinetics. These findings indicate that incorporation of mutagens during DNA synthesis was required for mutation.

DISCUSSION

Synchronized human fibroblast cultures were obtained by the amino acid deprivation method of Milo and DiPaolo (1978). New DNA synthesis began 16 h after release from nutrient stress and peaked at 20 h. This permitted division of S phase into eight 1-h periods. The cellular effects of direct perturbation of DNA during various portions of the DNA synthetic period were examined. Mutation frequency was found to depend on the portion of S phase during which treatment was administered. Similar results have been reported for the HGPRT locus in Chinese hamster cells (Aebersold and Burki, 1976; Rasko et al., 1976) and Syrian hamster cells (Tsutsui et al., 1978). Treatment of S-phase human fibroblasts with MMS, NA-AAF, and MNNG induces

TABLE 3. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY
REPEATED TREATMENT WITH N-ETHYL-N-NITROSOUREA AND
N,N-BIS(2-CHLOROETHYL)-N-NITROSOUREA

Conc. ($\mu\text{g/ml}$)	Relative cloning effic. (%)	P_0	No. of mutants per dish	No. of viable cells per dish	Mutation freq. ($\times 10^{-6}$)	Induced mutation ($\times 10^{-6}$)
<u>N</u> -ETHYL- <u>N</u> -NITROSOUREA						
0	100	0	0	2866	0	0
0.78	97	0.975	0.025	2766	9.04	9.04
1.56	95	0.975	0.025	2713	9.22	9.22
3.13	91	0.950	0.051	2600	19.62	19.62
6.25	89	0.831	0.186	2547	73.03	73.03
12.50	91	0.808	0.213	2613	81.52	81.52
25.0	73	0.658	0.418	2100	199.05	199.05
<u>N,N</u> -BIS(2-CHLOROETHYL)- <u>N</u> -NITROSOUREA						
<u>Experiment 1</u>						
0	100	0.941	0.061	2373	25.71	0
0.006	89	0.928	0.075	2113	35.50	9.79
0.012	97	0.890	0.117	2313	50.58	24.87
0.024	94	0.858	0.153	2220	68.92	43.21
0.048	83	0.882	0.126	1980	63.64	37.93
<u>Experiment 2</u>						
0	100	0.983	0.017	3690	4.60	0
0.006	100	0.967	0.034	3680	9.20	4.6
0.012	81	0.922	0.081	2980	27.2	22.6
0.024	72	0.894	0.112	2670	41.9	37.3
0.048	78	0.928	0.075	2888	26.0	21.4

mutation (Huang et al., 1981). The present report and others show that only S-phase cells are sensitive to mutagens and that S-phase cells must be included in mutagenesis experiments (Taylor, 1960; Bader et al., 1963; Kikuchi and Sandberg, 1964; Mueller and Kajiwar, 1966; Mukherjee et al., 1968; Braun and Willis, 1969). Mutation can be induced in logarithmically growing human cells (Albertini and DeMars, 1973; Cox and Masson, 1974; Van Zeeland et al., 1974; Kuroda, 1975; Maher and Wessel, 1975; Jacobs and DeMars, 1978; Huang and Lieberman, 1978). Such cultures contain various proportions of S-phase cells. Cell culture synchrony was used here to accumulate larger numbers of S-phase cells for mutagen treatment and thus to increase the sensitivity of the assay.

TABLE 4. INDUCTION OF 6TG-RESISTANCE IN HUMAN FIBROBLASTS BY REPEATED TREATMENT WITH CYCLOPHOSPHAMIDE AND MELPHALAN

Conc. ($\mu\text{g/ml}$)	Relative cloning effic. (%)	P_0	No. of mutants per dish	No. of viable cells per dish	Mutation freq. ($\times 10^{-6}$)	Induced mutation ($\times 10^{-6}$)
CYCLOPHOSPHAMIDE						
<u>Without Activation</u>						
0	100	0.958	0.043	2293	18.8	--
0.014	105	0.992	0.008	2407	3.3	--
0.028	114	0.955	0.046	2620	17.6	--
0.056	103	0.992	0.008	2353	3.4	--
0.110	102	0.983	0.017	2340	7.3	--
0.220	116	1.000	0	2667	0	--
0.440	91	0.983	0.017	2080	8.2	--
<u>With S-9 Activation</u>						
0	100	0.983	0.017	2760	6.2	0
0.014	91	0.975	0.025	2500	10.0	3.8
0.028	100	0.974	0.026	2760	9.4	3.2
0.056	89	0.967	0.034	2460	13.8	7.6
0.110	82	0.967	0.034	2267	15.0	8.8
0.220	93	0.926	0.077	2573	29.9	23.7
0.440	83	0.918	0.086	2290	37.6	31.4
MELPHALAN						
<u>Without Activation</u>						
0	100	0.854	0.157	4030	39.1	--
0.00078	99	0.900	0.105	3980	26.4	--
0.00156	102	0.912	0.092	4110	22.4	--
0.00313	100	0.890	0.162	4030	40.2	1.1
0.00625	101	0.851	0.161	4080	39.5	0.4
0.01250	99	0.866	0.144	3990	36.1	--
<u>With S-9 Activation</u>						
0	100	0.954	0.047	2360	19.9	--
0.00078	89	0.951	0.050	2093	23.9	4.0
0.00156	78	0.925	0.078	1852	42.1	22.2
0.00313	82	0.914	0.090	1933	46.6	26.7
0.00625	83	0.908	0.097	1955	49.6	29.7
0.01250	81	0.838	0.176	1920	91.7	71.8

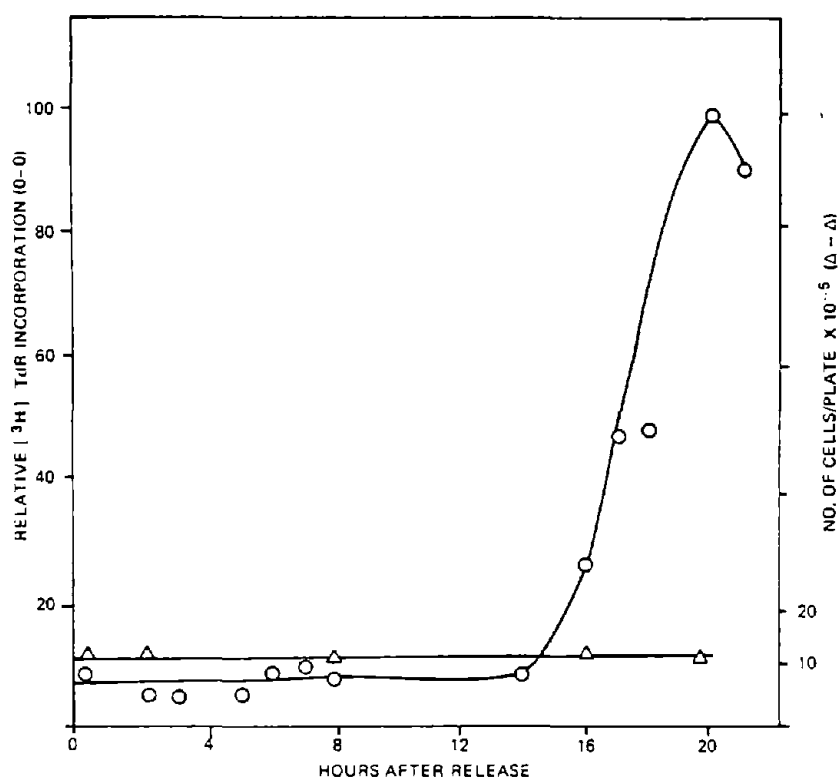


Figure 3. Relative DNA synthesis by human fibroblasts at different phases of the cell cycle after release from blockage.

TABLE 5. INDUCTION OF MUTATIONS IN HUMAN FIBROBLASTS BY NA-AAF DURING DIFFERENT PHASES OF THE CELL CYCLE

Hours after release	cpm	Number of cells/dish (x 10 ⁵)	Mutation frequency (x 10 ⁻⁶)	
			Control	Treated
7	263	1.31	3.9	0
8	215			0
16	804	1.44	11	29
17	1427	1.39		
18	1448			96
20	3065	1.39		127
22	2600		10	76

TABLE 6. INDUCTION OF MUTATIONS IN HUMAN FIBROBLASTS BY MMS DURING DIFFERENT PHASES OF THE CELL CYCLE^a

Cell phase	Conc. (µg/ml)	Survival (%)	Corrected no. of mutant clones	Total no. of viable cells (x 10 ⁵)	Mutants per viable cells (x 10 ⁶)	Induced mutation frequency (x 10 ⁶)
G ₁ + S	0	32.4	1.91	2.66	7.18	
	6.25	32.6	3.52	2.97	11.86	4.68
	12.50	28.8	6.47	2.53	25.58	18.40
	25.00	28.2	14.66	2.43	60.34	53.16
	50.00	22.8	16.18	1.98	81.72	74.54
S + G ₂	0	46.8	6.73	3.70	18.19	
	6.25	47.0	15.74	3.85	40.88	21.81
	12.50	47.4	23.62	3.93	60.10	41.91
	25.00	44.0	40.55	3.83	105.87	87.68
G ₂ + G ₁	0	21.6	5.08	2.38	21.36	
	3.13	19.0	5.74	2.09	27.44	
	6.25	20.2	0	2.22	0	
	12.50	12.4	0	1.36	0	
	25.00	27.6	0	3.04	0	

^aLinear regression: G₁ + S phases, $\ln(y) = -0.653 + 1.339 \ln(x)$
S + G₂ phases, $\ln(y) = -0.49 + 0.98 \ln(x)$

Our choice of methods for the present studies (single treatment of synchronized cells and multiple treatment of nonsynchronized cells) reflects previous experience with these and other combinations of culture growth state and treatment regimen. With synchronized cell cultures, our previous studies comparing single and multiple treatment have led us to discontinue work with the latter treatment regimen. The differential effects of chemical and/or concentration on cell cycling make it very difficult to predict, following multiple treatment, the time at which a synchronized culture will pass into S phase. With nonsynchronized cultures, we have observed that known mutagens are more likely to cause dose-dependent mutations when administered by multiple treatment. For example, three or more treatments with MMS are needed to elicit dose-dependent mutations in nonsynchronized cells (Huang et al., 1982). MMS is considered a weak mutagen and, in that respect, is representative of most environmental mutagens of interest. Thus, our work with nonsynchronized cells has increasingly emphasized multiple treatment.

As shown in Table 1, the effective chemical concentrations of DMBA and 4AB differed markedly between single treatment of synchronized cultures and multiple treatment of nonsynchronized cultures. Multiple treatment caused more cell death, and the effective doses were much lower. Because more

mutations were induced at lower concentrations, multiple treatment of nonsynchronized cells appears to be the more sensitive method. Results obtained with N-ethyl-N-nitrosourea, N,N-bis(2-chloroethyl)-N-nitrosourea, melphalan, and cyclophosphamide (Tables 3 and 4) tend to confirm that multiple treatment of nonsynchronized cells is the more sensitive of the two methods reported here. All four chemicals caused dose-dependent mutation when administered to nonsynchronized cells by multiple treatment, but two (melphalan and cyclophosphamide) failed to do so when administered to synchronized cells in a single treatment (Huang, unpublished data).

MMS induces 6TG resistance in CHO cells (Couch et al., 1978), mutations at the TK locus in mouse lymphoma cells (Cole and Arlett, 1978) and sister-chromatid exchanges and chromosomal aberrations in cultured cells (Palitti and Becchetti, 1977; Raff and Sugahara, 1980). MMS does not induce Oua-resistant variants in mouse lymphoma cells when tested by conventional methods (Cole and Arlett, 1978). However, the ability of MMS to induce Oua-resistance in mouse lymphoma cells was demonstrated through the use of the fluctuation test (Cole et al., 1976). This result was attributed to the effects of prolonged treatment. Similarly, in our experiments, MMS did not induce mutation in human fibroblasts when tested by conventional methods (Jacobs and DeMars, 1977), but prolonged treatment with MMS produced significant dose-dependent mutation, as did the use of the Luria and Delbrück experimental design. Using the conventional protocol, we were also able to demonstrate mutagenicity of MMS in synchronized human fibroblast cultures (Huang et al., 1981). These results are explained by the necessity of including S phase cells in mutagenicity assay. Both cell synchronization and prolonged treatment increase the sensitivity of the test system, by increasing the opportunity for S-phase cells to be exposed to the mutagen, thus increasing the number of mutants per culture.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Human Teratoma Cell Culture System for the Prescreening of Environmental
Chemicals That May Initiate or Promote Tumor Formation

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Human Teratoma Cell Culture System for the
Prescreening of Environmental Chemicals that
May Initiate or Promote Tumor Formation

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Abbreviations:

B[a]P, benzo(a)pyrene;

B[e]P, benzo(e)pyrene;

BPDE, 7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene;

CFC, colony-forming cells;

DMBA, 7,12-dimethylbenz[a]anthracene;

HGPRT, hypoxanthine-guanine phosphoribosyl transferase;

IMP, inosine-5'monophosphate;

MCA, 3-methylcholanthrene;

MNNG, N-methyl-N'-nitro-N-nitrosoguanidine;

PAH, polycyclic aromatic hydrocarbon;

TG, 6-thioguanine;

trypsin-EDTA, trypsin-ethylenediaminetetraacetic acid.

INTRODUCTION

The ultimate objective of many in vitro bioassays is to predict the carcinogenic risk to man of exposure to environmental chemicals. Because most chemical carcinogens are mutagenic, it is believed that the process of malignant transformation may be initiated by chemically inducing damage to the genes controlling cell growth and differentiation (Bouck and DiMayorca, 1973; Cairns, 1975; Huberman et al., 1976). Mutagenic activity as measured in in vitro assays, therefore, is widely used as an indicator of carcinogenic hazard from chemicals. Most mutagenesis assays use bacterial or rodent cells as targets; human cells, however, may respond differently to environmental chemicals. This differential response may arise from differences in cell permeability, carcinogen metabolism, or DNA repair. Therefore, the use of human cells as the targets in mutagenesis assays may enable more accurate predictions of risk to human health.

Various human fibroblast strains have been used in mutagenesis assays to detect the mutagenicity of direct-acting chemicals and radiation (Glover et al., 1979; Gupta and Siminovitch, 1978; Jacobs and DeMars, 1978; McCormick and Maher, 1981), as well as chemicals requiring metabolic activation in order to express their mutagenic activity (Aust et al., 1980; Michalopoulos et al., 1981; Strom et al., 1981; Tong et al., 1981). In general, human fibroblast strains exhibit low saturation densities and a limited lifespan in culture (Hayflick and Moorhead, 1961), factors that restrict their usefulness in routine testing. Human lymphoid cells, which do not have these limitations, have been developed by Thilly and associates (1980). However, since most human cancers are carcinomas, target cells derived from human epithelia could provide the most relevant data in mutagenesis assays. To achieve such a goal, we have isolated and characterized a cell clone, designated P3, from a human

teratocarcinoma cell line for use as targets in mutagenesis studies to identify potential carcinogens and elucidate the genetic events most likely to result in malignant transformation. Most chemicals are not carcinogenic per se but become carcinogenic after metabolic activation. However, the mammalian cells used as the target cells in mutagenesis assays are usually unable to metabolize carcinogens. To overcome this limitation, an exogenous metabolic activation system such as a liver microsomal or S9 preparation, is added. These activation systems, however, will not necessarily metabolize compounds in a manner analogous to that of intact cells (Bigger et al., 1980; Newbold et al., 1977; Selkirk, 1977).

Mutagenesis assays in which viable normal cells are used for metabolic activation yield a chemical activation resembling that observed in vivo (Huberman and Sachs, 1974; Newbold et al., 1977; Sebtì et al., 1982). Indeed, studies with polycyclic aromatic hydrocarbons (PAHs), nitrosamines, and aflatoxins have shown that results obtained in cell-mediated mutagenesis assays can reflect cell/organ specificity and the carcinogenic potency of the tested chemicals (Gould, 1980, 1982; Huberman and Sachs, 1976; Huberman and Slaga, 1979; Jones et al., 1981, 1983; Kuroki et al., 1982; Langenbach et al., 1978a,b, 1981a,b; Newbold et al., 1979; Raveh et al., 1982; Reiner et al., 1983). Cells growing attached to the surface of tissue-culture vessels are extremely useful in the cell-mediated mutagenesis assay, which requires contact between the target and metabolizing (mediating) cells for efficient transfer of the active intermediates to the target cells (Kuroki and Drevon, 1978). In the present study, human breast carcinoma BJ cells were incorporated into the P3 system to supply the necessary enzymes for chemical activation (Huberman et al., 1984).

METHODS

Cell Culture

The P3 cells, which exhibit an epithelial cell morphology, were derived from a clone isolated from a human teratocarcinoma cell line (Zeuthen et al., 1980) kindly supplied by Dr. P. Giovanella (The Stehlin Foundation for Cancer Research, Houston, TX). The epithelial BJ (BJ-015) cells, also with an epithelial cell morphology, obtained from a pleural effusion of a human breast carcinoma, were received from the Cell Culture Laboratory, University of California, Naval Bioscience Laboratory (Naval Supply Center, Oakland, CA). Cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum plus penicillin (100 units/ml) and streptomycin (100 µg/ml) (GIBCO, Grand Island, NY). Cell numbers were determined after the exclusion of nonviable cells, which were stained by trypan blue.

Determination of hypoxanthine-guanine phosphoribosyltransferase (HGPRT (E.C.2.3.2.8) activity. Cell-free lysates from the parental P3 cells and the TG-resistant cells were prepared according to the procedure described by Capechi et al. (1974). HGPRT activity was assayed according to Gillin et al. (1972) and the reaction products were separated by descending paper chromatography (Huberman et al., 1984).

Chromosome Analysis. Exponentially growing parental and mutant P3 cells were harvested after treatment for 1 h with a final concentration of 0.1 µg/ml Colcemid (Grand Island Biological Company, Grand Island, NY). The cells were fixed and stained for G-banding as described (Seabright, 1971). For each type of cell, a total of fifty well-spread metaphase cells were photographed and analyzed for chromosome number and chromosome abnormalities according to the Paris Conference (1972) and International System for Human Cytogenetic Nomenclature (1978).

Induction of 6-thioguanine resistance by chemically reactive mutagens or carcinogens and by chemicals activated in a cell-mediated assay. To test for the induction of TG-resistance by direct-acting mutagens or carcinogens, P3 cells were seeded in 100 mm Petri dishes (10^6 cells per 10 ml growth medium). Forty-eight hours later, the exponentially growing cultures were treated for three hours with different concentrations of the mutagens. In cell-mediated assays to detect proximate mutagens/carcinogens, 3×10^5 P3 cells were seeded over lethally irradiated BJ cells (10^6 per 60 mm Petri dish) seeded twenty-four hours earlier. The cultures were incubated for two days in medium containing the chemicals.

After the incubation periods, the cultures were dissociated with trypsin/EDTA and the cells seeded at 200 cells in 5 ml of growth medium per 60 mm Petri dish to determine the cloning efficiencies of control and treated P3 cells. These efficiencies are indicative of the toxic effect of the tested chemicals. Cells were seeded also into 10-20 Petri dishes at 3×10^5 cells in 10 ml of growth medium per 100 mm Petri dish to allow an optimal phenotypic expression of the induced mutations (Bradley et al., 1981; Diamond et al., 1982; O'Neill et al., 1977; van Zeeland and Simons, 1976). At different times after this cell seeding, the cultures were dissociated and seeded at 200 cells in 5 ml growth medium per 60 mm Petri dish to determine cloning efficiencies and at 2×10^4 cells in 5 ml of selective medium containing an optimal concentration of $40 \mu\text{M}$ TG per 60 mm Petri dish to determine the number of TG-resistant mutants. The cloning efficiencies were determined by counting the number of Giemsa-stained colonies in 6-8 dishes per type of treatment at 10 days after cell seeding, and the number of TG-resistant mutants was determined by counting Giemsa-stained colonies in 36-60 Petri dishes per type of treatment at 14-16 days after cell seeding. Mutation frequencies for

TG-resistance were calculated per 10^6 colony-forming cells (CFC), based on the cloning efficiency and the number of cells seeded for mutant selection.

RESULTS AND DISCUSSION

We have developed a human epithelial cell mutagenesis system that can be used to detect a variety of chemical mutagens. This system uses an epithelial cell-line, designated P3, which was cloned from a human teratoma cell-line (Zeuthen et al., 1980). The majority of the cells have a stable diploid karyotype with 46 (including XX) chromosomes (Fig. 1). Less than 10% exhibit a tetraploid or nearly tetraploid karyotype. The diploid karyotype contains one translocation between chromosomes 15 and 20 (Fig. 1), which remains stable. P3 cells grow well in culture with a cloning efficiency of $65 \pm 10\%$ and a generation time of 16 h.

The induction of mutations at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus, which is situated on the X-chromosome, renders the cells resistant to the nucleic acid analogue 6-thioguanine (TG) (Caskey and Kruh, 1979). The loss of HGPRT enzyme activity can arise from a variety of genetic insults at this locus including frameshift mutations, base pair substitutions, chromosomal aberrations, and deletions (Caskey and Kruh, 1979; Cox and Masson, 1978). We have tested the mutability of the P3 cells for TG-resistance by a number of chemical mutagens including benzo(a)pyrene [B(a)P] and N'-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Table 1). We analyzed a series of TG-resistant clones induced by MNNG or B(a)P and found that the level of HGPRT enzyme activity was reduced by more than 10-fold in these clones, compared to parental P3 cells (Table 2). Analysis of the karyotype of these mutants indicated that the mutant cells continued to express a karyotype essentially unchanged from that of the parental P3 cells,

including the translocation between chromosomes 15 and 20 (Table 3, Fig. 1). Therefore, the chemically induced resistance to TG is not associated with any detectable gross structural change in the X chromosomes in which the HGPRT gene is located.

We have sought to optimize the assay conditions for the most efficient recovery of TG-resistant mutants following chemical treatment. The time required for maximal expression of TG-resistance was investigated in P3 cells treated with B(a)P as activated by human breast carcinoma BJ cells in a cell-mediated assay, with 7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene (BPDE), the ultimate mutagenic metabolite of B(a)P and MNNG. The optimal expression time proved to be 6-7 days, irrespective of the type or dose of mutagen (Fig. 2). Other human cell types can have phenotypic expression times for the optimal expression of the resistance that differ from that determined for P3 cells (Jacobs and Demars, 1978; McCormick and Maher, 1981; Michalopoulos et al., 1981; Thilly et al., 1980). An interesting observation was the reduced detection of TG-resistance at expression times longer than two weeks. Similar observations at prolonged expression times were described also by others in human and Chinese hamster cells (Bonatti et al., 1978; Diamond et al., 1982; Jacobs and Demars, 1978). It was suggested that the decreased detection of the TG-resistant mutants is the result of their growth disadvantage relative to the parental cells (Bonatti et al., 1978; Diamond et al., 1982).

Human cells, including the P3 cells, are sensitive to the phenomenon of metabolic cooperation, i.e., the reduced recovery of mutant cells during selection in TG due to uptake by the mutant cells of the toxic phosphorylated TG product transferred from the neighboring wild-type cells by intercellular communication (van Zeeland et al., 1972; Bradley et al., 1981). This problem

can be avoided by seeding cells at low densities during the selection procedure. When P3 cells are seeded at densities below 2×10^4 cells/60-mm Petri dish, the effect of metabolic cooperation is minimized (Fig. 3).

The P3 cells do not contain the enzymes required to activate proximate chemical mutagens or carcinogens. Therefore, to enable this mutagenesis system to detect these chemicals as well, we have combined the P3 cells with other types of cells that contain the necessary enzyme systems. After cocultivating the P3 cells with other human cells (BJ cells), that are capable of metabolizing polycyclic hydrocarbons, we were able to show an induction of TG-resistant mutants by 7,12-dimethylbenz(a)anthracene (DMBA), 3-methylcholanthrene (MCA), B(a)P, and chrysene, with the degree of mutant induction directly related to the carcinogenic potency of the polycyclic aromatic hydrocarbons (PAH) (IARC, 1973). Pyrene and benzo(e)pyrene (B(e)P), which are not considered to be carcinogenic, were either inactive or exhibited a limited induction of TG-resistance (Table 4). We have also shown that P3 cells can be used to detect the mutagenic response of B(a)P in a cell-mediated assay with Syrian hamster embryo cells (unpublished results).

Currently, we are developing an hepatocyte-mediated assay (Jones and Huberman, 1980; Langenbach et al., 1978a), which will include P3 cells as the targets. Use of hepatocytes as metabolizing cells will broaden the scope of chemicals detectable by the P3 mutagenesis assay to include nitrosamines and aromatic amines. Recent studies have shown that P3 cells can also be used to detect genetic damage caused by both nonionizing and ionizing radiations. A preliminary action spectrum has been obtained for the induction of mutations, DNA-protein crosslinks, and DNA strand breakage in P3 cells exposed to near ultraviolet light (Jones et al., 1983; Peak et al., unpublished information). We are also developing a second marker of mutagenic damage,

namely the induction of resistance to diphtheria toxin. Preliminary studies suggest that this marker is a more sensitive indicator of mutagenic activity than the induction of TG resistance.

In conclusion, we believe that the human P3 epithelial cell mutagenesis assay constitutes a useful tool for the detection of genetic damage induced both by physical agents and by direct-acting and proximate mutagens or carcinogens.

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Table 1
Susceptibility of Parental and Mutant
P3 Cells to the Cytotoxic Effect of TG

Cell type	Relative percentage of CFC after treatment with different TG concentrations (μ M)			
	4	12	40	120
P3	2.8	0.3	< 0.03	< 0.03
NG-1 ^a	97	92	94	92
NG-2	92	93	100	95
NG-3	96	94	94	74
BP-1	101	100	97	67
BP-2	97	103	108	88
BP-3	98	102	95	87
BP-4	97	96	93	92
BP-5	98	95	99	73
BP-6	95	96	107	93

^aNG-1,-2,-3 cells are the progeny of TG-resistant colonies isolated from P3 cells after treatment with 7 μ g MNNG and BP-1,-2...-6 from P3 cells treated with 4 μ M B(a)P activated in a cell-mediated assay with BJ cells. The cells were used in the cytotoxicity studies after the mutant cells were subcultured for 4-6 weeks in the absence of TG. The cloning efficiency of the P3 cells was 69%, whereas the cloning efficiency of the mutant cells varied from 45-75%. These cloning efficiencies were considered as 100% for the relative percentage of CFC.

Table 2
HGPRT Activity in Parental P3 and
Six TG-Resistant Mutant Cells

Cell type	Specific activity (pmol/min/ μ g protein)
P3	4.6 $\pm$ 1.0
NG-1	0.2 $\pm$ 0.1
NG-2	0.2 $\pm$ 0.1
NG-3	0.3 $\pm$ 0.1
BP-1	0.1 $\pm$ 0.1
BP-2	0.2 $\pm$ 0.1
BP-3	0.2 $\pm$ 0.1

The origin of the NG-1,-2,-3 and BP-1,-2,-3 mutants is described in Table 1. The results are the mean $\pm$ SD of 7 measurements.

Table 3
Modal Chromosomal Number and Chromosomal
Changes in Parental P3 and Two
TG-Resistant Mutant Cells

Cell type	Cells counted	No. of chromosomes									
		44	45	46	47	48	53	86	87	90	92 (tetraploidy)
P3	50	0	2	41	4	0	1				2
NG-1	50	0	1	44	3	0			2		
BP-1	50	0	2	45	0	0		1		2	

Cell type	No. of metaphases analyzed	Karyotype
P3	8	46,XX,t(15p20p),t(15q20q),-15,-20,
NG-1	8	46,XX,t(15p20p),t(15q20q),-15,-20
	2	46,XX,t(15p20p),t(15q20q),-15,-20,del(4)(q26)
BP-1	6	46,XX,t(15p20p),t(15q20q),-15,-20

Table 4
Induction of TG-Resistant P3 Mutants
by Carcinogenic PAH Activated
in a Human Cell-Mediated Assay

PAH	Concentration (μ M)	No. of TG resistant mutants/ 10^6 CFC
Control	--	2 $\pm$ 2
Pyrene	4	2 $\pm$ 2
	12	5 $\pm$ 2
B(e)P	4	3 $\pm$ 2
	12	7 $\pm$ 3
Chrysene	4	12 $\pm$ 4
	12	24 $\pm$ 5
B(a)P	0.15	26 $\pm$ 5
	0.4	47 $\pm$ 8
	1.3	103 $\pm$ 32
	4.0	124 $\pm$ 60
MCA	0.15	45 $\pm$ 12
	0.4	188 $\pm$ 25
	1.3	196 $\pm$ 30
DMBA	0.05	56 $\pm$ 21
	0.15	106 $\pm$ 18
	0.4	211 $\pm$ 26
	1.3	181 $\pm$ 47

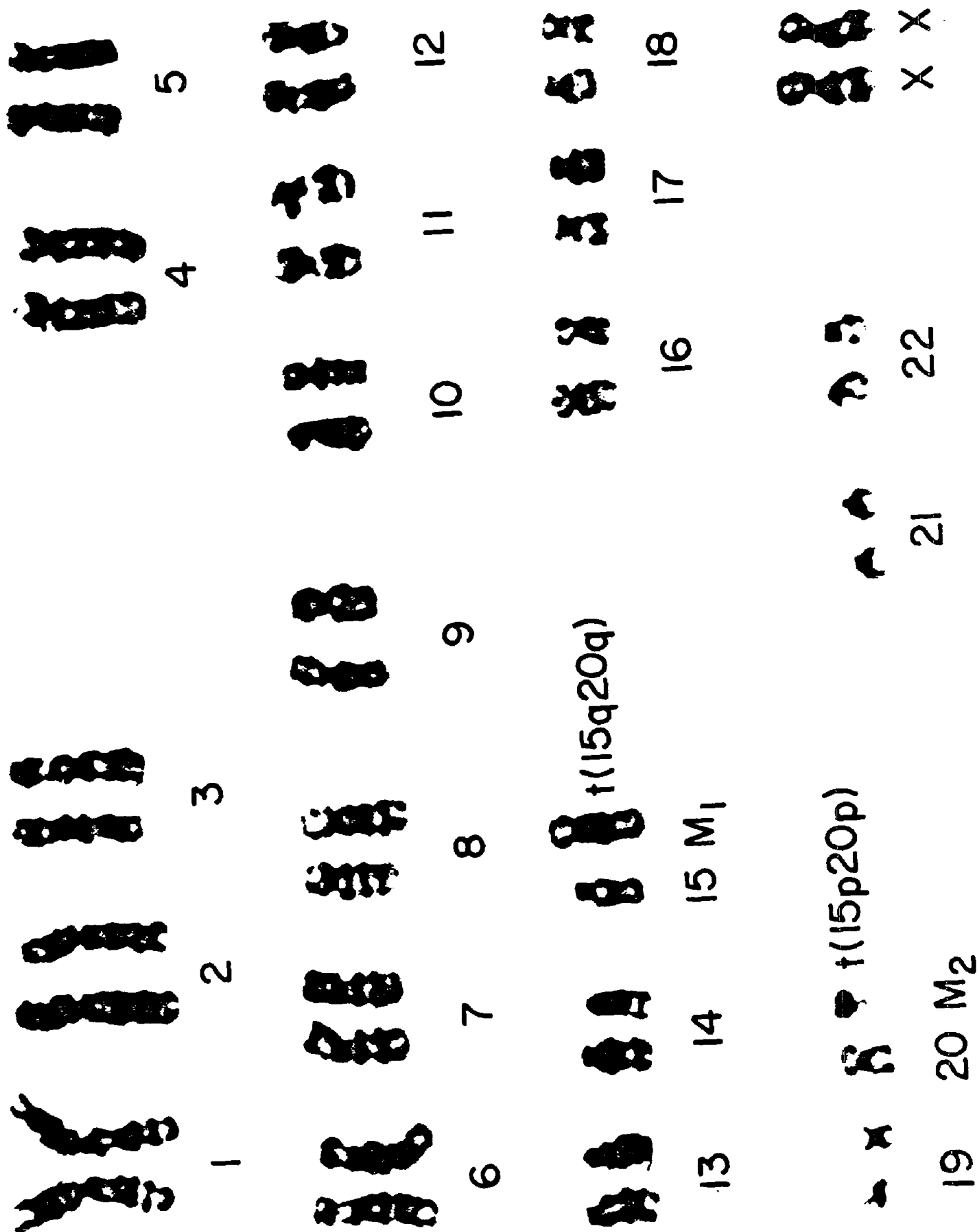
Two days after treatment with DMBA, BP, and MCA, there were $0.3-1.6 \times 10^6$ cells/Petri dish. The cloning efficiencies of these treated cells varied from 7-48%. In all other cases there were $1.4-1.8 \times 10^6$ cells/Petri dish and the cloning efficiencies of these cells varied from 31-52%. The results are the mean $\pm$ SD of 4-6 measurements.

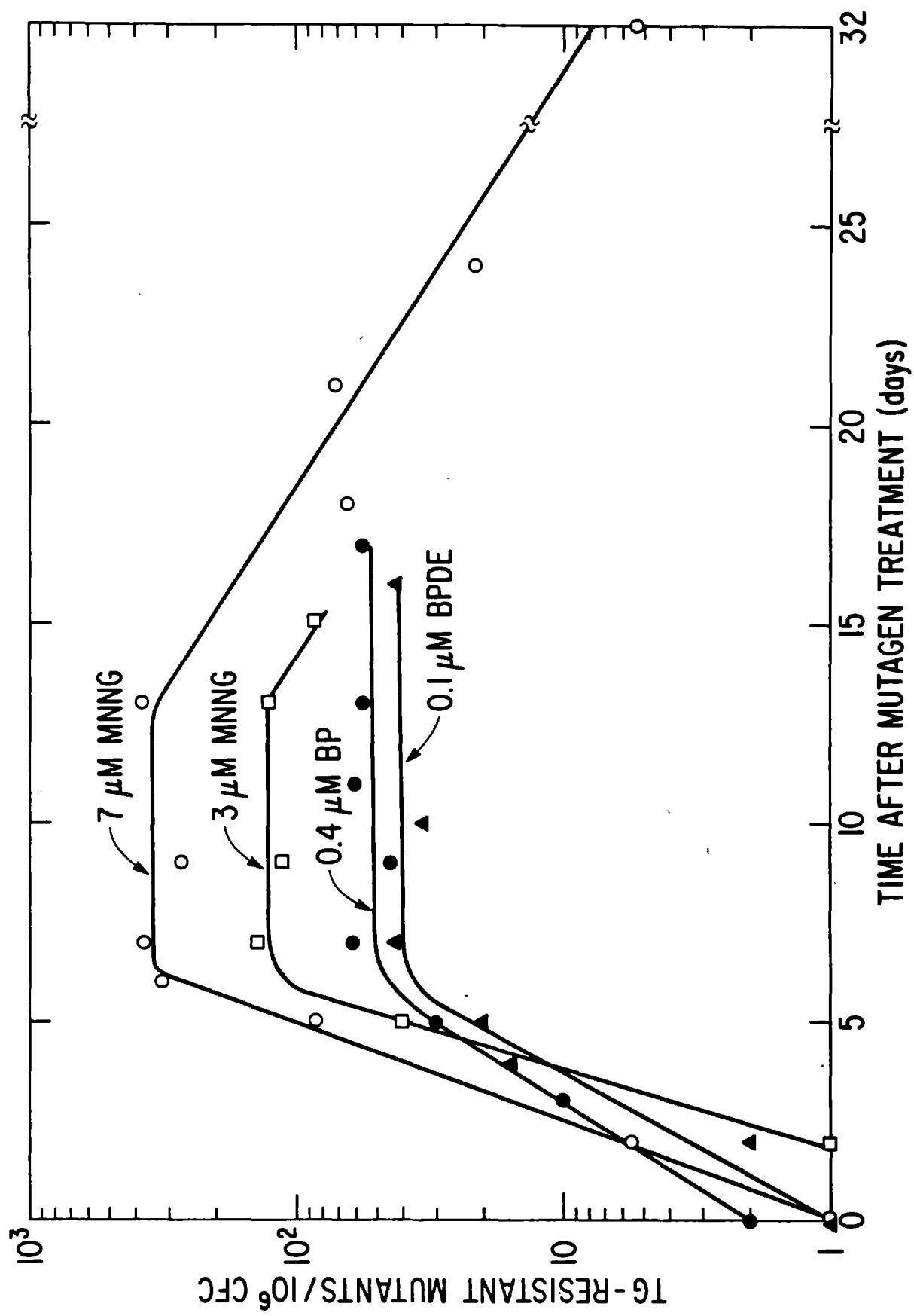
Legend to Figures

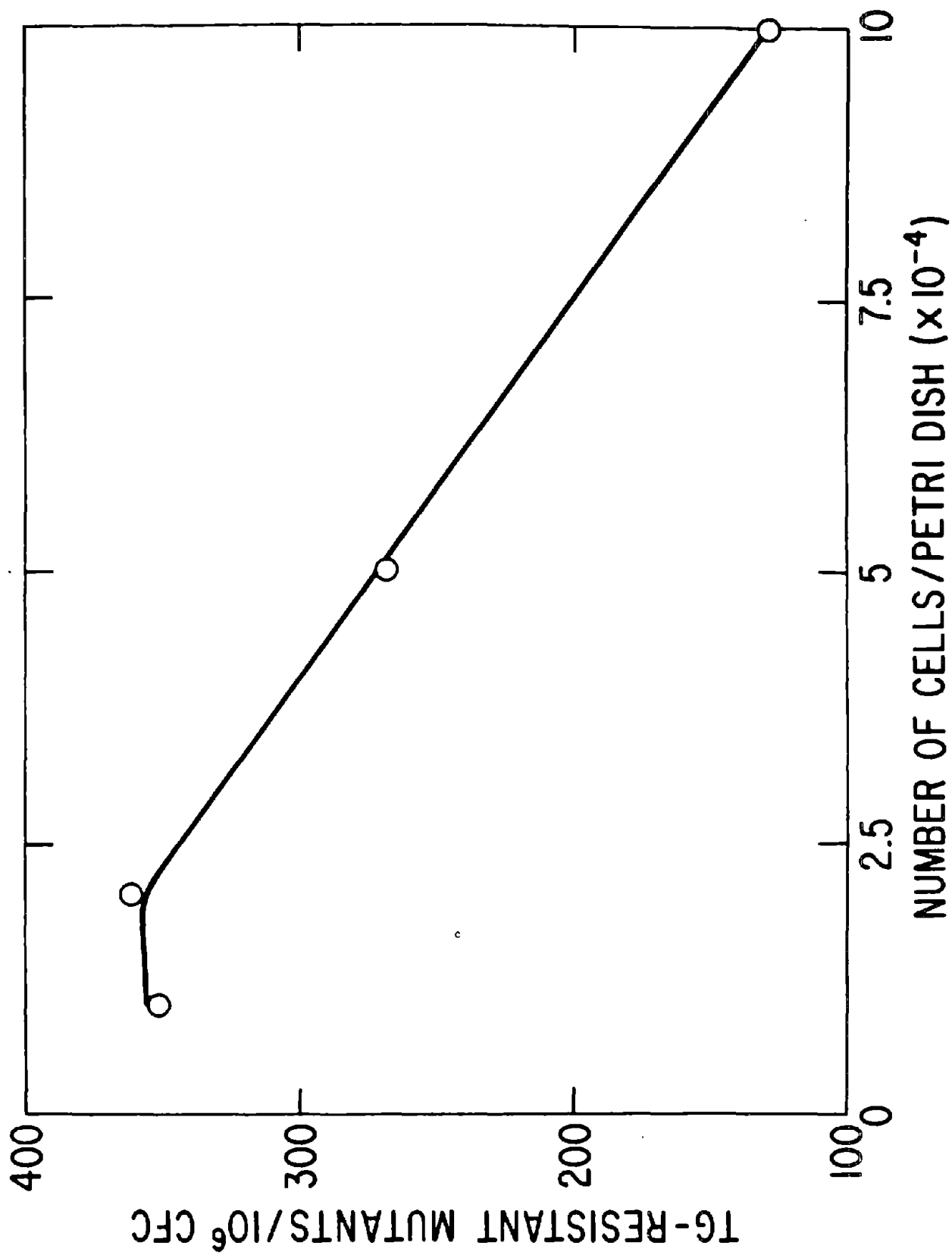
Fig. 1. Karyotype of untreated P3 cells.

Fig. 2. The recovery of TG-resistant mutants from P3 cells at different times after treatment with MNNG and BPDE and with B(a)P activated in a cell-mediated assay with the human breast carcinoma BJ cells. At different times after treatment, the control and mutagen-treated cells were seeded at 2×10^4 cells/60-mm Petri dish and incubated with TG. The cloning efficiencies of the control cells varied from 35%-72% and of the treated cells from 9-53%. The lower cloning efficiencies were detected in the early time points (0-4 day). The mutation frequencies of control cells varied from 1-2 TG-resistant mutant/ 10^6 CF.

Fig. 3. The recovery of TG-resistant mutants from P3 cells seeded at different cell densities. Seven days after treatment with 7 μ M MNNG, the cells were seeded at different cell densities and incubated with TG. The cloning efficiency of the control cells was 68% and of the MNNG treated cells 61%. Control cells yielded at a seeding level of 10^5 cells/60-mm Petri dish <1 mutant/ 10^6 CFC. At seeding levels of 5×10^4 and 10^4 cells/dish, 2 mutants/ 10^6 CFC were found; at the cell density of 2×10^4 cells/dish, 1 mutant/ 10^6 CFC was found.







PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Further Evaluation of the C3H 10T 1/2 Cell Transformation Assay
for Use in Screening Chemicals for Carcinogenic Potential

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Further Evaluation of the C3H 10T 1/2 Cell
Transformation Assay for Use in Screening
Chemicals for Carcinogenic Potential ^{1,2}

by

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1.0 Introduction

Over the past several years, considerable effort has been directed at establishing batteries of tests which together or individually offer reliable indications of the genotoxic potential of chemical and physical agents. Although in vivo bioassays have classically served to examine the carcinogenic potential of chemicals, the number of such agents and the financial burden imposed makes large scale in vivo screening untenable. In vitro test methods have therefore emerged to fulfill the need to screen large numbers of compounds in a relatively brief period of time, economically, yielding reliable data which can identify potentially hazardous chemicals and aid in establishing priorities for in vivo bioassays. The available in vitro test systems can be broadly categorized as (a) those which detect mutagenic or chromosomal alterations in prokaryotic or eukaryotic cells, (b) those which assess physical or chemical interaction between the test agent and cellular macromolecular targets (e.g. DNA), and (c) those which examine the potential oncogenic activity of agents by induction of neoplastic transformation of mammalian cells in culture. Experimental evidence is continuously being compiled to verify the usefulness of such in vitro screening tests; indeed, a positive correlation between the in vivo carcinogenicity of many chemicals and their capacity to elicit responses in a variety of in vitro systems has been documented (Kolber et al., 1983; IARC, 1980; Mishra, et al., 1980; de Serres and Shelby, 1981).

In a comprehensive review of short-term test methods by Hollstein and co-workers (1979), approximately one hundred different model test systems were identified as having been used, to varying degrees, for assessing the carcinogenic and/or mutagenic potential of chemicals. These systems encompassed eight general areas, i.e. assays with prokaryotic micro-organisms, assays with eukaryotic micro-organisms, mutagenesis assays with mammalian cells, assays measuring DNA repair and DNA replication, assays with insects, in vivo and in vitro mammalian cytogenetics assays, and in vivo mammalian tests. In some instances, limited numbers of chemicals have been tested and in many cases they have been restricted to well-characterized and direct-acting agents. Furthermore, methodological approaches used in a given test often differed, among laboratories. Consequently, in the absence of standardized procedures, inter-laboratory reproducibility may suffer for any given test. Despite these shortcomings, such retrospective reviews and data compilations serve as valuable resources for assessing the efficiency of specific tests in identifying genotoxins. Still, tests being performed on agents for which full knowledge of in vivo carcinogenic activity is available can introduce a bias in data evaluation. To circumvent such limitations, prospective screening programs have been organized which mandate the "blind" testing of coded compounds in order to establish objective assessments of the validity and reliability of specific in vitro and in vivo short-term tests. Some of these systems, originally developed for other purposes such as mechanistic studies of

genotoxicity, have had to undergo methodological changes to accommodate screening programs and/or to enhance their sensitivity to diverse genotoxins. For any test of this nature to serve as a valid indication of the carcinogenic potential of chemicals it is essential that (a) the methodology be well defined and standardized, (b) the results (both intra- and inter-laboratory) be reproducible, (c) the system be evaluated against a broad spectrum of chemicals of diverse structure and biological activity, (d) the data derived be quantifiable, and (e) in vivo relevancy be demonstrated.

Efforts sponsored by various federal agencies and the private sector have been ongoing to evaluate the suitability of in vitro transformation as a relevant endpoint which parallels in vivo responses to chemical carcinogens and effectively discriminates between carcinogens and non-carcinogens, irrespective of the mechanism(s) by which the aberrations(s) is manifested (Sivak and Tu, 1982a). most widely used mammalian cell transformation systems has been the C3H 10T 1/2 clone 8 mouse embryo fibroblast cell line, developed by Reznikoff, et al, (1973 a,b). This system has been employed to examine a number of mechanistic questions regarding carcinogenesis, such as initiation/promotion (Mondal, et al, 1976; Kennedy, et al, 1978; Boreiko, et al, 1982), the effect of vitamins (Benedict, et al, 1980; Bertram, 1980), cell phase dependence of oncogenic transformation (Bertram and Heidelberger, 1974), and correlations between neoplastic transformation and other genotoxic lesions (Landolph and Heidelberger, 1979; Gehly, et al, 1982). Despite

the extensive use of the C3H 10T 1/2 cell system for mechanistic studies, its utility as a reliable screen for potential carcinogens has been examined only recently through a major collaborative effort between two independent laboratories, i.e. Microbiological Associates and Arthur D. Little, Inc.

The purpose of the program reported herein was to evaluate the utility and reliability of an in vitro transformation assay using C3H 10T 1/2 clone 8 mouse embryo cells as a candidate for one of a battery of short-term assays for the initial determination of the carcinogenic potential of chemicals. The intra- and inter-laboratory reproducibility of the system was assessed through this collaborative effort. Part of the effort was also devoted to modifying and improving the standard C3H 10T 1/2 cell transformation assay (Reznikoff, et al, 1973 a,b) in order to expand its responsiveness to a broad spectrum of xenobiotics.

2.0 Characterization of the Standard C3H 10T 1/2 Cell Assay

2.1 Studies with Model Compounds

The intra- and inter-laboratory reproducibility of the basic C3H 10T 1/2 cell transformation assay (Figure 1) was demonstrated using the polycyclic aromatic hydrocarbon (PAH) 3-methylcholanthrene (3-MC). Positive dose responses were obtained for the induction of transformed foci (Schechtman, et al, 1982a). Similarly, the responsiveness of the C3H 10T 1/2 cell system to a set of known model compounds was evaluated

collaboratively (Schechtman, et al, 1982a). Positive responses were obtained with the known PAH carcinogens 3-MC, 12-dimethylbenz(a)anthracene (7,12-DMBA), and benzo(a)pyrene (BaP). The response to the PAH dibenz (a,h)anthracene (DB{a,h}A), however, was marginal. The two non-carcinogenic PAH's phenanthrene and anthracene were not active in this transformation system. The potent short-lived direct-acting alkylating agent N-methyl-N'nitro- N-nitrosoguanidine (MNNG) induced little to no focal transformation using the standard assay. Induction of oncogenic transformation by this compound has been shown to be cell cycle dependent (Bertram and Heidelberger, 1974; Tu and Sivak, 1980a). C3H 10T 1/2 cells were also non-responsive to certain agents requiring metabolic activation, such as the aromatic amines 2-fluorenylacetamide (2-FAA) and 2-aminoanthracene, and the nitrosamines dimethylnitrosamine (DMN) and diethylnitrosamine (DEN). These results suggested major limitations associated with the archetypal C3H 10T 1/2 cell assay as published (Reznikoff, et al, 1973 a,b) when subjected to diverse classes of chemicals. This was apparent both with direct-acting agents as well as those requiring metabolic conversion to bioactive intermediates.

2.2 Studies with Coded Compounds

Subsequent to these preliminary studies, in-depth validation of the C3H 10T 1/2 cell transformation assay was attempted using a series of coded chemicals. The initial set of coded samples issued for "blind" testing independently by both collaborating

laboratories consisted of 30 chemicals, of which, when the code was broken, 20 were considered to be carcinogenic in vivo, 8 were considered non-carcinogenic in vivo, and 2 were of undetermined in vivo activity at the time these agents were tested in vitro. Specific details and complete assay data for all compounds can be found in Tu and Sivak (1983) and Schechtman, et al (1983). The criteria employed for determination of the validity of a given test are listed in Table 1. Criteria established for a positive response, a negative response, suspect activity, or a "no test" are listed in Table 2. Based on these criteria, the comparative responses obtained for those samples which satisfied the criteria for a valid test were evaluated for inter-laboratory agreement and for corroboration of in vivo carcinogenic activity. The results in Table 3 show that of the 20 carcinogens tested, less than 50% elicited a clear positive transformation response in either lab using the established C3H 10T 1/2 cell transformation assay. A dichotomy in results between the collaborating laboratories became apparent when the "positive" and "suspect" categories were considered together. Specifically, 45% (20 valid tests) of the known carcinogens were identified as inducing either positive or suspect activity in lab A, whereas 85% (13 valid tests) were identified as either positive or suspect agents in lab B. Additionally, examination of the responses to the known non-carcinogens revealed a similar dichotomy (Table 3.) Thus, of the 8 non-carcinogens tested, 25% of the agents were observed to evoke suspect activity in lab A, while 67% were categorized as suspect or positive in lab B. Of the 2 compounds

of unknown in vivo activity, no agreement could be reached between laboratories regarding their activity in C3H 10T 1/2 cells. Taken together, these data indicated that the established C3H 10T 1/2 cell transformation assay of Reznikoff, et al (1973b) was apparently insensitive to numerous carcinogenic chemicals, with respect to their ability to elicit unequivocal responses. The extent to which the C3H 10T 1/2 cell system could identify non-carcinogens as such is probably a reflection of the fact that typically the results obtained tended toward being negative, lending a bias in this direction. Overall, then, it became apparent that for the C3H 10T 1/2 cell transformation assay to serve as a screen for detecting chemical carcinogens of a diverse structure and biological activity, major revisions of the established assay would be required.

3.0 Assay Modifications

To enhance the sensitivity of C3H 10T 1/2 cells to a broader spectrum of chemicals, efforts in both laboratories were directed toward modifying the basic transformation assay protocol. These procedural modifications were then evaluated in order to determine which would be suitable for routine screening of genotoxins. Independent endeavors of the two participating labs were focused on several main areas. These included (a) variations in target cell density for chemical exposure, (b) increased duration of treatment, (c) synchronization of target cells prior to treatment, (d) delayed treatment of plated target cells, (e) amplification of phenotypic transformation by delayed

replating of treated target cells, (f) addition of exogenous metabolic supplements (intact hepatocytes and hepatic 9000 X g post-mitochondrial supernatant (S-9) preparations).

3.1 Target Cell Density

Although the archetype C3H 10T 1/2 cell transformation assay called for a cell seeding density (prior to treatment) of 1×10^3 cells/dish, subsequent studies (Haber, et al, 1977) demonstrated that focus transformation was inversely related to cell density. Thus, for the PAH BaP, transformation frequency (TF) expressed in terms of the number of transformed foci per viable cell was found to decrease with increasing plating densities over the range of 1×10^3 to 4×10^4 cells/dish. Studies among the collaborating laboratories for the effort reported herein have examined this parameter in depth (Tu and Sivak, 1980b; Schechtman, et al, 1982a). It was determined that for the PAH 3-MC, moderate increases in the absolute number of transformed foci were dependent upon the target cell seeding density over the range of 1×10^3 to 1×10^4 cells/dish. When expressed in terms of number of transformed foci per dish, the TF was invariably higher (2-10-fold) at the plating density of 1×10^4 cells/dish, despite the decline in TF expressed on the basis of viable cells at this higher density (Tu and Sivak, 1983). Similar results were independently observed by the other participating laboratory in this joint effort. Further studies suggested a plateau of TF (foci/plate) was achieved at $\sim 1 \times 10^4$ cells seeded/dish, with a decline in TF at densities exceeding this optimal level. It was

the consensus, based upon these experiments, that for the purposes of screening unknown potential genotoxins, it was essential to optimize assay conditions to allow for the expression of the maximum number of transformed foci. Consequently, a majority of the experiments in both participating laboratories were performed with a target cell seeding density of 1×10^4 cells/plate.

3.2 Treatment Period

The influence of exposure time was examined for the PAH 3-MC and for the metal salt beryllium sulfate. The treatment periods ranged from 24-72 hours in 24-hour intervals. For 3-MC (2, 5, and 10 ug/ml), it was determined that virtually all of the responsiveness of the C3H 10T 1/2 cells to the transforming effects of this PAH were manifest after the first 24 hours of treatment for both Types II and III foci. Additional exposure (48 or 72 hours) failed to enhance the TF any further (Tu and Sivak, 1983). For the metal salt, however, some differences in focal responses were encountered which appeared to be time dependent. A 24 hour treatment yielded a total of 3 Type III foci in 71 dishes (all treatment groups, 200 - 12.5 ug/ml in 2-fold dilutions); however, numerous Type II foci were present in every treatment group (69 Type II foci/71 dishes, spontaneous background of 3 Type II foci subtracted). Interestingly, with a 72-hour treatment the total yield of Type II foci declined (9 Type II foci/42 dishes) and the total Type III focus yield increased (14 Type III foci/42 dishes), although the difference

was insufficient to account for a transition (maturation?) of the Type II to the Type III focus phenotype (Schechtman, et al, 1983).

Other investigators have reported it to be advantageous to extend the C3H 10T 1/2 cell treatment period to 6 days in order to induce focus transformation by certain chemicals, such as aflatoxin B₁, benz(a)anthracene and 4-nitroquinoline-1-oxide (Oshiro and Balwierz, 1982). This technique was not successful, however, with the procarcinogenic aromatic amines 2-FAA and 2-naphthylamine, both of which have since been shown to require exogenous metabolic activation (discussed below) in order to transform C3H 10T 1/2 cells. Inasmuch as the general applicability of extended exposure periods requires further investigation for this transformation system, the 24-hour exposure period originally established for the basic C3H 10T 1/2 cell assay was employed for subsequent experiments performed in the participating laboratories.

3.3 Cell Synchronization

The cell cycle dependence of C3H 10T 1/2 cell transformation by certain direct-acting carcinogens (e.g. MNNG) was demonstrated by Bertram and Heidelberger (1974), and later by Tu and Sivak (1980a). The sensitive phase was determined to be between 4 hours prior to S and the G₁-S boundary. The two laboratories participating in the effort reported on herein further examined three methods by which synchronize C3H 10T 1/2 cells, i.e. release from confluence, serum deprivation and isoleucine

deprivation. Of these methods, the one which seemed to offer the most promise was the release of cells from confluence. Employing this technique, some success was achieved in transforming C3H 10T 1/2 cells with MNNG and N-acetoxy-2-fluorenylacetamide, provided elevated densities of synchronized cells (e.g. 1×10^5 cells/dish) were used at the time of chemical treatment (Sivak and Tu, 1982b). However, transforming activity by MNNG was also obtained using asynchronous target cells at densities of 1×10^4 - 1×10^5 cells/dish. At the lower cell density of 1×10^3 cells/dish (employed also in the basic assay), cell synchronization failed to enhance MNNG-induced transformation (Schechtman, et al, 1983). Three other chemicals selected from the set of 30 coded samples used to validate the original C3H 10T 1/2 cell transformation assay were also examined in the cell cycle studies. Only one of these, i.e. 7-bromomethyl-12-methylbenz(a)anthracene (BMBA) exhibited some cell-cycle dependency; the two other agents, i.e. lead acetate and 2-nitro-p-phenylenediamine (2-NPD) failed to display any such dependence (Tu, et al, 1983).

The possible use of synchronized cultures to improve the sensitivity of C3H 10T 1/2 cell transformation assay and in the routine screening of genotoxins thus appears to have its limitations. Generally, it appeared that absolute cell synchrony might be difficult to achieve repeatedly, since the precise period of maximal DNA synthesis may vary by as much as 4 hours in different experiments. Further, since most chemicals are relatively stable in cell culture medium, exponentially growing

asynchronous cells are just as likely to be exposed to these chemicals during their period of maximum sensitivity over a 24 hour period. Higher cell seeding densities prior to treatment may help increase the size of the susceptible population in a given portion of the cell cycle, thereby providing sufficient numbers of transformable target cells to avoid the need for cell synchronization. For these reasons and the inconsistencies encountered, cell synchronization was not pursued as a viable modification of the C3H 10T 1/2 cell transformation assay.

3.4 Delayed Treatment Post-Seeding

It has been reported (Nesnow, et al, 1982) that exposure of target C3H 10T 1/2 cells to certain test chemicals five days after seeding resulted in an elevated transformation response beyond that obtainable with the original experimental conditions described by Reznikoff, et al (1973b), which employed a 24 hr treatment of cells one day after plating. Compounds which were found to induce transformation in this modified assay included BaP and aflatoxin B₁, and the direct-acting carcinogens MNNG and propane sultone. The increase in transformation (foci/dish and fraction of dishes with foci) was accompanied by a reduction in cytotoxicity as measured by colony formation in concurrent toxicity assays. It is not clear whether the responses observed using the delayed treatment procedure were the result of (a) higher number of target cells available ($\sim 1.4 \times 10^4$ cells/dish derived from an initial seeding density of 1×10^3 cells/dish five days earlier), or (b) partial cell synchrony established

sometime during the interval between cell seeding and chemical treatment, or (c) some less evident intercellular interactions imposed by the more highly populated cultures.

The general applicability of this methodology in identifying other classes of potential carcinogens was examined as part of the present effort detailed in this report. A greater transformation response was observed for MNNG with treatment delayed for five days, confirming the data of Nesnow, et al (1982). A slightly enhanced response was obtained with the metal salts beryllium sulfate and lead chromate and no enhanced response was found with the PAH 3-MC (Tu and Sivak, 1983). Thus, some advantage was realized in modifying the C3H 10T 1/2 cell transformation assay to accomodate a delay in chemical treatment five days post-seeding. In theory, a delay in treatment allows the initially plated cells to increase in number such that the resulting target cell population places a higher number of cells at the risk to chemical treatment when imposed. In fact, the estimated cell density at the time of treatment very closely approximates that which is employed by our laboratories (i.e. 1×10^4 cells per dish), but seeded one day prior to treatment (see section 3.1, above). It remains to be determined if there are significant differences attributable to plating the final desired cell concentration vs. reaching a similar cell density via exponential growth.

3.5 Amplification Transformation Assay

In view of (a) the known in vivo carcinogenic activity of many of the agents tested in the C3H 10T 1/2 cell system, in which they failed to elicit a transformation response, (b) the reported genotoxic activity of these agents in other in vitro systems, and (c) the capacity of many such agents to induce other biological responses in C3H 10T 1/2 cells, e.g. cytotoxicity, mutagenicity, chromosomal aberrations, or nonspecific DNA damage, including single strand breaks or alkaline-labile sites (Lubet, et al, 1983), it was reasoned that transformed C3H 10T 1/2 cells were perhaps present in any given population of chemically treated C3H 10T 1/2 target cells, but that due to some restrictive culture conditions these cells were unable to express the transformed phenotype. Previous studies of retrovirus enhancement of chemical transformation of Fischer rat embryo cells (Freeman, et al, 1973; reviewed in Heidelberger, et al, 1983) suggested that transformation which was induced in the originally treated cultures may require numerous cell divisions and cell transfers in order to manifest the aberrant phenotype. Based upon these findings, we felt that transformed C3H 10T 1/2 cells might similarly express their altered morphologies in the form of transformed foci if permitted to undergo more rounds of replication by releasing the originally treated cells from confluence and by replating at lower density to allow additional expression time for transformed cells to yield aberrant foci. To this end, the Amplification/Level II C3H 10T 1/2 Cell Transformation Assay was developed (Schechtman, et al, 1984).

Preliminary reports of the use of this methodology in expanding the responsiveness of C3H 10T 1/2 cells to various genotoxins have been published (Schechtman, et al, 1982a; 1982b; 1982c). Detailed discussion of the technique, its development with model compounds, its utility in identifying potential carcinogens tested under code, and its further refinement in making use of an exogenous metabolic supplement (discussed further below) can be found elsewhere (Schechtman, et al, 1983). Further details related to inter-laboratory utilization of the Level II assay are reported by Tu and Sivak (1983).

The Level II assay (depicted in Figure 2) involves the post-confluence subcultivation of previously treated cells. Replating occurs either at confluence (~2 weeks post-treatment) or 2 weeks post-confluence (4 weeks post-treatment). Thus, "incipient transformed clones" are disrupted, thereby permitting each constituent transformed cell to generate a separate aberrant focus upon replating.

Studies with the model compounds MNNG and BMBA, neither of which elicited transformation in the basic ("Level I") transformation assay, served to develop conditions which would maximize the Level II focus response. These conditions include (a) chemical treatment of 1×10^4 cells/dish, (b) reseeding confluent cultures 4 weeks post-treatment, and (c) a replating density of 2×10^5 cells/dish (Schechtman, et al, 1984).

Using this technique, a number of important points were determined (Schechtman, et al, 1984). First, chemical carcinogens which failed to elicit a transformation response in the basic Level I assay (Figure 1) were detected as potential carcinogens in the Level II assay (Table 4). Such compounds included MNNG, BMBA, 2-NPD, lead acetate, aflatoxin B₁, DB(a,h)A, actinomycin D, 2-naphthylamine, and ethyl methanesulfonate (EMS). Second, chemical carcinogens capable of inducing Level I transformation were likewise capable of eliciting a Level II transformation response (Table 4), but significantly more amplified in the latter. Such compounds included 3-MC, BaP, Bap-7,8-dihydrodiol and 4,4'-bis-(dimethylamino)-benzophenone (DABP). Third, the Level II assay was capable of discriminating between carcinogens and non-carcinogens. Thus, the non-carcinogenic chemicals phenanthrene, anthracene, cyclohexamide, ouabain, 4-nitro-p-phenylenediamine (4-NDP) and the organic solvents acetone and dimethylsulfoxide (DMSO) failed to induce Level II (or Level I) responses (Table 4).

The Level II assay was then subjected to validation using coded compounds supplied by the NCI Chemical Repository. Of 18 chemicals tested (Table 5), the results of two were inconclusive ("no test"), leaving 16 whose results could be compared with their in vivo carcinogenic activity. Of these, 14 compounds (88%) yielded results which agreed with the available in vivo data. One false negative (pararosaniline) was found and one agent (dimethylnitrosamine) yielded suspect activity, which when later re-tested in the Level II assay in the presence of an

exogenous metabolic (S-9) supplement induced a positive C3H 10T 1/2 cell response (Lubet, et al, 1984). Thus, the overall accuracy of the Level II assay was 94%.

Overall, the results obtained with the Level II/Amplification C3H 10T 1/2 Cell Transformation Assay indicate this to be a necessary and reliable adjunct to the original C3H 10T 1/2 cell assay. Its degree of accuracy, its sensitivity to diverse classes of chemical carcinogens, and its ability to limit false negative results obtained in the basic assay all argue in favor of its use. For the purposes of screening potential carcinogens, the combined Level I/II assays offer a degree of confidence heretofore unattainable with the C3H 10T 1/2 cell system.

3.6 Metabolic Activation

The limited sensitivity of the C3H 10T 1/2 clone 8 cells to numerous procarcinogenic chemicals was in part attributed to its limited inherent metabolizing capacity. In studies conducted by Schechtman and colleagues (1983) and by Tu and Sivak (1983) it was determined that the capacity of C3H 10T 1/2 cells to metabolize PAH's such as BaP or 3-MC to water-soluble products over a 24 hour period was markedly lower than that of primary Syrian hamster embryo cells (~3-fold) or primary rat hepatocytes (~30-fold). Although this reduced metabolic capacity was determined for PAH's, it may reflect other metabolic limitations of C3H 10T 1/2 cells as far as their ability to metabolically activate procarcinogens of other classes, at least those whose

primary metabolic alteration relies on the family of cytochromes P-450/P-448. In view of the limited availability of endogenous mixed-function oxidase activity (and perhaps other enzymatic capabilities required in the primary and secondary metabolism of procarcinogenic chemicals), it was mandated that a significant portion of the studies conducted in this collaborative effort be devoted to the development of a suitable exogenous metabolic activation system compatible with the C3H 10T 1/2 clone 8 transformation system. To this end, two approaches were pursued, i.e. one which employed X-irradiated (3000 R) intact primary rodent (rat and mouse) hepatocytes (Tu and Sivak, 1983; Tu, et al, 1984), and one which employed Aroclor 1254-induced sub-cellular enzyme preparations derived from the 9000 x g post-mitochondrial supernatant (S-9) fraction of rodent liver homogenates (Schechtman, et al, 1983; Lubet, et al, 1984; Tu et al., 1984).

3.6.1 Primary Hepatocytes

Four different procedures were attempted in order to utilize the metabolic capacity of Fischer 344 rat hepatocytes to supply bioactive metabolites to indicator C3H 10T 1/2 cells with which they were co-cultured (Tu and Sivak, 1983). In the first method, target cells were seeded at 1×10^4 cells/dish and one day later hepatocytes (usually 20 X the number of target cells) were plated and allowed to attach for 2 hours; chemical was added and the mixture was incubated for 24-72 hours. The remainder of the method paralleled that of the basic transformation assay. In

the second method, confluent or near confluent target cells ($5-6 \times 10^5$ cells/plate) were plated prior to the addition of $2-3 \times 10^6$ hepatocytes/plate. Following chemical treatment or sometime later, cells were harvested and replated ($1-2 \times 10^4$ cells/plate) and monitored for development of transformed foci. In the third method, a slight modification of the second method, target cells were seeded 1×10^5 cells/plate (dense but subconfluent, in order to allow the target cells to divide during treatment), to which was added 1×10^6 hepatocytes/plate. Cell harvesting and replating was as described in the second method. The fourth method was a suspension assay in which target cells and hepatocytes were co-incubated in the presence of test chemical for 4 hours. Cells were then diluted and seeded at 1×10^4 cells/plate and incubated for 6 weeks to allow focus formation.

The first procedure proved to be unsuccessful in activating 2-AA, DMN, DEN and 2-FAA to products capable of transforming C3H 10T 1/2 cells. Cyclophosphamide yielded equivocal results, i.e. at a dose of 10 ug/ml, numerous Type III foci (10 foci/20 plates) were observed in the presence of hepatocytes while several Type II foci (1 Type III and 6 Type II foci/20 plates) were observed in the absence of hepatocytes.

The second and third procedures were likewise unsuccessful in providing rat hepatocyte-mediated transformation of C3H 10T 1/2 cells by 2-AA, 2-FAA, 2-aminofluorene, DMN and DEN. Cyclophosphamide again yielded equivocal results as it did

in the first procedure. The fourth procedure failed to activate 2-FAA to metabolites which could transform C3H 10T 1/2 cells.

Additional attempts at hepatocyte-mediated C3H 10T 1/2 cell transformation by procarcinogens focused on use of C3H mouse primary hepatocytes (Tu, et al, 1984). Two approaches were pursued using as the basis the first procedure described above. After a three day treatment of co-cultivated C3H 10T 1/2 cells and mouse hepatocytes, cells were maintained in situ for six weeks with regularly scheduled medium changes to promote focus formation (standard Level I methodology). Alternatively, a slightly modified Level II (replating) methodology was also employed. Cyclophosphamide (1-10 ug/ml dose range) induced a dose-dependent, hepatocyte-dependent transformation of C3H 10T 1/2 cells using the Level I (in situ) approach (Table 6). In the absence of hepatocytes, cyclophosphamide failed to transform the indicator cells. The mouse hepatocytes also mediated transformation of C3H 10T 1/2 cells by DMN, although the TF was generally lower than that observed for cyclophosphamide (data not shown). Employing the Level II assay procedure (replating of 2×10^5 cells/dish 4 weeks post-treatment), DMN was observed to induce significant Type III focus transformation (20 foci/10 plates) at one dose (20ug DMN/ml) against a zero spontaneous background. In the same dose range (0.8-100 ug/ml) DMN failed to transform C3H 10T 1/2 cells in Level I or Level II assays in the absence of hepatocytes, or in a Level I assay in the presence of hepatocytes.

The general applicability of this technique to focus transformation systems will warrant further investigation. At least two fundamental problems associated with the use of intact cell-mediated activation systems need be recognized. First, since indicator cells employed in transformation assays are seeded at rather low densities (e.g. 10^3 - 10^4 cells/dish), their proximity to hepatocytes (which tend to attach in clusters) may not be sufficient to allow effective cross-feeding of active metabolites. This assumes that intercellular communication provides a more efficient mechanism of transferring bioactive products between metabolizing cells and target cells. Second, hepatocytes (whether or not they were irradiated) tended to remain adherent to the culture dish growing surface for the duration of the (six week) transformation assay. The resulting confluent monolayers therefore tended to appear non-uniform or mottled, interfering with the focus formation or the interpretation of morphologies of the foci. Future success with cell-mediated transformation assays whose endpoints rely on development of foci upon confluent monolayers of contact-inhibited cells will require issues of this nature to be addressed.

3.6.2 Hepatic S-9

The utility of rodent hepatic S-9's to metabolically activate promutagens/procarcinogens has been demonstrated for both bacterial and mammalian cell mutagenesis assays (Ames, et al, 1975; O'Neill, et al, 1979; Bartsch, et al, 1980) and

mammalian cell transformation assays (Schechtman and Kouri, 1977). Compatibility of S-9 metabolic supplements with C3H 10T 1/2 cells has been shown by both Benedict, et al, (1978) and ourselves (Schechtman, et al, 1982a). The initial studies performed as part of this collaborative effort centered on optimizing assay conditions for use of an exogenous S-9 to metabolize model genotoxins to forms cytotoxic and transforming to C3H 10T 1/2 cells. To this end, a chemical known to transform C3H 10T 1/2 cells and known to require metabolic activation, i.e. BaP, was employed. Assay conditions were optimized (cell seeding density, S-9 concentration, cofactor components, exposure period, etc.) such that the transforming effects of BaP were substantially enhanced by the addition of S-9 (Table 7). The use of BaP, however, had certain limitations. First, BaP is metabolized by the C3H 10T 1/2 cells themselves. Second, BaP is highly lypophilic and difficult to remove from cells. Thus, the concentration of S-9 which routinely and substantially enhanced BaP-induced transformation (1.25 ul S-9/ml) gave no significant evidence of activating aromatic amines such as 2-AA or 2-FAA. It was therefore necessary to reinvestigate the metabolic capacity of the Aroclor-1254-induced hepatic S-9 preparation employed in the C3H 10T 1/2 cell assays. To this end, BaP served as the primary substrate and the results indicated that S-9 mediated BaP metabolism was (1) proportional to the S-9 protein concentration, (2) dependent on the concentration of substrate, (3) achieved in the presence of 2.5% fetal bovine serum, (4) maximally active for

up to 60 minutes, and (5) inhibited by 7,8-benzoflavone (Schechtman, et al, 1983).

Attempts were then made to optimize conditions for the activation of other compounds employing cytotoxicity as an endpoint. The rationale for this approach was that metabolic activation of a chemical to cytotoxic products would likely be associated with its activation to potentially mutagenic and/or carcinogenic moieties. Furthermore, the time required to induce a cytotoxic response was considerably shorter than that required to yield a transformation response. Results from these studies showed that although conditions had been optimized for the activation of agents such as BaP-7,8-dihydrodiol, 2-AA, 2-FAA and cyclophosphamide to cytotoxic metabolites, minimal phenotypic transformation was obtained employing assay conditions which paralleled the more standard C3H 10T 1/2 cell assay (e.g., treatment of 1×10^3 cells/dish, without subsequent replating post-confluence). Nevertheless, these same mammalian cell culture treatment conditions were capable of activating 2-AA, BaP-7,8-dihydrodiol and DMN (but not DEN or cyclophosphamide) to moieties mutagenic to Salmonella typhimurium (Table 8).

At about the same time, significant progress had been made in development and optimization of the Level II Amplification assay in the absence of supplementary metabolic activation (section 3.5, above). The logical adaptation of this procedure to include an S-9 metabolic supplement soon followed. The methodology is essentially that of the Level II assay

depicted schematically in Figure 2, except that a 4 hour treatment period is employed in the presence of S-9 and a NADPH regenerating system. Using this modified transformation procedure in conjunction with rat (Fischer 344) or hamster (Syrian golden) hepatic S-9, (20-100 ul/ml, 20-25 mg protien/ml), positive C3H 10T 1/2 cell transformation responses have been achieved with a variety of chemical carcinogens (Schechtman, et al, 1983; Lubet, et al, 1984) including PAH's, aromatic amines, mycotoxins and nitrosamines (Table 9). Only a marginal response at best was obtained with 2-FAA under these conditions, even though this agent could be activated by S-9 to cytotoxic metabolites. Likewise, S-9 mediated, cyclophosphamide induced Level II transformation of C3H 10T 1/2 cells could not be achieved in spite of the generation of cytotoxic metabolites in the presence of this metabolic supplement.

4.0 Conclusion

Overall, the basic C3H 10T 1/2 clone 8 cell transformation assay, originally developed for use with polycyclic aromatic hydrocarbons and to address certain mechanistic questions related to chemical carcinogenesis, did not perform as expected for other chemical classes. To broaden the responsiveness of these cells to diverse genotoxins and to limit false negative results, the original assay has been modified. The improvements introduced include (a) increasing the number of cells at risk to chemical treatment, (b) varying exposure time, (c) development of a method by which to amplify the presence of chemically transformed cells

(Level II assay), and (d) use of an exogenous source of metabolic activation (hepatocytes, S-9's). Together, these procedural changes have enhanced the sensitivity of the C3H 10T 1/2 cells to various classes of both direct-acting chemical carcinogens and those requiring metabolic activation. This improved sensitivity was realized without sacrificing the discriminatory ability of these cells to distinguish between carcinogenic and non-carcinogenic compounds. The results obtained suggest that the modified C3H 10T 1/2 cell transformation assays (in the absence and presence of metabolic supplements) will permit this system to be employed as a reliable screening test to complement other in vitro mammalian cell assays in the identification of potential chemical carcinogens.

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FIGURE 1. BASIC ASSAY FOR CHEMICALLY-INDUCED
MORPHOLOGICAL TRANSFORMATION OF C3H 10T $\frac{1}{2}$ CLONE 8 CELLS

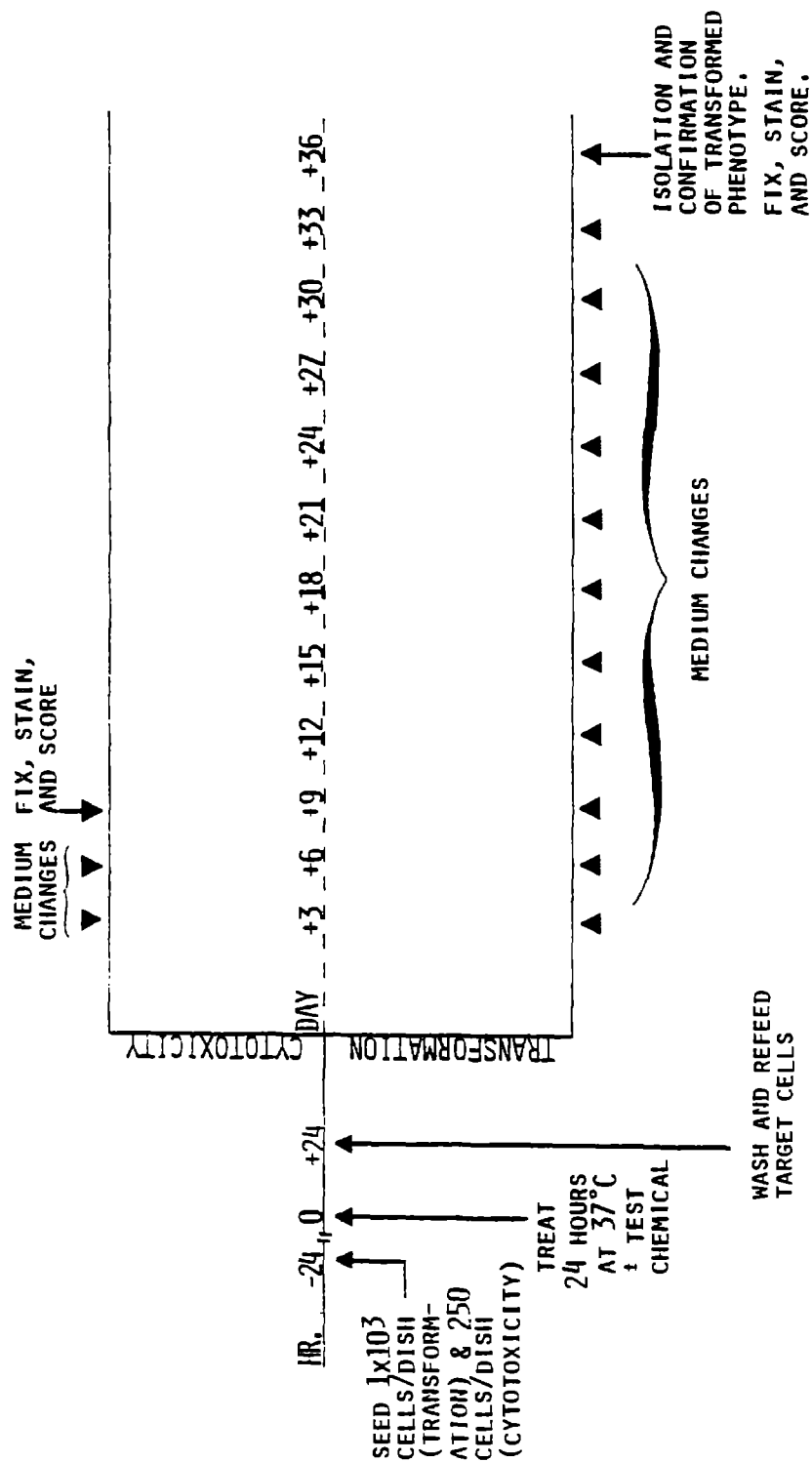


FIGURE 2. C3H 10T $\frac{1}{2}$ CL. 8 AMPLIFICATION TRANSFORMATION ASSAY IN THE
ABSENCE OR PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

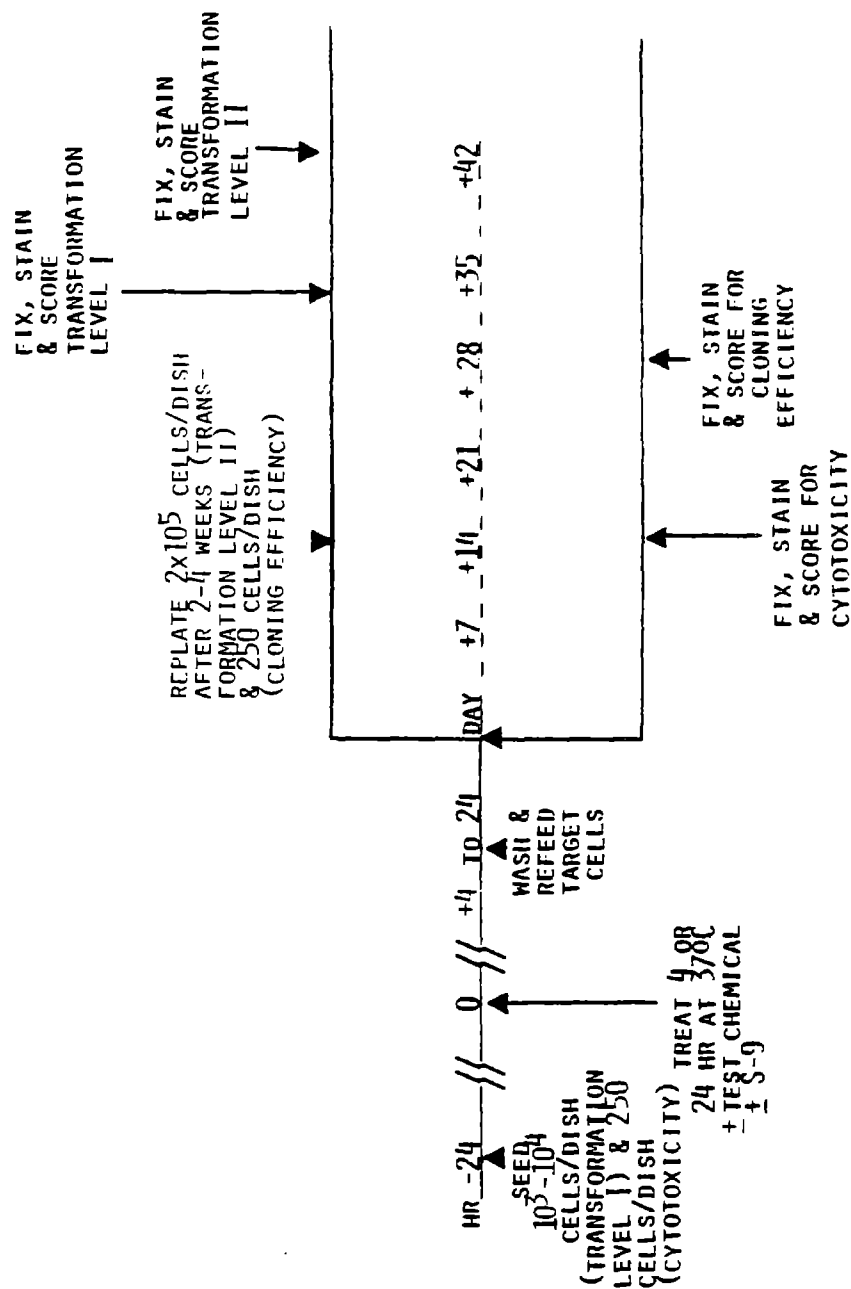


Table 1

Criteria for a Valid C3H 10T $\frac{1}{2}$ Transformation Test

-
1. Cloning efficiency, i.e. number of colonies per number of cells seeded per condition in concurrent cytotoxicity assay, of $\geq 15\%$ for the negative (solvent-vehicle) control.
 2. Number of replicates per condition of ≥ 12 plates.
 3. Number of test chemical doses ≥ 3 .
 4. Spontaneous transformants in the negative (solvent-vehicle) controls of ≤ 1 Type II or Type III focus.
 5. Number of chemically-induced transformants in the positive control of ≥ 4 Type II or Type III foci above background.
 6. Chemically induced cytotoxicity relative to the negative control $\geq 50\%$ for at least one dose of test chemical.
-

Table 2

Response Criteria for C3H 10T $\frac{1}{2}$ Cell Transformation Assay

I. Positive

- A. A minimum of 4 Type II and/or Type III transformed foci (all doses) over background.
- B. Type II and/or Type III foci in >1 test chemical dose.
- C. Transformed foci (Type II and/or Type III) in >4 total dishes (all doses).

II. Suspect

- A. Fails to meet criteria for a positive response.
- B. One or more (but <4) Type II and/or Type III foci in >1 test chemical dose.
- C. Two or more foci in a single plate counted as one focus.

III. Negative

- A. Less than or equal to 1 transformed Type II or Type III focus (background subtracted).
- B. Cytotoxicity of ≤25% in accompanying toxicity assay.

IV. No Test

- A. Fails to meet criteria for a valid test.
 - B. Fails to meet criteria for a negative response.
 - C. Less than 25% cytotoxicity in concurrent toxicity assay when ≤1 transformed Type II or III focus is present.
-

Table 3

Summary of Results on 30 Coded Samples:
Basic C3H 10T $\frac{1}{2}$ Cl. 8 Cell Transformation Assay^a

IN VIVO ACTIVITY	# OF COMPOUNDS	C3H 10T $\frac{1}{2}$ CELL TRANSFORMATION RESPONSE ^b	
		LAB A	LAB B
Carcinogenic	20	5P 4S 11N	6P 5S 2N
Non-carcinogenic	8	6N 2S	2N 2S 2P
Unknown	2	1P 1N	1P

^a Complete details of assays performed on all compounds are available in Tu and Sivak (1983) and Schechtman, et al, (1983).

^b Data are given in terms of the actual numbers of positive (P), suspect (S), and negative (N) responses. Response criteria employed are found on Table 2. Tests which failed to meet the criteria for an acceptable test (Table 1) were excluded.

Table 4

Transformation Level I and II Response^a
of C3H 10T $\frac{1}{2}$ Cells to Various Genotoxins

COMPOUND	TRANSFORMATION	
	LEVEL I	LEVEL II
3-MC	+	+
BaP	+	+
BaP-7,8-dihydrodiol	+	+
DABP	+	+
DB(a,h)A	0	+
BMBA	0	+
Lead acetate	0	+
MNNG	0	+
2-NPD	0	+
2-Naphthylamine	0	+
Aflatoxin B ₁	0	+
EMS	0	+
Actinomycin D	0	+
Phenanthrene	0	0
Anthracene	0	0
4-NPD	0	0
Cyclohexamide	0	0
Ouabain	ND	0
Acetone	0	0
DMSO	0	0

^a + indicates a positive response; 0 indicates no response; ND = not determined. Further details are available in Schechtman, et al (1984).

Table 5
Level II Transformation Activity
of Coded Chemical Carcinogens^a

COMPOUND	LEVEL II C3H 10T $\frac{1}{2}$ ASSAY RESULT ^b	IN VIVO CARCINOGENECITY
Ethylene dibromide	NT	positive
4,4'-bis-(Dimethylamino)- benzophenone	+	positive
p-Quinone dioxin	+	positive
Dimethylnitrosamine	?(+) ^c	positive
5-Nitro-o-toluidine	NT	positive
Lead acetate	+	positive
7-bromomethyl-12-methylbenz- (a)anthracene	+	positive
2-Nitro-p-phenylenediamine	+	positive
Cinnamyl anthranilate	+	positive
Aniline	+	positive
Propyleneimine	+	positive
Pararosaniline	0	positive
tris(2,3-dibromopropyl)- phosphate	+	positive
N-hydroxy-2-fluorenyl- acetamide	+	positive
Benzidine	+	positive
7,12-Dimethylbenz(a)anthra- cene	+	positive
Dyrene	0	negative
Lithocholic acid	0	negative

^a Level II results were analyzed in terms of "treatment groups" where a group consists of all replicate dishes of a given condition. A group was called positive if $\geq 20\%$ of the component dishes displayed Type III foci and/or $\geq 33\%$ of the dishes contained Type II and/or Type III foci.

^b Symbols used are:

- + = positive; $\geq 2 / \leq 8$ treatment groups displaying transformation.
- 0 = negative; 5-8 treatment groups with no transformation.
- ? = suspect; $1 / \leq 8$ treatment groups displaying transformation.
- NT = no test; ≤ 4 treatment groups available, none of which exhibit transformation.

^c Positive in the presence of an S-9 metabolic supplement (Lubet, et al, 1984).

Table 6

Mouse Hepatocyte-Mediated Cyclophosphamide Transformation of C3H 10T $\frac{1}{2}$ Cells

TREATMENT	DOSE (ug/ml)	RELATIVE SURVIVAL ^a		TRANSFORMATION FREQUENCY ^b	
		CELLS	COLONIES	T-II + T-III/td	(dwF)
Control	---	1.00	1.00	0 + 0/15	(0)
Cyclophosphamide	10.0	0.22	0	0 + 13/15	(8)
	7.5	0.14	0	2 + 7/15	(7)
	5.0	0.19	0	1 + 7/15	(7)
	2.5	0.50	0.11	0 + 0/15	(0)
	1.0	0.92	0.26	0 + 0/15	(0)
3-MC	5.0	0.66	0.30	9 + 10/14	(9)

^a Survival is expressed relative to the medium control in terms of cell count $\times 10^5$ /plate and as colony forming efficiency upon replating 100 cells/plate after treatment.

^b Transformation frequency is expressed as the number of Type II (T-II) and Type III (T-III) foci per total dishes (td) per condition; numbers in parentheses are the numbers of dishes with transformed foci (dwF) per condition.

Table 7

S-9 Mediated Benzo(a)pyrene Induced Transformation of C3H 10T $\frac{1}{2}$ Cells

TREATMENT ^a	DOSE (μ g/ml)	RCE ^b	TRANSFORMATION		FREQUENCY ^c
			T-II + T-III/td	(dwT-III)	
S-9	--	1.00	0 +	0/12	(0)
BaP	10.0	0.36	2 +	2/11	(2)
BaP + S-9	10.0	0.71	6 +	13/12	(13)
BaP + S-9	5.0	0.79	1 +	11/11	(11)
BaP + S-9	2.5	>1.00	0 +	1/10	(1)
BaP + S-9	1.0	>1.00	1 +	0/12	(0)

^a Cells were seeded at 1×10^3 cells/dish and treated for 4 hr + test chemical, + S-9 (1.25 μ l S-9/ml, 20 mg protein/ml) and an NADPH regenerating system (0.5 mg NADH/ml, 0.75 mg/ml each of NADP, NADPH and glucose-6-phosphate in EBME + 5% FBS, pH 7.4. Cells were washed, resupplied with medium and cultivated 6 weeks for focus formation.

^b RCE= relative cloning efficiency, i.e. number of colonies per condition relative to the negative control.

^c Transformation frequency is expressed as the number of Type II (T-II) + Type III (T-III) foci per total dishes (td) per condition; numbers in parenthesis are the number of dishes with Type III foci (dwT-III) per condition.

Table 8

Activation of Various Procarcinogens to Mutagenic Moieties by Hepatic S-9 Fractions^a

STUDY NUMBER	TIME (MINUTES)	CHEMICAL TREATMENT	CONCENTRATION		AVERAGE NUMBER REVERTANTS/PLATE	
			CHEMICAL (ug/ml)	S-9 (ul/ml)	RAT S-9	HAMSTER S-9
1	0	2-AA	5.0	20	0	ND
	7.5	2-AA	5.0	20	327	ND
	15.0	2-AA	5.0	20	726	ND
1	0	BaP-7,8-dihydrodiol	0.5	20	0	ND
	7.5	BaP-7,8-dihydrodiol	0.5	20	113	ND
	15.0	BaP-7,8-dihydrodiol	0.5	20	334	ND
2	0	DMN	2.0	100	20	13
	60	DMN	2.0	100	13	70
	120	DMN	2.0	100	13	83
2	0	DEN	2.0	100	10	6
	60	DEN	2.0	100	13	16
	120	DEN	2.0	100	6	11
3	0	Cyclophosphamide	5.0	20	15	ND
	30	Cyclophosphamide	5.0	20	18	ND
	60	Cyclophosphamide	5.0	20	16	ND

^a Mutagenesis was performed by incubating at 37°C 1 X 10⁸ TA100 (Study #1) or TA1535 (Studies #2 & 3) bacteria per ml + 20-100 ul/ml Aroclor 1254 induced hepatic S-9 + NADH (0.5 mg/ml), NADP, NADPH and glucose-6-phosphate (0.75 mg/ml of each) in EBME + 2.5% FBS, pH 7.4. Following incubation for the indicated times mutagenized bacteria were added to 9 ml cold phosphate buffered saline, centrifuged, and 1-3 X 10⁸ bacteria were added to 2 ml molten agar supplemented with 0.6% NaCl and 0.05 mM histidine - 0.05 mM biotin. The tubes were vortex-mixed and the contents poured onto base plates of Vogel-Bonner minimal medium. Plates were incubated 48 hr at 37°C, after which time the number of histidine revertant auxotrophic mutant colonies were counted. Each point represents triplicates per condition. The background mutagenic level was between 7-10 revertants per plate for TA1535 and between 156-178 revertants per plate for TA100.

Table 9

S-9 Mediated C3H 10T $\frac{1}{2}$ Cell Transformation
by Various Procarcinogens^a

COMPOUND	CYTO- TOXICITY ^b	TRANSFORMATION ^c	
		LEVEL I	LEVEL II
BaP	+	+	+
BaP-7,8-dihydrodiol	+	+	+
2-AA	+	0	+
2-FAA	+	0	?
2-Naphthylamine	+	0	+
6-Aminochrysene	+	+	+
Benzidine	+	0	+
Aflatoxin B ₁	+	0	+
DMN	+	0	+
DEN	0	0	+
Cyclophosphamide	+	0	0

^a Cells were seeded at 1×10^4 cells/dish, treated for 4 hr with test chemical in the presence of hepatic S-9 and an NADPH regenerating system, washed and cultured 4-6 weeks. Level I plates (15/condition) were scored for transformation after 6 weeks. Level II plates (derived from cells harvested from 5 Level I plates/condition and replated 4 weeks post-treatment into 15 daughter plates/condition) were similarly scored 6 weeks later.

^b Reduction in cloning efficiency relative to negative control. Symbols are: $\geq 50\%$ toxicity (+); non-toxic (0).

^c Transformation responses were as detailed in Table 5, footnotes 'a' and 'b'.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Comparative Carcinogenesis Database and Quantitative Species Comparison

Lois Gold
Bruce Ames

University of California, Berkeley

Morris I. Kelsey*
National Cancer Institute

* Dr. Kelsey presented for Drs. Gold and Ames.

Presentation of "Comparative Carcinogenesis Database and Quantitative Species Comparison" by Dr. Morris I. Kelsey (NCI) for Drs. Lois Gold and Bruce Ames (U. of California, Berkeley)

Quantitative information on the capacity of various chemicals to cause cancer is desirable in setting priorities with respect to risk/benefit judgements and establishing regulatory policy. Although such quantitative information is not available for humans, there are a plethora of such data from animal experiments. The problem with interpreting experimental studies in animals is that such studies are not necessarily conducted in a standard method, and diverse experimental protocols causing a variety of toxicities, including cancer are difficult to compare between individual experiments and/or laboratories.

In order to rectify this situation, the authors (Drs. Lois Gold and Bruce Ames) have created a comprehensive database which incorporates animal bioassay data from the world's literature that satisfy certain minimal criteria and summarizes such information in a standardized uniform format. The database calculates an index of carcinogenic potency, the TD₅₀, for a diverse number of chemicals of different classes tested in various species, sexes and strains of animals and, these TD₅₀ values are calculated for all target sites reported in each series of experiments. It should be stressed that while this information will be quite useful to those individuals who attempt to make predictions of carcinogenic potency of various compounds in humans, this potency database does not perform risk assessments for humans but will be valuable in comparing results from different animal experiments. Therefore, this presentation describes the features and content of this carcinogenic potency database which will appear as a special issue of Environmental Health perspectives in the summer of 1984.

SLIDE#1

COMPARATIVE CARCINOGENESIS DATABASE AND
QUANTITATIVE SPECIES COMPARISON

- ANNOUNCEMENT OF THE EXISTENCE OF DATABASE WHICH SUMMARIZES QUANTITATIVE TUMORIGENICITY DATA IN A SYSTEMATIC WAY FOR 3000 EXPERIMENTS ON 775 CHEMICALS.
- THIS STANDARDIZED DATABASE CONTAINS QUANTITATIVE RESULTS OF PUBLISHED ANIMAL CARCINOGENESIS BIOASSAYS SO THAT COMPARISONS CAN BE MADE ACROSS:
 - COMPOUNDS
 - SPECIES/STRAINS
 - TARGET SITES
 - LABORATORIES TESTING SAME COMPOUNDS
- NEED TO DEVELOP SUCH A DATABASE TO SUMMARIZE IN A STANDARD WAY RESULTS FROM DIVERSE EXPERIMENTAL PROTOCOLS.
- DEVELOPED INDEX OF CARCINOGENIC POTENCY ESTIMATES, THE TD₅₀.

SLIDE #2

CARCINOGENIC POTENCY AS EXPRESSED BY TD_{50} IS DEFINED AS:

THE CHRONIC DOSE EXPRESSED AS MG/KG BODY WEIGHT/DAY THAT CAUSES TUMORS IN 50% ANIMALS FOR A GIVEN TARGET SITE. THIS VALUE IS STANDARDIZED FOR THE LIFESPAN OF THE SPECIES AND ACCOUNTS FOR:

1. TUMOR INCIDENCE IN CONTROL ANIMALS
2. INTERCURRENT MORTALITY CAUSED BY TOXICITY OR DISEASE. VALUE CALCULATED FOR EACH WEEK OF EXPT.

SLIDE #3

SOURCES OF DATA

1. NATIONAL CANCER INSTITUTE/NATIONAL TOXICOLOGY PROGRAM
BIOASSAY TECHNICAL REPORTS
2. AROMATIC AMINES (20) TESTED BY WEISBURGER ET AL., 1978 AND RUSSFIELD
ET AL., 1973, 1975.
3. OPEN LITERATURE ON CHEMICALS REVIEWED IN 1 AND 2 ABOVE.

REFERENCES

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2: 325-356, 1978.

RUSSFIELD, A.B., HOMBURGER, F., BOGER, E., VAN DONGEN, C.G., WEISBURGER,
E.K., AND WEISBURGER, J.H. THE CARCINOGENIC EFFECT OF 4, 4'-METHYLENE-
BIS-(2-CLOROANILINE) IN MICE AND RATS. TOXICOLOGY AND APPLIED PHARMACOLOGY,
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MA: BIO-RESEARCH CONSULTANTS, INC., JULY 1973.

SLIDE 4A.

CRITERIA FOR STUDIES INCLUDED IN DATABASE

- A. ANIMALS ON TEST WERE MAMMALS;
- B. ADMINISTRATION WAS BEGUN EARLY IN LIFE (BEFORE 100 DAYS OF AGE FOR RATS AND MICE);
- C. ROUTE OF ADMINISTRATION WAS DIET, WATER, GAVAGE, INHALATION, INTRAVENOUS OR INTRAPERITONEAL INJECTION (I.E. WHERE THE WHOLE BODY WAS MORE LIKELY TO HAVE BEEN EXPOSED RATHER THAN ONLY A SPECIFIC SITE, AS WITH SUBCUTANEOUS INJECTION OR SKIN PAINTING);
- D. TEST AGENT WAS ADMINISTERED ALONE, RATHER THAN IN COMBINATION WITH OTHER CHEMICALS;
- E. EXPOSURE WAS CHRONIC, WITH NOT MORE THAN 7 DAYS BETWEEN ADMINISTRATIONS;

SLIDE 4B

CRITERIA (CONT'D)

- F. DURATION OF EXPOSURE WAS AT LEAST ONE-FOURTH THE STANDARD LIFESPAN FOR THAT SPECIES;
- G. DURATION OF EXPERIMENT WAS AT LEAST HALF THE STANDARD LIFESPAN FOR THAT SPECIES;
- H. RESEARCH DESIGN INCLUDED A CONTROL GROUP;
- I. RESEARCH DESIGN INCLUDED AT LEAST 5 ANIMALS PER GROUP;
- J. PATHOLOGY DATA WAS REPORTED FOR THE NUMBER OF ANIMALS WITH TUMORS RATHER THAN THE TOTAL NUMBER OF TUMORS;
- K. RESULTS REPORTED WERE ORIGINAL DATA, RATHER THAN SECONDARY ANALYSES OF EXPERIMENTS ALREADY REPORTED BY OTHER AUTHORS.

SLIDE #5

SELECTION OF TISSUE AND TUMOR TYPES FOR
CALCULATION OF CARCINOGENIC POTENCY

A TD₅₀ WAS CALCULATED WHENEVER DOCUMENTATION WAS ADEQUATE FOR EACH OF THE FOLLOWING CATEGORIES:

1. EACH CATEGORY EVALUATED BY THE AUTHOR AS TREATMENT RELATED.
 - ANY NEOPLASM (BENIGN/MALIGNANT) RELATED TO TREATMENT REGARDLESS OF STATISTICAL/BIOLOGICAL BASIS FOR AUTHORS' EVALUATION.
 - HYPERPLASIA AND NON-NEOPLASTIC LESIONS WERE NOT INCLUDED IN THE DATABASE.
2. ALL TUMOR BEARING ANIMALS (TBA)
 - THIS CATEGORY IS A BASIS OF COMPARISON ACROSS EXPERIMENTS
 - EXCLUDES INTERSTITIAL-CELL TUMORS OF TESTIS FOR F₃₄₄ RATS.
3. MANDATORY SITES
 - TISSUES WERE SELECTED BECAUSE THEY OCCURRED MOST OFTEN IN A FREQUENCY COUNT OF THE TREATMENT-RELATED TISSUES IN NCI/NTP BIOASSAYS.
 - THESE SITES ARE:
 - LIVER, LUNG - MOUSE, HAMSTER
 - LIVER - RAT

SLIDE #6

SPECIAL CONSIDERATIONS - SELECTION PATHOLOGY FOR TD₅₀

NCI/NTP TECHNICAL REPORTS

1. REPORT TARGET SITE - POSITIVE EVALUATION
2. SITES IN STATISTICAL TABLES ("STATISTICAL SITES")
 - NOT CONSIDERED TREATMENT RELATED
 - INCLUDED ONLY IF TD₅₀ IS SIGNIFICANT AT P0.05 LEVEL
3. MANDATORY SITES
 - A. FOR EACH MANDATORY SITE, A TD₅₀ IS ESTIMATED FOR A COMBINATION OF TUMORS:
 - LIVER - NEOPLASTIC NODULE, HEPATOCELLULAR ADENOMA,
HEPATOCELLULAR CARCINOMA
 - LUNG - ALVEOLAR/BRONCHIOLAR ADENOMA OR CARCINOMA
 - B. IN ALL CASES THE INCIDENCE REPRESENTS THE NUMBER OF ANIMALS WITH ANY OF THE TUMORS; ANIMALS WITH MULTIPLE TUMORS ARE COUNTED ONLY ONCE.

$$\begin{array}{l} \text{(11)} \\ 100\dots:\cdot\text{lUG}\dots:\cdot 10\dots:\cdot 100\dots:\cdot\text{lMG}\dots:\cdot 10\dots:\cdot 100\dots:\cdot\text{lG}\dots:\cdot 10 \end{array}$$

- (1) (2)(3) (4) (5) (6) (7) (8)(9)(10) (13) (14) (15) (16)

Accession	Strain	Sex	Age	Location	Host	Pathogen	Isolate	Conc.	Time	Result
843	M	F	B6C	GAV	MXB	MXB	53w78	SV	3.74MG	* P<.0005
A	M	F	B6C	GAV	STO	SQC	53w78	SV	4.07MG	* P<.0005 C
B	M	F	B6C	GAV	LUN	MXA	53w78	SV	15.4MG	* P<.04 C
C	M	F	B6C	GAV	LUN	A/A	53w78	SV	17.3MG	* P<.03 C
D	M	F	B6C	GAV	TBA	MXB	53w78	SV	3.52MG	* P<.0005
E	M	F	B6C	GAV	LIV	MXB	53w78	SV	NO DRE	P=1.
F	M	F	B6C	GAV	LUN	MXB	53w78	SV	15.4MG	* P<.04
844	M	F	B6C	INH	MXB	MXB	22m24	S	9.60MG	/ P<.0005

SLIDE 7B

SLIDE / B										PATHOL	BERKELEY	
										CITAT.	CODE	
										(18)	(27)	(28)
(17)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)				
1,2-DIBROMOETHANE (ETHYLENE DIBROMIDE, EDB, c00522 is NCI TR#86; c00523 is NCI/NTP TR# 210) 106-93-4												
843	c00522	2.56MG	6.93MG	0/20	26.0MG	46/50	52.0MG	30/50	LUN:A/A,A/C; STO:SQC. C			
A	c00522	2.81MG	8.09MG	0/20	26.0MG	46/50	52.0MG	28/50				
B	c00522	8.06MG	N.S.S.	0/20	26.0MG	11/50	52.0MG	06/50	LUN:A/A,A/C			
C	c00522	8.85MG	N.S.S.	0/20	26.0MG	10/50	52.0MG	06/50				
D	c00522	2.42MG	6.31MG	0/20	26.0MG	47/50	52.0MG	31/50				
E	c00522	N.S.S.	N.S.S.	0/20	26.0MG	01/50	52.0MG	00/50	LIV:HPD,HPC,NND			
F	c00522	8.06MG	N.S.S.	0/20	26.0MG	11/50	52.0MG	06/50	LUN:A/A,A/C			
844	c00523	6.41MG	14.8MG	6/50	23.9MG	31/50	95.6MG	47/50	HEM,HES; LUN:A/A,A/C,ADN,			
										APN,CAN; MGL:ACN;		
										NAS:---,ADN,APN,CAN;		
										SUB:FDS		
										C		

SLIDE #8

CONCLUSIONS

- PRESENTATION OF TD₅₀ DATA IN A GRAPHIC DISPLAY PROVIDES SYSTEMATIC MEANS OF DISTINGUISHING AMONG CARCINOGENIC POTENCIES OF VARIETY OF CHEMICALS.
- RANGE OF TD₅₀ VALUES IS MORE THAN MILLION-FOLD
 - TCDD - 526 NG
 - 4-CHLORO-O-PHENYLENEDIAMINE 2.24G
- RANGE OF CARCINOGENIC POTENCY FOR FEMALE MICE (FIGURE)
- FUTURE WORK WILL USE DATABASE TO COMPARE POTENCY BETWEEN:
 - SPECIES
 - AMONG STRAINS
 - BETWEEN MALES AND FEMALES
 - DIFFERENT BIOASSAYS OF SAME CHEMICAL

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION II - EXPERIMENTAL STUDIES
Chairman: Dr. Frode Ulvedal
Environmental Protection Agency

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. ULVEDAL: This session is now open for discussion. I would like the speakers to come forward to be able to use the microphone. Again, I would like for the questioners to identify themselves when they come to the microphone.

DR. LONGFELLOW: I am Dave Longfellow, from NCI. I was interested in Dr. Waters' paper particularly. Were those animals that you were using for getting your S-9 Aroclor-induced?

DR. WATERS: I believe they were Aroclor-induced. Dr. Huang is here.

DR. HUANG: Yes.

DR. LONGFELLOW: And is there any effect on the synchrony of the cells when you are using those S-9 fractions?

DR. HUANG: Not sure, we hope not. S-9 treatment was brief and it took place after cell synchrony and accumulation of S-phase cells.

DR. LONGFELLOW: So, you are getting an increase in the tritium incorporation?

DR. HUANG: We didn't try that. Tritium incorporation increases when the cells enter S-phase. We did not try to determine when the second S-phase occurs. Often in the synchronized cells we can identify the S-phase again. We didn't do a systematic study to determine when the second S-phase occurs.

DR. LONGFELLOW: I was wondering, too, from the nonsynchronized population that you're looking at, what is the relative density on the plate? In other words, do you expect a high percentage of those cells to be in the cell cycle in this?

DR. WATERS: I am going to have to defer to Dr. Huang.

DR. HUANG: We have a 2.5×10^5 cells per T-60. They're not confluent cells.

QUESTION: Has that been logged?

DR. HUANG: Yes. Some cells are in log phase.

DR. LONGFELLOW: I might suggest that on those, if you're going to correlate with the S-phase, it might be interesting to follow those cells autoradiographically so that you can see relatively what portion of the cells are actually in S. I mean, just doing tritium incorporation doesn't really tell you anything about the number of cells that might be in there.

DR. HUANG: Yes. I agree with you.

DR. LONGFELLOW: If I can beg one more question from Dr. Jones. In that population, I am not sure whether or not these cells inactivate one of the X's. Do you know what happens in male human cells? Is that possibly a more sensitive assay since the TG locus was on the X?

DR. JONES: The P3 cells resemble the CHO cells in that one of the X chromosomes is functionally inactive, therefore I don't think a male cell-line would prove to be more sensitive.

DR. BRESNICK: My name is Bresnick, Eppley Institute. A collective question to Dr. Waters and Dr. Schechtman and also a question to Dr. Jones.

The collective question is: I wonder if it would be advantageous to use rat seminal vesicle microsomes as an activating system as well as using S-9? I mean, you are showing data with benzidene, you are showing data with 4-aminobiphenyl. Both these compounds are probably activated more by a prostaglandin endoperoxide synthetase reaction than they are by the P-450 dependent monooxygenases?

DR. WATERS: Let me just say, with regard to Dr. Huang's assay, I believe our approach is slightly different from the other assay that has been described.

We are trying to systematically work out the bugs in a rather straightforward human cell assay system that could be applied on a more routine basis, whereas the assay that you have just heard described is oriented towards improving metabolic activation systems doing the kinds of things that you are suggesting. So I think the question would be more appropriately addressed to Dr. Jones.

DR. BRESNICK: No. I disagree. I think the question is more appropriately addressed to you. You are using an activating system. If you are using an activating system, then use the appropriate one.

DR. WATERS: Well, I think the appropriate activating system might be a range of different primary cell types. But I think S-9 will be a usable activating system, as has been demonstrated in any number of systems. If we were trying to optimize for each side, for each chemical, we would optimize by using a whole series of different cell types, a whole series of activating systems.

Maybe I am missing your point.

DR. BRESNICK: Well, I am not sure I would use an intact cell -- and that is a question for Dr. Jones -- as an activating system.

DR. WATERS: Okay.

DR. BRESNICK: Because if you want an activating system --

DR. WATERS: We could go either way -- intact cell or various types of homogenates. The point is that we are trying to standardize a simple fibroblast system as opposed to trying to optimize a system for detection of an agent that's site-specific or metabolism-specific.

DR. SCHECHTMAN: I believe I can add a further comment addressing this issue. A number of these systems that will ultimately be used for the blind screening of potentially genotoxic agents. If an exogenous source of metabolic activation is to be employed in a situation in which the class of compounds being tested is unknown, it becomes feasible only to consider a rather limited number or set of exogenous metabolic activation systems in order to expedite testing and remain within reasonable financial boundaries.

The choice of metabolic supplement(s) would require judicious selection of one (or more) activation systems with the broadest applicability with respect to the spectrum of chemicals each is capable of metabolizing to bioreactive forms. To demand of each assay, which constitutes a battery of such genotoxicity tests, that it be performed in conjunction with metabolic supplements which have been derived from a variety of sources and/or have been induced with a variety of inducers (which would only by chance serve to elevate the specific and metabolically relevant enzymes) places unrealistic expectations on the philosophy of short-term testing. I would say then, in such a case, that without knowing whether a given S-9 would be useful in the metabolic activation of a given class of chemicals, we are probably going to be limited logistically to only a few (perhaps up to two) S-9's in conjunction with a given test protocol.

DR. BRESNICK: I would agree with that. My suggestion is that one would use an S-9 from an Aroclor-treated animal and that one would use an RSV, rat seminal vesicle microsomal preparation, just to pick up the endoperoxide synthetase co-oxygenation dependent types of mechanisms that could go on.

DR. WATERS: Well, I agree with the general trend of what you are saying, but wouldn't that depend on the prevalence of that type of compound in the series of compounds that you plan to investigate?

DR. BRESNICK: The trouble is we don't know what -- I mean, you're taking blind compounds --

DR. WATERS: Well, what would the prevalence be among compounds that we do not know about?

DR. BRESNICK: I don't know. I'm not sure anybody knows that. Do you know that for sure?

DR. WATERS: No, I don't. But I think you should like to be able to answer that sort of question.

DR. BRESNICK: I don't want to engage in a dialogue. May I ask -- the second question really is kind of a flip side of that coin. If you are going to use an activating system only for the purpose of activation, then why interpose a membrane, although it be a natural membrane, between your target cell and your activating system? In other words, I am saying why use an intact system as an activating system?

DR. JONES: The choice of activating system really depends on the question one is trying to answer. We are interested in the organ specificity exhibited by chemical carcinogens and therefore we want to develop in vitro mutagenesis assays which will resemble as closely as possible the situation for a particular organ in vivo -- especially in terms of metabolic competence. For

example, primary intact hepatocytes contain the full complement of drug metabolizing enzymes -- involved both in carcinogen activation and inactivation -- and these enzymes are operating under the normal cellular control mechanisms that exist *in vivo*. Therefore this is not the most "efficient" activation system to use in routine testing since competing inactivation pathways are present, but on the other hand, the degree and type of activation of a particular chemical will more accurately indicate the fate of the chemical *in vivo* in that organ from which the activating cells were derived.

DR. BRESNICK: No. In an *in vitro* assay, you are not measuring the susceptibility of that activating system to your mutagen, you are using it as an activating system.

DR. JONES: True. However, the advantage of using an intact activation system is that this approach is more representative of what is happening *in vivo* and, while it may not be the most sensitive system to indicate the frank mutagenic potential of a chemical, it may be the most realistic. It may also not be the most economic for the reasons that Len has said. I would not recommend these kinds of assays as routine screening procedures, but as a means to examine the role of metabolism in the expression of organ/tissue specificity in chemical carcinogenesis, it might constitute a more valid approach. Although the activation takes place in one cell type and the mutagenic response is determined in a different cell type, the existence of cell membranes does not appear to be a problem. Kuroki (1978) showed that if the activating and target cells are in close proximity, the transfer of activated chemical intermediates between cells is very efficient. Leila Diamond (1982) also showed that the spectrum of DNA adducts generated in the target cells is exactly the same as the present in the cells where the chemical activation occurred. Ideally, it would be nice to look at carcinogen activation and the induction of mutations in the same cell type and we are trying to do this.

DR. SCHECHTMAN: Excuse me. Before you go on -- I would just add one more comment on the use of hepatocytes in mammalian cell transformation and mutation assay systems. One of the critical factors which dictates the feasibility of using hepatocytes as the source of exogenous metabolic activation is the endpoint being monitored, such as colony formation versus focus formation on a background monolayer of confluency.

We have found, generally, that hepatocytes tend to interfere with the uniform establishment of the contact-inhibited monolayer of target cells. Thus, even though the hepatocytes might be functional metabolically, generating those metabolites required to transform the target cells, at times the focus endpoint becomes unreadable against the mottled background, making interpretation of results much more difficult. On the other hand, in mutation and transformation assays whose endpoint relies on colony formation, hepatocytes interspersed among target cells are seemingly quite compatible with one another. Should residual hepatocytes fail to detach during the course of the assay, they do not interfere with the clonal development of the mutated or transformed target cells. Colonies can be readily quantified and examined for morphological aberrations.

DR. RICE: Rice, NCI. Both Dr. Schechtman and Dr. Waters presented positive results for carcinogenic metal salts, including compounds for which the putative carcinogenic metal is present either as a cation, a nickel sulfate, acetate, or as part of a complex anion. I wondered if you would want to offer any comment or hypothesis about how those compounds are exerting their effects in your system, in particular whether they are acting directly on the DNA, or whether there is some sort of indirect mechanism involved?

DR. WATERS: That is a difficult question and it might be dependent on the specific metallic salts with which one is dealing.

I would just like to say in response that we have reviewed the literature on the chromium salts, which was representative of the compound that we did evaluate in the human fibroblast system. The data, very interestingly, on the chromium salts in the Ames test, would indicate that for the most part they are demonstrating mutagenic activity as frameshift mutagens. In fact, if you do a comparison of the profile of the results that are obtained in a variety of different short term genetic assay systems -- in fact we have looked at over a hundred -- it turns out that the profile is very similar to aflatoxin. Now, I don't have any explanation as to why that might be. But statistically that association is showing up. So, that particular metal is displaying its mutagenic effects in a rather peculiar way for such a simple material.

Other metals we know a little bit more about -- nickel, for example. Maybe Lenny would care to address that one.

DR. SCHECHTMAN: Studies examining the genotoxic effects of various nickel salts have been reported by Sunderman and other investigators. Nickel compounds have been shown to cause localized sarcomas and carcinomas. Epidemiologic observations of cancers acquired by nickel refinery workers suggest that early-stage by-products of nickel refining, such as nickel subsulfide, nickel oxide and nickel carbonyl, may be the reactive derivatives. I believe there is a 1975 IARC publication which reviews the carcinogenic risks associated with nickel. Is that right?

DR. WATERS: Yes.

DR. SCHECHTMAN: With regard to the possible mechanism of the transformation event induced by metals, this is a subject which requires further analysis. Whether the insult imparted is a macromolecular one in which DNA is the critical target, or the event is mediated via an effect on one or more enzymes associated with DNA synthesis, is as yet an open question. Bruce Casto, using an *in vitro* assay that monitors the chemical enhancement of simian adenovirus SA7 transformation of Syrian hamster embryo cells, has suggested that the genotoxic effects imparted by inorganic metal salts require cellular DNA synthesis and may mediate transformation by interfering with the fidelity of DNA replication. Sirover and Loeb have suggested that some metals may interfere with DNA polymerases, resulting in copy errors during DNA synthesis.

DR. RICE: Well, it is particularly interesting that, in the case of your compound, lead subacetate with its pronounced organ specificity for the kidney epithelium, that you pick it up in the C3H 10T 1/2 assay.

DR. SCHECHTMAN: Although organotropism may be important in vivo, the pharmacokinetics operative in vivo are certainly dramatically altered in vitro, as are other related physiological conditions. Admittedly, as screening tools, the fibroblastic systems are being asked to respond to a wide range of chemical classes. Ordinarily these cells would not be expected to respond to many of these agents. However, often these fibroblastic systems have, in fact, responded. Possibly, this responsiveness is a reflection of some basic mechanism of transformation common to both fibroblasts and epithelial cells, such as some pre-neoplastic early stage aberrant phenotype.

Nevertheless, the C3H 10T 1/2 cell system was responsive to this metal salt, but the induced transformation was manifest only upon amplification of the aberrant phenotype using the Level II/Amplification procedure. It is possible, then, that manifestation of the transformed phenotype induced by such metal salts in this cell system requires an extended expression period. The Level II replating procedure thereby provides that opportunity for further cell proliferation and DNA replication which may be necessary beyond that afforded prior to the establishment of the initial confluent state.

DR. ULVEDAL: Dr. Weisburger?

DR. WEISBURGER: Elizabeth Weisburger, NCI. I have a question of Dr. Jones. Have you characterized your cell line by any other means than just the chromosome numbers?

DR. JONES: No. We know that the wild type P3 cells have a stable diploid karyotype with double X chromosomes and a translocation between chromosomes 15 and 20. In all the TG-resistant mutants we have analyzed, this karyotype was unaltered by the mutagen treatment. That is all we have done so far.

DR. WEISBURGER: You don't know what type of cell it's actually derived from? I mean, teratoma can be derived from many different cell types?

DR. JONES: No, we don't. The cell-line was originally cloned from a human ovarian teratocarcinoma by Dr. Giovanella. We have subcloned a cell population which is suitable for mutagenesis studies. We have tried to differentiate the cells by treatment with chemicals such as phorbol-12-myristate-13-acetate and dimethylsulfoxide but without success. However the untreated cells did react with monoclonal antibody that is directed against a neuronal membrane antigen, so perhaps the P3 cells were derived originally from a neuronal cell type.

DR. ULVEDAL: Any other questions?

DR. CAMERON: Cameron, NCI. Len, you recently were an invitee at an IARC meeting on the Balb/C 3T3 and the C3H 10T 1/2 cell transformation systems. Would you share some of the conclusions?

DR. SCHECHTMAN: Essentially, the workshop involved an in-depth analysis of the Balb/C 3T3 clone A-31 and C3H 10T 1/2 clone 8 cell transformation systems with special emphasis on mechanisms and application to screening for potential carcinogens.

The systems were evaluated in terms of their advantages and disadvantages, ability to detect carcinogens, cellular and molecular mechanisms of the transformation process, available protocols, procedural modification and improvements, requirements for supplemental metabolic activation, relationships between *in vitro* cell transformation and *in vivo* carcinogenicity, and the criteria employed in scoring the transformed phenotype.

One of the primary goals of the workshop was the generation of recommended standardized protocols for each system. It is the working group's hope that these standardized protocols, once published, will then be used by various testing laboratories in order to validate the assay systems and to limit interlaboratory variability.

There are many different laboratories using many different approaches even with the same target cell systems. There are also many different laboratories using different subclones of a given target cell system. So, in order to help standardize each of the systems, the working group recommended the specific clones, specific assay methodologies, specific treatment periods, specific scoring procedures, specific ways of interpreting the results, etc.

There will be an IARC scientific publication detailing the various aspects of the workshop. In addition, a Meeting Report submitted to Cancer Research will briefly outline the efforts of the workshop participants and will include the recommended assay protocols for each system.

DR. ULVEDAL: Are there any other questions? Then I would like to thank the speakers and the audience, and we will see each other again at 1:30 after lunch. Thank you.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Thursday Afternoon, March 22

SESSION III - EPIDEMIOLOGY
Chairman: Dr. Howard M. Hayes
National Cancer Institute

SESSION III - EPIDEMIOLOGY
Chairman: Dr. Howard M. Hayes, NCI

DR. HAYES: This afternoon we will first begin with Dr. Philip Landrigan, who will be substituting for Dr. Robert Rinksy, who had an illness in the family and could not be with us today.

PROCEEDINGS OF THE
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PRESENTATION:

Mortality and Industrial Hygiene Study of Benzene Workers

Dr. Robert A. Rinsky
National Institute for Occupational Safety and Health

Presented by:

Dr. Philip J. Landrigan
National Institute for Occupational Safety and Health

Mortality and Industrial Hygiene Study of Benzene Workers

Dr. Robert A. Rinsky, NIOSH

This afternoon I am going to summarize the work which has been done over the past decade at NIOSH to evaluate the relationship between occupational exposure to benzene and death from leukemia.

The association between benzene exposure and leukemia was first recognized in a series of case reports published from the 1890's to the 1960's. To evaluate this association more systematically, NIOSH undertook a combined epidemiologic and industrial hygiene study. The epidemiologic study employed a retrospective cohort design. It examined a population of 1,006 non-salaried white male workers who had been exposed to airborne benzene vapors at two rubber plants in Ohio. The only chemical present at these plants which was known to be associated with blood dyscrasias was benzene.

The study population was divided into two groups. Group 1 consisted of 748 men who worked at least 1 day between January 1, 1940 and December 31, 1949, in a department with benzene exposure. Group 2 consisted of 258 men who first worked in a department with benzene exposure between January 1, 1950 and December 31, 1959.

Vital status follow-up was conducted to June 30, 1975. Vital status was ascertained for 98% of the cohort. The remaining 2% were considered to be alive as of the study end-date. Death certificates for all known deaths were obtained and coded by a qualified nosologist according to the rules of the International Classification of Disease Adapted for Use in the United States (ICDA) in effect at the time of death, and were then converted to 7th revision codes. A NIOSH life-table computer program was used to calculate expected numbers of deaths by cause within 5-year age and 5-year calendar time periods. Age- and calendar-specific U.S. white male death rates were used as referents. Observed and expected cause-specific mortalities were compared using a two-tailed test of significance based upon the Poisson distribution.

INDUSTRIAL HYGIENE BACKGROUND

As knowledge of benzene toxicity increased over the years covered by the NIOSH study, recommended levels for occupational exposure were continually revised downward as follows:

<u>Year</u>	<u>Recommended Standard</u>
1941	100 ppm
1947	50 ppm 8-hour time weighted average (TWA)
1948	35 ppm 8-hour TWA
1957	25 ppm 8-hour TWA
1963	25 ppm ceiling
1969	10 ppm 8-hour TWA

Industrial hygiene information concerning atmospheric concentrations of benzene vapor at the plants was available to NIOSH from several sources. This information enabled us to compare actual occupational exposure to benzene with those standards in force at the time of the exposure.

Our analysis of the available industrial hygiene data led us to the conclusion that although there were occasional excursions above recommended limits, for the most part, employees' 8-hour time-weighted average exposures to benzene were within the recommended standard in effect at the time of the exposure.

RESULTS OF THE MORTALITY STUDY

Group 1

Among the cohort who worked at least 1 day in a benzene-exposed department at either location between 1940 and 1949, there were 180 deaths from all causes, compared with an expected number of 161. Although not statistically significant, this increased number of deaths from all causes was nevertheless unanticipated, since in an industrial population one normally observes fewer than the expected total number of deaths.

Moreover, there was a statistically significant excess of deaths from malignancies of the lymphatic and hematopoietic system (ICDA codes 200-205). Ten such cases were observed vs. 3.03 expected ($p < 0.01$) (Table 1). When leukemias (ICDA code 204) were considered alone, seven cases were observed vs. 1.25 expected ($p < 0.001$). Although the ICDA code for leukemia encompasses lymphatic, myeloid, and monocytic leukemias, all seven cases were of the myeloid or monocytic cell types, as ascertained by death certificate designation. Five of these seven cases were seen by one hematologist, who confirmed the diagnosis.

Of the 748 workers included in Group 1, 437 (58%) had exposure to benzene for less than a year. Because only 1 day of exposure qualified a worker for entry into the cohort, individuals with short duration of exposure contributed a large proportion of the total person-years at-risk used to calculate the expected number of deaths. Inclusion of these persons with brief exposures served to lower the calculated risk of death from leukemia from what it would have been if a requirement for minimum duration of exposure had been imposed. When exposure to benzene is examined in terms of duration, a sharp increase in the SMR is seen among the group of workers exposed for longer than 5 years. For those persons who had attained 5 or more years of exposure, 5 leukemia deaths were observed, while only 0.23 was expected (SMR = 2100). Also for those with more than 10 years of employment, 3 leukemia deaths were observed, whereas only 0.09 were expected (SMR = 3300).

The observed excess of leukemia deaths remained evident when the Group 1 cohort (1940-1949) was divided according to location and the locations examined separately. At Location One, two cases of leukemia were observed vs. 0.58 expected, yielding an SMR of 345. At Location Two, five cases were observed vs. 0.67 expected, yielding an SMR of 746. Thus, both locations considered as independent cohorts experienced an excess of leukemia deaths.

Group 2

Among the cohort who first worked in a benzene-exposed department between 1950 and 1959, there were 49 observed deaths from all causes compared to 56 expected (SMR = 87). In group 2, one case of leukemia was observed vs. 0.46 expected (SMR = 217).

CONCLUSION

These data show a strong association between occupational exposure to benzene and death from leukemia. They suggest strongly that the association is causal. They therefore form a basis for preventive action.

From the combined epidemiologic and industrial hygiene data obtained in this study it was possible to undertake a quantitative assessment of the risk of leukemia in workers exposed for a working lifetime (40 years) to benzene vapors. In this risk assessment, it was calculated that in a population of 1000 workers exposed for a working lifetime to 100 ppm benzene vapor, 140 excess deaths from leukemia would occur. Similarly, at a lifetime exposure of 10 ppm, it was calculated that 14 excess leukemia deaths would occur.

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OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Mortality and Industrial Hygiene Study
of Workers Exposed to Dyes

Marie K. Haring-Sweeney
National Institute for Occupational Safety and Health

Mortality and Industrial Hygiene Study of Workers Exposed to Dyes

Marie K. Haring-Sweeney, NIOSH

Approximately five years ago, there was a general concern in the scientific and the lay communities at large about the relative safety of hair dyes. This concern was based on the observation that some chemicals in permanent and semipermanent hair dyes were mutagens by the Ames test.

Since then more extensive research using animal models has determined a few of these same chemicals to be carcinogens. Recently, in the Third Annual Report on Carcinogens by the National Toxicology Program, two components of hair dyes, 2,4-diaminotoluene and 2,4-diaminoaniline sulfate, also known as paraphenylenediamine, were reported to be suspect carcinogens based on sufficient animal data.

In humans, exposure to hair dyes has been associated with increased mortality due to aplastic anemia, leukemia, and cancers of the bladder, breast, and lung. These result from use of hair dye or from hair dressing as an occupation.

However, it is important to note that the epidemiologic studies which ascribe the above causes of death to hair dye use are fairly limited in their ability to causally relate mortality with a specific dye product or substance.

Due to the common concerns among consumers and workers who both use and manufacture products containing hair dyes, we initiated this project in the effort to quantify the risk of exposure using cause of death as an indicator of health outcome.

During the planning phases of the project our first inclination was to assemble for study a cohort of beauticians and cosmetologists because their profession specializes in the application of hair care products and we assumed that they would have had some exposure to hair dyes. However, we found this group to be unsuitable for a cohort mortality study because of their general mobility--they tend to move from shop to shop and place to place frequently--and because of the lack of a single group large enough to study.

We then identified another population whose exposure to hair dye chemicals--and you might want to put that in quotes--has been reported in the literature since the early part of this century.

Fur workers have used phenylenediamines as fur colorants since the late 1890's. Phenylenediamines were used primarily in the dark color for a tint. Until the late 1970's, paraphenylenediamine and the 2,4-diaminoaniline sulfate, as well as the 2,4-toluene diamine, were used as basic ingredients in brunette, brown, black shades of permanent type hair dyes. They were taken out of most of the hair dyes before 1980.

The study population of fur workers was identified through the pension program of the Joint Board of the Fur, Leather and Machine Workers Union, otherwise known as the FLM. This union has represented fur workers in the New York City area since approximately 1930.

The pensioned workers were members of separate union locals under the FLM and they included fancy fur, lamb and rabbit dyers, fur dressers, and fur service workers. Since these job categories are a bit unusual and not well known in the modern industry, a brief description of each is in order.

Fur dyers tint or color the furs by using a semi-automated vat process or by actually brushing the dyes onto the furs. Exposures are reported to be to the permanent or oxidative dyes--phenylenediamines and toluene diamines--as well as other dyes containing lead and chromate.

Fur dressers remove the excess flesh and fat from skins. They also tan the leather portion of the skin prior to the dyeing process. Their reported exposures include the tanning chemicals, such as chromate and arsenicals, and wood dust, which is used to absorb the excess tanning chemicals from the fur and the leather.

Just to give you an idea of what some of the working conditions in the past were and actually are now -- this is a fur service worker. These people clean, grade, match and bale raw and dressed fur. Their potential exposures include residual tanning chemicals if they grade and match the furs after the tanning operation.

It was with these three groups of workers that we conducted a historical prospective mortality study. The 807 fur workers included in the cohort were pensioned by the FLM between January 1, 1952 and December 31, 1977, the beginning and ending dates of our study.

All study participants were required to have 20 years of continuous active membership in the union and to be 62 years of age or older when they entered the cohort. All demographic data and information pertaining to usual occupation prior to retirement was abstracted from the pension application and other union records.

Because neither detailed job histories nor work-place exposure information were available from company or union records, we used the fur worker category dyer-dresser-service worker as the best indicator of occupation and potential for dye exposure.

The vital status was determined as of December 31, 1977 and the underlying cause of death was coded using the rules and rubrics of the International Classification of Diseases revision in effect at the time of death. Using the NIOSH Modified Life Table Analysis System, cause-specific expected numbers of deaths were calculated based on mortality rates of white males both in the U.S. and in New York City and adjusted for 5-year age groups and calendar time periods to control for any confounding of each of these variables. Mortality risks were estimated using the standardized mortality ratios.

Although we calculated SMRs for both U.S. and New York City white male population comparisons, any further descriptions and discussion of the results are based on the New York City population. We felt that the New York City population was a more appropriate group to use for comparison because of the differential in cancer mortality rates among urban, suburban and rural residents.

Vital status was ascertained for 100 percent of the 807 members in the cohort, and death certificates were obtained for 96 percent of the 468 decedents.

In the Life Table analysis, we found that among fur dyers total mortality was lower than expected due to decreased mortality from cardiovascular diseases and nonmalignant respiratory diseases. However, there were slight increases in cancers of the stomach, lung, and colo-rectal systems.

Among fur dressers mortality from all malignant neoplasms was significantly higher than expected. This excess appears to be due to a two-fold increase in lung cancer deaths and a slight statistically nonsignificant increase in mortality from colo-rectal neoplasms.

Fur service workers were the only group to experience a statistically significant increase in cardiovascular-related deaths and they also had a slight elevation in colo-rectal cancers, and slight elevations in stomach cancer.

We also found that approximately 80 percent of the cohort was foreign-born. Because mortality patterns for lung, stomach and colo-rectal cancers are reported to be differentially distributed among immigrant groups residing in the U.S., we evaluated these causes by place of birth. Four place of birth categories were constructed for this purpose. They included people born in the United States, those in Eastern European countries, Italy, and an aggregate group of other countries.

To compare fur worker categories by place of birth, we calculated a series of rates directly adjusted for age and standardized by the person-years at risk for the entire cohort. The rate ratios for cause, occupation and place of birth were calculated by dividing the directly age-adjusted rates for the cohort by the age-adjusted rates for New York City.

We observed elevated rate ratios for lung cancer primarily among Eastern European fur dressers. Although the numbers are small, it appeared there was an increase in stomach cancer mortality among Italian-born fur dyers. Finally, the Eastern European fur workers appeared to have the greatest risk of death for colo-rectal cancers. This finding has corroborated the results of other studies of male immigrants from Russia and Eastern European countries. These other studies reported consistently elevated mortality from colon and rectal cancers among immigrants compared to white native-born males.

In summary, the primary purpose of this investigation was to assess the health effects of long-term human exposure to permanent or oxidative hair dye chemicals. The results of this cohort study suggest that, after controlling for urban residence and ethnic effects, the increased stomach cancer mortality observed in the fur dyers may be influenced by occupational exposures.

These findings are merely suggestive, inasmuch as there were no objective measurements of dye exposure, only historical counts of heavy repeated contact with these types of dyes.

The excess lung cancer observed among the fur dressers was an unexpected finding but seemed plausible because fur dressers were exposed to tanning chemicals containing chromates and arsenicals.

Lastly, we also recognize the limitations of this study. The evaluation of a retiree or a survivor population has advantages and disadvantages. One of the distinct disadvantages is the potential loss of individuals who left the industry prior to achieving retirement age. This may be the case in this occupation.

The phenylenediamines are known respiratory and dermal irritants causing delayed hypersensitivity among people who are sensitive. There are reports in the literature of people becoming sensitized and leaving the industry prior to reaching retirement age. But the advantages of using a retired population are also pretty good in that follow-up is usually high. Latency is long and these people had at least 20 years of exposure. There is also a reduced healthy worker effect. All of these factors are noteworthy.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Study of the Dupont Chambers Works Plant
Bladder Cancer Screening Program

Dr. Thomas J. Mason
National Cancer Institute

STUDY OF THE DUPONT CHAMBERS WORKS PLANT BLADDER CANCER SCREENING PROGRAM

PRESENTER/PROJECT OFFICER: Dr. Thomas J. Mason, NCI

First of all, I would like to acknowledge the cooperation and support of Dr. Bruce W. Karrh, Vice President for Safety, Health and Environmental Affairs of the DuPont Company, who facilitated the collaboration of Dr. Philip Prorok, who is now in the Division of Cancer Prevention and Control, and myself with Dr. William Neeld, who is the Medical Director of the DuPont Chambers Works, and William Vogler P.A., the Medical Administrator. We have been abstracting information from the medical records on a large group of individuals who have been screened by urinary cytologic methods for bladder cancer, as well as persons who developed their bladder cancer before the cytology program started.

Today I will present a progress report on our study. At present there are no completed analyses, but we do have some rather provocative preliminary tabulations which I will share with you.

In 1954 DuPont instituted a urinary cytologic screening program. We have been able to abstract information on all persons ever enrolled in the screening program, whether or not they went on to develop bladder cancer, as well as all historic bladder cancers for the entire history of this particular plant. Persons were enrolled in the cytology program if it was likely that they were exposed to either beta naphthylamine or benzidine.

There are 1,901 persons in our study, 316 of them have been diagnosed with bladder cancer. It is primarily a male work force, and almost all are white, although 6 bladder cancers have been identified among the black workers. We have information on 142 bladder cancer cases with cytology, and 174 bladder cancer cases without cytology.

What we have done over the past few years is develop an approach for abstracting information for all of these persons independent of whether or not they subsequently developed bladder cancer. From lifetime occupational histories we have, by job title/location on the plant and calendar time an independent assessment of the potential for exposure to beta naphthylamine and/or benzidine with some several hundred persons exposed only to benzidine. The potential exists within this particular data set to calculate a person months cumulative exposure score by job title and location through calendar time.

We have abstracted data from physical exams, on all persons by type of exam, whether it was pre-employment, annual, special, retirement, or post-retirement, for these persons are indeed entitled to a post-retirement physical examination.

Data have been abstracted from all urinalyses which were ever performed on all of these persons, with specific emphasis given to the notation of hematuria. The clinical contention is that hematuria is the better predictor of early

bladder disease, and that the cytology results may play a role in the complete workup of a particular individual. Cytologic readings were performed by one individual, and all readings have been abstracted.

Of specific interest to us are the early signs and symptoms of bladder disease. We utilized all of the nurses in the clinic at the DuPont Chambers Works and have successfully abstracted the entire lifetime clinical histories of all bladder cancer cases. Particular emphasis was given to genitourinary complaints such as frequency, burning, abdominal pain, coming to clinic because of blood in the urine, noting whatever procedures were followed and their findings as well as recommendations for follow-up. We will have the potential--and I will share some of that with you as I close--for looking at the timing of all of these clinical events.

The importance of this particular data set is that it is a very rich one. In addition to the above mentioned data we have pathologic confirmation on every case with specific emphasis given to type, location, grade, stage, multicentricity, evidence of metastatic spread, multiple primary tumors, and sites of secondary tumors.

What are some of the characteristics of this study population? If one looks at the number of hematurias found per person in the clinical histories, among our screened bladder cancer cases the median number of hematurias is 6 with an

extreme of 167 mentions for one person of discrete episodes of hematuria. Among our non-screened cases the median is 4 with an extreme of 61. Among our noncases the median is 2 with an extreme of 55.

What about timing? If hematuria is sentinel to early bladder disease and if time between subsequent hematurias is a reasonable clinical indicator, then one might a priori posit that among the nonscreened cases, because the potential exists for their having realized greater exposure, you might find a shorter time interval between subsequent hematurias.

Purposefully, I have considered the extremes. I have examined the times between the tenth and the eleventh mention of hematuria. These are persons who had at least 10 prior episodes. The median time between the tenth and the eleventh hematurias for the nonscreened case is 79 days, for the screened cases it is 115 days, and for the noncases 160 days.

What about the number of cytologies? The screened cases have a median of 10, the non-cases of 13. The non-cases have continued working, and we have a few additional cytologies per person.

What about the first cytology reading? Simply looking at the proportions of the first cytology readings, for which there was a finding of frankly malignant cells in the urine, the proportion among the persons who subsequently developed bladder cancer is ten times that of the persons who did not.

Now, where are we at present? We are finishing the editing of all of these data. By the end of April 1984 we will have in place programs which will allow calculation of a person months exposure score. Once that is done, we will have the ability to stratify our study population as a function of intensity of exposure and to utilize all of the clinical signs, whether they are hematuria or other GU signs, as well as the timing of the first diagnosis of bladder cancer, the extent of the disease and timing from first diagnosis to death.

All of you are cordially invited to hear me present the results of a number of these analyses at a meeting jointly sponsored by the NCI and NIOSH in Cincinnati in July. It is going to be called a Conference on Medical Screening and Biological Monitoring for Exposure in the Work Place.

At that time, hopefully we will be able to report that there is some indication, first of all, that the level of severity of cytology responds to increasing exposure to the two compounds in question and answer some fundamental questions concerning the time sequence of genitourinary signs and/or symptoms, related to exposure to beta naphthylamine and benzidine in the subsequent development of bladder cancer. We also will address some of the differences between persons who went on to develop bladder cancer and those who did not.

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PRESENTATION:

Mortality Surveillance Among Metalworkers

Michael Silverstein, M.D., M.P.H.
Robert Park, M.S.
Neil Maizlish, M.P.H.
Franklin Mirer, Ph.D.

International Union, United Automobile Workers

MORTALITY SURVEILLANCE AMONG METALWORKERS

MICHAEL SILVERSTEIN, M.D., M.P.H.
ROBERT PARK, M.S.
NEIL MAIZLISH, M.P.H.
FRANKLIN MIRER, PH.D.

INTERNATIONAL UNION, UNITED AUTOMOBILE WORKERS

The United Automobile Workers (UAW) represents 1.2 million workers in more than 2500 workplaces throughout the U. S. and Canada. These workers face an enormous range of potential health hazards on jobs ranging from forging and welding to spray painting and salt mining.

In recent years many UAW members have expressed intense concern with apparent excesses of cancer mortality in various workplaces. It became clear that the Union needed the independent capacity to investigate these concerns promptly so that threshold decisions about health and safety strategy could be made on sound grounds. For example, what is the appropriate response to a reported cluster of colon cancer deaths among workers in a plant—a comprehensive mortality study, immediate industrial hygiene evaluation and environmental control, a medical surveillance program, or worker education? Epidemiologic tools needed to be adapted for field use to assist in choosing between such alternate courses of action. Over the past 3 years the UAW has begun to develop the pieces of a mortality surveillance system as part of such a triage process.

Since this effort began we have reviewed over 50 situations where some epidemiologic judgment was needed for first level health and safety evaluation. In six of these we have decided that additional, more substantial in-house epidemiologic study

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was the next step. Our method of choice has been proportional mortality with nested case-control analysis. This strategy has inherent limitations, but also has two major advantages. First this approach is often feasible using readily available records, thereby permitting studies with relative speed and economy of scarce resources. Second, these methods reveal mortality patterns and associations which, though not necessarily complete and definitive, are meaningful and provide a rational basis for step by step public health decision making. Indeed, these six studies, in various stages of completion, have already yielded valuable information.

The first study, which is being completed this month, is an investigation of mortality among workers in a large ferrous foundry. Statistically significant excess proportional mortality due to non-malignant respiratory disease (PMR=177), lung cancer (PMR=148) and lymphopoietic cancer (PMR=198) was found among 221 white male deaths. (Table 1) Case control analysis further demonstrated a significant association between non-malignant respiratory disease and finishing department work after controlling for the effects of age, prior occupations in coal mining or foundries and smoking. (Table 2) A significant excess in proportional mortality for lung cancer was also found for white males who worked in the finishing departments. This association was also found in case-control analysis, but after control for age, prior work and smoking was no longer statistically significant. (Table 3) Among a smaller group of black male deaths there was a significant excess of proportional mortality due to cardiovascular disease (PMR=143) which was, in some but not all analyses, associated with work in the melting and pouring areas of the plant. (Table 4)

These foundry findings are generally consistent with other reports. They do not, however, clarify the relative contributions of silica, other mixed particulates and

polynuclear aromatics to the development of respiratory disease in foundry workers. More progress on this front will probably have to come from toxicologists and industrial hygienists. The cardiovascular findings are new and need validation. If validated, a potential association with carbon monoxide exposure should be investigated.

A substantial amount of industrial hygiene data from this foundry was reviewed. Although the epidemiologic findings reflect historic conditions in this foundry, there is ample evidence to suggest that conditions have not changed appreciably enough over the past 25 years to warrant assurance to workers that the period of high risk has ended. In fact it is likely that substantial hazards still exist and that there is a need for full implementation of currently available control technology to reduce exposures of danger to workers.

The second study is an investigation of nearly 800 deaths among ball bearing plant workers potentially exposed to cutting fluids and abrasives associated with typical machining and grinding operations. The major findings are statistically significant excesses in proportional mortality from stomach cancer (PMR=198), rectal cancer (PMR=304) and stroke (PMR=135) among white men. (Table 5) In case control analysis there was a substantial association between stomach cancer and grinding but not machining work, after control for age and country of birth. (Table 6) It was not possible, however, to distinguish between those exposed primarily to straight oils or primarily to water emulsified oils. Other findings of interest included a cluster of lung cancer deaths among women grinders and a possible association between an ill-defined group of non-malignant central nervous system disorders and grinding work among white men.

Our third study offers an excellent opportunity to validate these bearing plant findings and to test hypotheses which have been generated. 2000 deaths among workers

at a second bearing plant located just several miles from the first are under review. The two plants have used similar manufacturing technology to produce similar products and have employed worker populations comparable in many respects. This second bearing plant study will be able to incorporate information from a more extensive set of job history and industrial hygiene records.

The fourth study was initiated following a report commissioned by the Motor Vehicle Manufacturers' Association which revealed excess proportional mortality from several cancer types among workers in automobile assembly plants. We have now completed a similar analysis of deaths from three auto and one truck assembly plant. (Table 7) Among 1217 white male deaths there were small proportional excesses of lymph, lung, and stomach cancer, only the latter being close to statistical significance. The prior MVMA study had found significant excesses of respiratory cancers, lymphomas, and some digestive cancers. Among 159 black male deaths we found small excesses of lung, colon and pancreas cancer. The MVMA study had found excesses for respiratory and liver cancers among black men.

Although we have, with employer assistance, gathered complete work history records for deceased workers from two of the four assembly plants we have not yet decided the extent to which we will proceed with further analysis. One finding to pursue may be the marked deficit in stroke mortality among the black male decedents. Only 4 stroke deaths occurred while 15 were expected ($PMR=.27$). Stroke mortality among the white men was about what was predicted.

Preliminary results from our fifth investigation are particularly interesting when compared with results from a recent NIOSH mortality study. (Table 8) Both are proportional mortality studies among workers in plants manufacturing electromechanical

equipment for the aerospace industry, including gyroscopes, guidance systems, and landing gears. The two plants are remarkably similar in physical appearance, product mix, and production technology. The NIOSH study was conducted in response to worker reports of a brain cancer cluster. A statistically significant threefold excess was demonstrated. Our study has not identified any brain cancer deaths but both studies unexpectedly have found substantial excesses of pancreatic cancer and, among white women, genital cancers. In our series these were predominantly ovarian tumors. We are now in the process of completing vital status ascertainment, coding job histories and reviewing the history of chemical exposures in the plant. Case-control analyses are being planned.

The final study in this series will examine about 400 deaths among workers from a large group of small auto repair shops and dealers. We are particularly interested in the mortality experience of the auto repair workers who are potentially exposed to asbestos, gasoline exhaust, and various other oils and solvents. Most of the death certificates have been obtained. Current efforts are directed at gathering job histories and smoking histories through questionnaires and interviews with co-workers of the deceased.

While these six investigations are the most rigorous we are currently doing, we have obtained some of our most useful health hazard information from rapid response surveillance techniques, confirming our view that epidemiologic principles have an important role in the day to day activities of an occupational health program.

One method which has been particularly valuable in assessing the relative urgency of many simultaneous worker concerns has been established in Michigan by making use of the State's computerized death registry. Three steps are involved:

First, when a potential problem is identified—as, for example, when a union representative recently noted that more than half of a series of retiree deaths coming

to his attention were due to cancer—the Local Union and International Union systematically search their records. The immediate goal is a listing of persons at risk, or known to have died. The full cohort of interest, all those who worked in the plant over an extended period, usually cannot be generated quickly. However, an inventory of available records such as seniority or dues lists generally gives a useful approximation. The fact that such a list might have gaps is not a deterrent to this triage process, as long as there is reasonable assurance that major selection bias has been avoided. This often means using a subgroup for which nearly complete and unbiased ascertainment is possible, such as all those hourly employees with at least ten years of credited pension service.

Second, names and social security numbers on the list of deceased or at-risk persons are matched against computerized mortality files obtained from the State of Michigan Department of Public Health. These files contain all in-state deaths since 1970 with underlying cause coded by the ICD classification in effect at the year of death.

Third, the resulting file of deaths with underlying cause and demographic data is used as input to the Monson program (with revisions) which generates standardized proportional mortality ratios. (1)

This system played a central role in the evaluation of suspicions of excess cancer mortality brought to our attention by workers at a large metal stamping plant.

A search of available union and employer insurance and pension records identified 98 deaths in a twelve year period among local union members ever-employed at the plant. A rapid review of 62 deaths in the Michigan death registry revealed possible cancer excesses. This was incentive for us to obtain the remaining 32 certificates for

Michigan deaths which had occurred before the State computerized its files.

Among 91 white male deaths there were statistically significant excesses of proportional mortality from all cancers combined (PMR=189), digestive cancers (PMR=226), lung cancer (PMR=220) and leukemias (PMR=428). (Table 9) Essentially the same pattern was found in the subgroups with five or more years seniority.

During a site visit it was noted that wood block floors in this plant are set and protected with hot coal tar in an operation that closely resembles roof tarring. Millwrights have been responsible for laying the floor tar. These millwrights, in addition, work closely with the maintenance welders who weld, torch metal parts and flame-cut floor bolts close to the floor level where tar and wood blocks sometimes ignite and generate smokey emissions. These activities had been done with a minimum of local exhaust ventilation or respiratory protection.

Using all non-cancer deaths as controls there was a strong association between cancer mortality and work as a maintenance welder or millwright, the odds ratio being 10.4. (Table 10) This association remained strong after stratifying for age and prior employment as a welder or millwright. Odds ratios for lung cancer and digestive system cancer followed a similar pattern, although statistical power was diminished with decreasing cell sizes and the ability to stratify on potential confounders was limited.

Our judgment is that these findings are important for two reasons:

First, hypotheses have been generated which are of both practical and conceptual interest. The workplace associations call for validation at another location where similar work is done in such a manner that the combined and independent effects of coal tar and welding exposures can be evaluated. The relatively short employment

durations associated with large apparent cancer risks suggest the possible presence of potent carcinogens during floor tarring and welding. The separation and bioassay of specific components of the emissions may provide insights in this regard.

Second, the findings served the Union's need as an effective and efficient guide to action. This exercise had several obvious limitations: it is small and unvalidated; there was no control for cigarette smoking; all the death certificates did not receive an independent review; SPMR's are subject to distortion. However, despite these cautions the statistical associations gain considerable credibility because they have biologic plausibility. Exposure of roofers and coke oven workers to coal tar pitch volatiles containing polyaromatic hydrocarbons has been convincingly associated with excess multiple-site cancer mortality. The group with high risk in this study uses similar materials under analogous conditions, and, in fact, substantial exposure to known coal tar pitch volatiles has been documented during tarring operations. (Table 11) In 1979 personal and area samples during tarring revealed CTPV concentrations in excess of 200 ug/cu.m. as an eight hour TWA with peak concentrations much higher. Just a few months ago source samples at the tar pot demonstrated the existence of six polyaromatic hydrocarbons with known or suspected carcinogenic properties in concentrations exceeding that found in many coke oven and roofing surveys.

There is little question that these epidemiologic findings are preliminary. Yet in cases such as this it is prudent to base vigorous public health interventions on just such preliminary information. We must, in other words, work with what we have before us, not what we hope to learn after several more years of study. In this situation the employer was convinced to install a new thermostatically controlled electric tar pot, to purchase numerous portable ventilation units, to construct several exhausted welding

booths, and to strengthen its respirator program. Area sampling of floor tarring has shown a 50 fold reduction in the generation of polyaromatic hydrocarbons following the purchase of the new tar pot.

In summary, 3 years ago the UAW added an epidemiologic component to an existing occupational health and safety program. The progress and preliminary results in the area of mortality surveillance have already proven practically useful on the shop floor and promise to generate information of general scientific interest. We are encouraged by this experience and plan to extend these efforts to the investigation of disabling injuries and illness in addition to cancer mortality.

REFERENCE

1. Monson, R., "Analysis of Relative Survival and Proportional Mortality," Comput. Biomed. Res., 7: 325-332, 1974.

TABLE 1

FOUNDRY PROPORTIONAL MORTALITY

WHITE MALES

CAUSE	PMR
ALL CANCER	61/51.5=118
LUNG CANCER	28/18.9=148*
LYMPH CANCER	9/4.6=198*
NON-MALIGNANT RESPIRATORY	25/14.2=177*
CARDIOVASCULAR	105/113.7=92

* P < 0.05

TABLE 2

FOUNDRY CASE CONTROL			
WHITE MALE NON-MALIGNANT RESPIRATORY			
CLASSIFICATION	OR	EXPOSED CASES	P
FINISHING	3.7	15	.004
FOUNDRY	0.3	2	.10
CORE	2.0	5	.17
MIXED	0.3	2	.08
OTHER	---	0	.05

TABLE 3

FOUNDRY CASE CONTROL

WHITE MALE LUNG CANCER

CLASSIFICATION	OR	EXPOSED CASES	P
FINISHING	1.4	11	.27
FOUNDRY	0.9	6	.53
CORE	0.6	2	.40
MIXED	0.9	6	.56
OTHER	0.9	3	.57

TABLE 4

FOUNDRY PROPORTIONAL MORTALITY BLACK MALES

CAUSE	PMR
ALL CANCER	12/10.2=117
LUNG CANCER	3/3.5=85
LYMPH CANCER	2/0.7=307
NON-MALIGNANT RESPIRATORY	1/2.6=39
CARDIOVASCULAR	30/21=143*

* $P < 0.05$

BEARING PLANT PROPORTIONAL MORTALITY

WHITE MALES

TABLE 5

CAUSE	PMR	P
ALL CANCER	157/135.2=116	.03
DIGESTIVE CANCER	55/35.3=156	<.001
STOMACH CANCER	11/5.6=198	.02
RECTAL CANCER	11/3.6=304	.003
LUNG CANCER	59/47.3=125	.08
NEUROPSYCHIATRIC	13/3.2=409	<.001
CARDIOVASCULAR	323/332=97	NS
STROKE	60/44.5=135	.01
NON-MALIGNANT G.I.	22/26.1=92	NS

TABLE 6

BEARING PLANT CASE CONTROL
WHITE MALES
STOMACH CANCER

CLASSIFICATION	O. R.	NO. EXPOSED CASES	P
GRINDING	6.7	7	.01
WATER BASED FLUIDS	6.5	8	.04
MINERAL OIL	7.4	6	.04
MACHINING	0	0	---
TOOL & DIE	0	0	---
ASSEMBLY	---	1	---

TABLE 7

ASSEMBLY PLANT PROPORTIONAL MORTALITY

<u>CAUSE</u>	<u>PMR</u>	<u>P</u>
<u>WHITE MEN</u>		
ALL CANCER	227/253 = 109	
STOMACH CANCER	18/10.9 = 166	.06
LUNG CANCER	94/83 = 113	
LYMPH CANCER	27/22.9 = 118	
CARDIOVASCULAR	481/477 = 101	
STROKE	97/100 = 97	
<u>BLACK MEN</u>		
ALL CANCER	50/34.1 = 146	.002
PANCREAS CANCER	6/1.9 = 322	.02
COLON CANCER	7/2.2 = 322	.02
LUNG CANCER	17/11.4 = 149	
CARDIOVASCULAR	46/43.2 = 106	
STROKE	4/14.7 = .27	.005

TABLE 8

ELECTRONICS PLANT PROPORTIONAL MORTALITY

CAUSE	PMR (UAW)	PMR (NIOSH)
WHITE MEN		
PANCREAS CANCER	8/3=260	6/2.6=228
BRAIN CANCER	0/.37=0	5/1.6=313*
		4/1.3=303**
WHITE FEMALES		
PANCREAS CANCER	5/1=500	0/1.1=0
COLON CANCER	6/2.4=250	3/2.8=107
GENITAL CANCER	6/1.8=330	10/5.5=180
BRAIN CANCER	0/.25=0	2/1=200
* Hourly		
** Salaried		

TABLE 9

STAMPING PLANT PROPORTIONAL MORTALITY:

WHITE MALES

<u>CAUSE</u>	<u>PMR</u>	<u>P</u>
ALL CANCER	28/14.8 = 189	<.001
DIGESTIVE CANCERS	7/3.1 = 226	<.05
LUNG CANCER	10/4.8 = 220	<.01
LEUKEMIAS	4/.9 = 428	<.001
CARDIOVASCULAR	22/26.8 = 82	

TABLE 10

STAMPING PLANT
CASE CONTROL
WHITE MALES

CAUSE	CLASSIFICATIONS	O.R.	NO. EXPOSED CASES
ALL CANCER	WELDER/MILLWRIGHT	10.4	10
ALL CANCER	WELDER/MILLWRIGHT AGE 40 +	14.7	8
ALL CANCER	WELDER/MILLWRIGHT NO PRIOR EXPERIENCE	12.8	4
LUNG CANCER	WELDER/MILLWRIGHT AGE 40 +	13.2	3
DIGESTIVE CANCER	WELDER/MILLWRIGHT AGE 40 +	16.5	3

TABLE 11

STAMPING PLANT EXPOSURE DATA

CHEMICAL	OLD TAR POT VENT	OLD TAR POT FLOOR	NEW TAR POT FLOOR
PHENANTHRENE	500	50	0.32
ANTHRACENE	200	20	0.33
ACRIDINE	10	1	ND
PYRENE	100	10	0.10
CHRYSENE	20	1	ND
BENZ(A)PYRENE	1	ND	ND
ALL VALUES IN MG/M ³			

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION III - EPIDEMIOLOGY
Chairman: Dr. Howard M. Hayes
National Cancer Institute

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. HAYES: We have approximately 20 minutes for questions and discussion. If you would please identify yourself and to whom you are addressing your question.

DR. SMITH: Alan Smith, from the University of California, Berkeley. A question to Dr. Landrigan about the benzene and leukemia cases.

With the reduction in exposure over time one would hope to see some fall-off in the leukemia excess. You mentioned that you haven't yet ascertained cases '75 to '80, but I wondered if there had been any fall-off in excess out of the period studied and, in particular, if there were excess leukemia cases in the years '70 to '75, the last 5 years of the current data?

DR. LANDRIGAN: Operations at the last plant ceased in 1976. Therefore, exposure came abruptly to an end at that time. We do not yet know whether there has occurred a subsequent decline in leukemia.

DR. SMITH: No. I actually meant in the period before that, comparing say '60 to '65, '65 to '70, and '70 to '75. Has there been a reduction in the excess in those time periods?

DR. LANDRIGAN: We are analyzing those data now and hope to be able to publish them next year.

DR. LOWRY: Larry Lowry, from NIOSH. I would like to direct a question to Marie Haring. I realize that your fur study, if you wish, was not an industrial hygiene study, but did you have any means to document the levels of exposure that you might have seen in some of these plants and any indications of routes of exposure? Were they inhalation exposures? Were they in skin contacts? Did you have any feelings on that matter?

MS. HARING-SWEENEY: We didn't have any industrial hygiene studies. For the most part, the shops that the workers had worked in were closed and there were only a few places that actually had dyeing operations.

When we went into one it was obvious that inhalation could be an exposure and skin contact as well. The people did wear gloves but there was obvious dye on forearms and upper arms. So yes, there could be exposure.

Now, the only way we suspected they were exposed to any phenylenediamines was when we went through the literature. There is a voluminous amount of information on fur workers before 1950 on exposure to phenylenediamines. But that's all we had.

DR. WEISBURGER: Elizabeth Weisburger, NCI. Are you lumping all the phenylenediamines together? Because in animal tests, paraphenylenediamine didn't seem to be a carcinogen.

MS. HARING-SWEENEY: The indication is that they had some paraphenylene-diamine plus toluene diamine and so, since we didn't have any data, we don't know which was which. But we do know from the previous literature that both were around and being used.

DR. HAYES: I suppose that's it? If there are no more questions we can adjourn for an early break. Thank you for persevering with all of us.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Thursday Afternoon, March 22

SESSION IV - EXPERIMENTAL STUDIES
Chairman: Dr. Herman F. Kraybill
National Cancer Institute

SESSION IV - EXPERIMENTAL STUDIES
Chairman: Dr. Herman F. Kraybill, NCI

DR. KRAYBILL: We have a series of four papers I think are very exciting and three of them, of course, are in the area of aquatic animals. We haven't learned how to have the fish in an occupational setting. I asked Dr. Couch about that. That would take a bit of doing, I guess. But we are using them for other studies.

There is going to be, for those interested, during the first week of next June, a panel on some of the aquatic animal research at Bob Jolly's. I don't know whether it is the fifth or sixth conference on water chlorination, but we are going to have a series of eight papers there. It is going to be at Williamsburg.

I think we have come a long way. I have said that in using the aquatic animal we are probably today where they were 40 or 50 years ago with the rodent. But with no attempt to be slanderous, I think that there are still a lot of people out there who have not been convinced about the use of the aquatic animal, but it is the coming thing and it is attracting a lot of attention.

So, our first speaker today is really going to give somewhat of a terminal report, so to speak, on their work. We have heard Dr. Couch before. But this work went on for about five or six years down at Gulf Breeze and it has been a very important and profitable bit of research.

So, I would like to introduce to you Dr. Couch, from Gulf Breeze Research Laboratory, Gulf Breeze, Florida.

PROCEEDINGS OF THE
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PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Effects of Carcinogens, Mutagens, and Teratogens
on Nonhuman Species (Aquatic Animals)

John A. Couch, Ph.D.
W. Peter Schoor, Ph.D.
Lee Courtney
Will Davis, Ph.D.

Environmental Protection Agency
Environmental Research Laboratory
Gulf Breeze, Florida

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Final Report

NCI/EPA Collaborative Program

(August 1978 - September 1982)

Project 3

EFFECTS OF CARCINOGENS, MUTAGENS,
AND TERATOGENS ON NONHUMAN SPECIES (AQUATIC ANIMALS)

by

John A. Couch, Ph.D., Coordinator, Pathobiologist

W. Peter Schoor, Ph.D., Biochemist

Lee Courtney, Research Toxicologist

and

Will Davis, Ph.D., Research Biologist

U.S. EPA, ERL, GULF BREEZE, FLORIDA

EFFECTS OF CARCINOGENS, MUTAGENS, AND TERATOGENS

ON NONHUMAN SPECIES-AQUATIC ANIMALS

II. ABSTRACT

John A. Couch, Coordinator; W. Peter Schoor, Biochemist

Lee Courtney, Research Toxicologist; Will Davis, Biologist

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Aquatic systems and organisms have been under both laboratory and field study in order to develop indicator, screening, and modeling capabilities for detection and evaluation of risks of carcinogens, mutagens, and teratogens. Studies include both Gulf Breeze Laboratory projects and complementary, extramural cooperative agreements. During the course of the program, several advancements have been made in the development of laboratory and field carcinogen assay methods utilizing fishes, such as the sheepshead minnow (liver lesions via benzidine and aflatoxin exposures), Rivulus marmoratus (liver lesions via aflatoxin exposure), Menidia peninsulæ (liver tumor induction with aflatoxin exposure), the rainbow trout (tumor induction with benzo(a)pyrene B(a)P and methyl azoxymethanol acetate exposures), and freshwater catfish (papillomatous-like lesions via chlorinated effluent exposures). Emphasis has been placed on the development and utilization of critical life stage exposures (e.g., embryo and newly hatched fry exposures) in order to expedite carcinogen tests and minimize time required for detectable tumorigenic responses. Preneoplastic hepatic lesions development in Menidia, noted at 12 weeks and followed by hepatic tumors at 11 months, has shown promise for this species and exposure method. The first hepatic tumors induced in fishes with polycyclic aromatics (e.g., B(a)P) resulted in the further advancement of the rainbow trout carcinogen assay system. Biochemical studies involving metabolism of PAH's (e.g., perylene) by skin and liver tissues of the tiger salamander (Ambystoma tigrinum) have indicated induction of enzyme activity (MFO system); however, experimental perylene exposures have failed to elicit carcinogenic responses in this species. Advancements have been made in electrophoresis and serological methodology to study serum protein changes and humoral immune responses in non-exposed and exposed (e.g., benzidine) fishes (e.g., Cyprinodon variegatus). These advances have shown promise in detection of physiological responses to carcinogenic exposure. Furthermore, considerable field monitoring work on mollusks and carcinogenic PAH's along the coast of Oregon has revealed a positive correlation between prevalence of cellular proliferation disorders in shellfish and higher concentrations of certain PAH's in natural water.

Emphasis in biochemistry has been directed mostly toward the elucidation of the metabolism of mixed-function oxidases in marine organisms.

When mullet were treated with 3-MC and PB, and the effects on the metabolism of benzo(a)pyrene by isolated liver microcosms were evaluated, the most significant effect found in treated fish was an increase in the oxidation of

B(a)P at the bay region. This position-specific increase was not reflected in an increase in the total activity. When metabolite patterns of the dihydrodiols of B(a)P in the presence and absence of trichloropropene oxide (TCPO) were compared, the predicted inhibitions were observed in control as well as treated animals. No significant differences in metabolite patterns were found between the 3-MC- and PB-treated fish in either presence or absence of TCPO. Comparison with published data on similar position-specific effects in rats revealed closely parallel observations for both species.

Metabolites of benzo(a)pyrene (B(a)P) formed following incubation with hepatic S-9 protein preparations from control and induced goldfish (Carassius auratus), black bullhead (Ictalurus melas), brown bullhead (Ictalurus nebulosus) and rat have been separated by high-pressure liquid chromatography and identified by comparison of retention times with those of synthetic standards. In both bullhead species and the rat, three metabolites (B(a)P 9,10-dihydrodiol, B(a)P 7,8-dihydrodiol and 3-hydroxy-B(a)P) accounted for over 80% of the metabolite production. In the goldfish, however, these same metabolites yielded only 36% of the total; instead, B(a)P 1,6- and 3,6-dione, 9- and 3-hydroxy B(a)P each accounted for approximately 20% of the B(a)P metabolite production. Induced rat formed 8, 15 and 19 times as many total metabolites as induced brown bullhead, black bullhead and goldfish, respectively. Pretreatment of fish at 25-30° with 3-MC caused inducement of 10 to 20 times as much total benzo(a)pyrene hydroxylase activity as in control animals. However, no induction was observed in 3-MC-treated brown bullheads kept at 7°C. Metabolite production was inhibited by 30% in the presence of 0.1 mM α -naphthoflavone. Trichloropropene oxide (0.2 mM) decreased dihydrodiol production by 50%, but it had very little effect on the production of the remaining metabolites.

These results suggest that both the thermal history and type of fish must be considered when hepatic MFO activity is used as an indicator of environmental pollution. Furthermore, the large in vitro production of B(a)P 7,8-dihydrodiol by fish liver enzymes probably would favor the in vitro production of B(a)P 7,8-dihydrodiol-9,10-epoxide, the ultimate carcinogen for B(a)P in mammalian systems.

Attempts were made to define the oxygen species involved in the actual oxidation of B(a)P. Studies with various inhibitors of the O₂ superoxide anion and the •OH hydroxy radical indicate that each may be involved along with the P-450-bound oxygen species.

III. Introduction

A major problem faced by the National Cancer Institute and the U.S. Environmental Protection Agency (EPA) is that of aiding in determining the fate and effects of carcinogenic pollutants in the larger environment. One approach to solving this problem may be to study wildlife populations, particularly fishes, that are widespread, but which live in environments where exposure to ambient pollutants is certain. The use of wildlife populations as surrogates for human populations may be considered to be a novel expansion or logical extension of the use of laboratory animals and animal models of human diseases as alternatives to the use of human subjects. The sharing of biologic characteristics by phylogenetically diverse species makes certain comparative approaches possible.

Problems arise, however, when such factors as selection of sensitive indicative species, geographically adequate populations, and representative segments of the air, land, and water biosphere are considered. In this regard, EPA Office of Research and Development Laboratories, such as the Gulf Breeze Environmental Research Laboratory, have exemplary, pilot research programs that are investigating the use of aquatic animal species as indicators of carcinogens in the environment. The Gulf Breeze pilot research program has been underway since August, 1978 and is supported jointly by the Office of Research and Development (EPA) and the National Cancer Institute through an interagency agreement. The Gulf Breeze studies are based on the premise that the aquatic portion of the biosphere (water, biota, and sediment) is the ultimate "sink" for runoff, fallout, and the discharge of most toxic pollutants. In addition, animals living in the relatively efficient solvent, water, are more intimately exposed (total exposure through body surfaces, gills, alimentary tracts) than are species living in terrestrial or air environments. Aquatic species are also less likely to escape a dissolved or carried pollutant.

At Gulf Breeze, researchers have been studying species of fish and shellfish along the Northern Gulf of Mexico (Florida, Alabama, Mississippi) for the past four years in order to determine which are potential indicators of the role of carcinogenic agents in the environment. Selected species of fish have been used in both long-term and short-term laboratory exposures to determine their specific tissue, cellular, and biochemical responses to known chemical carcinogens which may occur in the environment. In addition, a significant number of cooperative agreements with principal investigators from around the nation support an extramural complementary and supplemental effort in the identification of aquatic species and systems that may serve as early warning mechanisms.

This is the final report for the four-year Interagency Agreement Project between the National Cancer Institute and the Gulf Breeze Environmental Research Laboratory (U.S. EPA).

IV. Summarized Objectives

The original objectives and goals of this project were as follows:

Objective 1:

Determine fate and effects of carcinogens, mutagens, and teratogens in aquatic species (individuals; populations).

Objective 2:

Determine role of aquatic species and systems in actual or potential exposure of man to carcinogens, mutagens, or teratogens (metabolism, accumulators).

Objective 3:

Identify and develop aquatic species as bioindicator, sentinel, and model systems for use in the study of risks of carcinogens, mutagens, and teratogens in the general environment (sensitive species and assay systems).

V. Methodological Approaches

In reference to the preceeding objectives, we have completed both in-house and complementary extramural research projects. The overall program was divided into two major disciplinary approaches: (1) Pathobiology and (2) Biochemistry. Therefore, methods outlined below and results in the next section will be reported under these two headings. Detailed methods and results of each cooperative agreement with academic colleagues will be included at the end of this report. The disciplinary area of each cooperative agreement will be identified as pathobiology or biochemistry.

1. Pathobiological Approaches and Methods

Fish carcinogen assay systems, toxicology, and histopathology of induced lesions.

Whole life-cycle fish exposure systems and methods were previously reported (Couch, Courtney and Foss, 1981).

In the last two years at Gulf Breeze, major emphasis was concentrated on short-term (single pulse) critical life-stage carcinogen exposures of several estuarine fish species. Cyprinodon variegatus (Sheepshead minnow), Menidia peninsulae (Silverside), and Rivulus marmoratus were exposed to the potent carcinogen, aflatoxin B₁. This compound, a natural product of the metabolic activity of fungi (genus Aspergillus), has been shown to be of high toxicity and carcinogenicity in mammalian studies (including humans) and in work with freshwater fish species. Selection of aflatoxin B₁ as a test compound was partly based on this high hazard background in order to test marine species with a proven and potent carcinogen. As such, special precautions and methods were needed to insure safety.

Aflatoxin B₁ exposures were conducted under static conditions within a glove box. Test organisms were exposed in 3.8ℓ chambers containing 1.5ℓ seawater, toxicant, and carrier. They were then rinsed and transferred to clean holding facilities for development and observation. A detailed description of the test system utilized is now in press (Courtney and Couch, 1982). Carrier controls without toxicant, were under identical conditions. Species, test concentrations, exposure duration, carrier, and life-stage tested varied. Ninety six-hour embryos and 120-hour newly hatched larvae of C. variegatus were exposed for 1 hour to 200 µg aflatoxin B₁/ℓ, using dimethylsulfoxide (DMSO) as a carrier. C. variegatus, R. marmoratus, and M. peninsulae were exposed to 1,000µg aflatoxin B₁/ℓ (using DMSO and triethylene glycol as carriers) for exposure durations ranging from 2 to 43.5 hours. Surviving test organisms were maintained for up to 18 months with periodic necropsy and histological evaluation.

Davis, at the Environmental Research Laboratory, Gulf Breeze, conducted exposures to assess the effects of water-borne teratogenic compounds (e.g., dibutyl phthalate (DBP) and hexazinone). Adult Rivulus marmoratus were exposed to multiple pulse-doses of waterborne compound three times per week at nominal concentrations of 1 and 2 mg/ℓ. Rates of egg production, viability, hatching, development, and frequency of deformed young were compared between offspring from DBP-treated fish and non-exposed controls.

In extramural efforts, Hendricks, at the University of Oregon, has completed histological evaluation of tissues from rainbow trout (Salmo gairdneri) exposed for 18 months to dietary benzo(a)pyrene (B(a)P, 1000 mg/kg), Aroclor 1260 (PCB, 5000 mg/kg), and toxaphene (50 mg/kg). Trout embryo exposures to methyl azoxymethanol acetate (MAMA) and benzidine dihydrochloride (BEN) were conducted, using embryos obtained from non-exposed or Aroclor 1254 (200 mg/ℓ for 2 months) pre-exposed females. Twenty-one-day old embryos were exposed for 24 hours to concentrations of MAMA or BEN ranging from 0.1 to 1000 mg/ℓ under static conditions.

At the University of Southern Mississippi, Martin has conducted long-term (1 mg/ℓ BEN weekly for 23 months) and pulse (15 mg/ℓ and 10 mg/ℓ BEN for 24 and 100 days, respectively) exposures, using C. variegatus. Maintenance and observation of these fish has continued with periodic necropsy and histological evaluation. Additionally, toxicological studies have been undertaken involving exposure of C. variegatus cell lines, previously established, to 3-methylcholanthrene, benzidine, and diethylnitrosamine. Then, soft-agar colony-forming efficiency has been studied as a tool to indicate cell transformation. Pilot studies, using rat liver postmitochondrial supernates to metabolize precarcinogens to active molecules, are underway with one of the Cyprinodon cell lines.

Furthermore, considerable effort has been directed toward determining changes in serum protein profiles in fish exposed to benzidine and to establish whether significant correlations exist between observed serum protein shifts and histological liver changes in exposed fish. Five analytical techniques were applied to separate, quantitate, and the serum protein profiles compared: (1) polyacrylamide gel electrophoresis, (2) analytical isoelectric focusing, (3) Graber-Williams immunoelectrophoresis, (4) crossed immunoelectrophoresis, and (5) tandem crossed immunoelectrophoresis.

Finally, further work has been conducted on development of microassays for monitoring the C. variegatus immune system. Several assays have been developed and evaluated, the most promising based on the use of 4-methylumbelliferone as a hapten, conjugated to a protein carrier to be immunogenic.

Grizzle, at Auburn University, has conducted laboratory and field experiments to examine causation and development of oral papillomas found on feral black bullheads (Ictalurus melas) from the final oxidation pond of a Tuskegee, Alabama sewage treatment plant. Objectives of the study were to: (1) determine the prevalence of papillomas on fish in the final oxidation pond; (2) describe the histology and ultrastructure of the papillomas; (3) determine if the papillomas would persist on black bullheads taken from the final oxidation pond and placed in clean water; (4) chemically identify mutagenic and carcinogenic chemicals in the water; (5) determine if the water was mutagenic; (6) determine if tumors would develop in new fish placed into the final oxidation pond; (7) determine if hepatic-enzyme induction occurred in fish exposed to the water; (8) determine if viruses were associated with the papillomas by ultrastructural examination, transmission by cell-free homogenate, and isolation of virus with cell-culture techniques; (9) determine if lesions would develop in brown bullheads exposed during embryonic development to a concentrate of the final oxidation pond water; and (10) determine if tumors would develop on black bullheads exposed to an organic concentrate of the final oxidation pond sediment injected or applied topically to the mouth.

Rose and Anderson at Texas Tech. Univ. have been involved in a two-part research project studying tiger salamanders (Ambystoma tigrinum) from a population inhabiting a sewage lagoon at Reese Air Force Base, Hurlwood Texas. This population, under investigation since 1969, has displayed a high incidence of integumental lesions, peaking in prevalence in 1975 and decreasing steadily since then. Anderson has been studying metabolic activation and/or detoxification of perylene and benzo(a)pyrene by A. tigrinum tissue enzymes. Rose has been conducting field studies to determine the usefulness of A. tigrinum as a sentinel organism and to monitor the existing population and its lesion frequency. Also, laboratory studies in tumor induction, using benzo(a)pyrene and/or perylene, tumor enhancement with in vivo perylene treatments, and melanocytoma subdermal transplant have been undertaken.

Mix, at Oregon State University, finished a long-term field study of mollusks in Oregon coastal waters and expanded laboratory research. Major emphasis last year included: (1) baseline measurements of arsenic, cadmium, and nickel, using either flame atomic absorption or neutron activation analysis; (2) evaluation of methods for measurement of trace polynuclear aromatic hydrocarbon (PNAH) concentrations in seawater, using a modification of EPA Method 610 (U.S. EPA. 1979). Polynuclear Aromatic Hydrocarbons-Method 610. Federal Register, 44 (223), 69514-69517) and an extraction process using C₁₈ mini-columns; (3) development and testing of an in vitro assay system, using the gooseneck barnacle, Pollicipes polymerus; (4) determination of possible viral association with a neoplastic disorder in Mytilus edulis; and (5) summation and evaluation of data previously collected on neoplastic disorders in M. edulis.

2. Biochemical Methods

Induction Studies

Aquatic species, such as mullet, killifish, flounder, sea catfish and others, have been exposed to inducers of microsomal mixed-function oxygenase (MFO) activity either by intraperitoneal injection or direct exposure in seawater. Since periods up to one year might be necessary to induce MFO activity by water exposure, the direct injection of inducer was used at the start of the investigations in order to optimize other parameters, such as metabolite and conjugation reactions.

Metabolite Identification

Metabolites from the MFO reactions were identified, using high pressure liquid chromatography coupled with fluorescence detection and confirmed by stopped-flow fluorescence scanning. Metabolite standards for benzo(a)pyrene [(B(a)P)] were obtained from the Illinois Institute of Technology through the courtesy of NCI. All the phenolic compounds were chromatographed and their excitation and emission spectra obtained in appropriate solvents. All spectra were stored on discs for later data manipulation.

Conjugation and Excretion Studies: Rats were used to make a series of conjugation products in vivo by injection of ^{14}C -labeled B(a)P. They include glucuronides, glutathiones, and sulfates. The occurrence in fish has been ascertained by comparison to the standards produced in the rat. This will be helpful in determining the final disposition of a carcinogen-like B(a)P within the animal and in what forms the parent compound is finally passed back into the seawater.

VI. Major Findings and Progress

As noted earlier under Methodological Approaches, results and findings of studies to date are reported under the two disciplinary areas of Pathobiology and Biochemistry, with extramural cooperative agreement reports at the end of this report.

1. Pathobiology

Table 1 summarizes the carcinogen assay/induction studies undertaken by the Gulf Breeze staff and investigators working under cooperative agreements for several years (1978-1982). This work represents an investigative effort encompassing a wide range of organisms and several selected carcinogens and suspect carcinogens. In addition to the data generated, and possibly of even greater value, is the methodology developed (both in exposure systems and support technology).

At Gulf Breeze, the static exposure system utilized in the aflatoxin studies has proven to be an efficient method for short-term exposures to high hazard compounds. Each species tested performed well and the use of various life-stages presented no problems. We consider this system a safe and inexpensive method of high hazard single short-term or pulse exposure for a wide range of life-stages and species (Table 2).

Data generated to date include some basic toxicity data with regard to aflatoxin and the three species tested, and two lesions related to the exposures. Gross necropsy and histological examination of C. variegatus exposed to 200 µg aflatoxin B₁/l for 1 hr have revealed benign hepatic polyps on several specimens. In addition, a suspect pre-neoplastic lesion has been noted in histologic sections of Menidia peninsulae exposed as fry for 2 hrs to 1000 µg/l aflatoxin (using both DMSO and TEG as carriers). The lesions, noted in specimens examined 12 weeks post-exposure, consist of foci of enlarged clear hepatic cells. Specimens of Menidia, from this study, taken at 11 months post-exposure, had developed nodular and trabecular hepatocellular carcinomas (Table 1). One Rivulus specimen from these studies also developed an incipient hepatocellular carcinoma (carcinoma in situ) (Tables 1 and 2). It is quite possible that the early lesions (foci of enlarged, clear hepatic cells) were precursor lesions or early altered foci leading to the later development of the carcinomas observed at 11 months post-exposure.

These results indicate that the tidewater silverside (M. peninsulae) was more responsive to aflatoxin than either the sheepshead minnow (C. variegatus) or Rivulus (See Table 1). Further work will be required to ascertain the sensitivity of Menidia to a broader spectrum of carcinogens. Rivulus has already shown sensitivity to known carcinogens such as n-nitro compounds (Koenig et al., 1982) (Table 1), but may not be as sensitive as Menidia to aflatoxin. Each of these species has advantages and disadvantages associated with its use as a laboratory carcinogen assay subject. The use, however, of all three for testing any given agent may provide a battery of estuarine fishes that, as a group, may be useful in carcinogen risk assessment in the general environment. The physical systems for laboratory tests and assessments are described and are available for more routine testing with either short-term or long-term exposure modes.

Other species tested during this cooperative NCI/EPA effort are presented in Table 1, including considerable work on the American oyster (Crassostrea virginica) as a useful carcinogen indicator. The results of these studies are available in published papers listed in the bibliography or collected reprints list at the end of this report.

Davis has noted rapid depletion of dibutyl phthalate (DBP) from systems containing Rivulus marmoratus and the accompanying microorganism complement compared to systems containing no organisms. This indicates uptake of the test compound by the experimental organism. Methods to determine body burden are now being developed. Parental exposure to DBP increases the frequency of skeletal deformity in surviving offspring. The offspring produced are still being examined; therefore, final results of this investigation are pending. Hexazone exposures resulted in inhibition of egg production.

In extramural projects, Hendricks (See attached report no. CR809344) has produced hepatocellular carcinomas, at a prevalence of 24%, in rainbow trout (Salmo gairdneri) exposed to 1000 mg/kg dietary benzo(a)pyrene. Hepatocellular carcinoma, fibrosarcoma of the liver and swim bladder adenomas have also been induced in trout injected with B(a)P. S. gairdneri exposed to dietary Aroclor 1250 and toxaphene have shown no carcinogenic response.

Table 1. Carcinogen Assays and Results from the NCI/EPA Gulf Breeze and Extramural Program

SPECIES	AGENT	EXPOSURE	RESULTS
OYSTER <u>Crassostrea</u> <u>virginica</u>	3-Methyl Cholanthrene	Flowing seawater, 1-5 µg/l for one year	*Incipient, perivascular blood cell proliferation
OYSTER <u>C. virginica</u>	Benzo(a)pyrene	Flowing seawater, 1-5 µg/l for one year	No tissue response
SHEEPSHEAD MINNOW <u>C. variegatus</u>	Trifluralin	Flowing seawater, 5 µg/l, 19 months	Focal benign vertebral growths; non-neoplastic
SHEEPSHEAD MINNOW <u>C. variegatus</u>	Aflatoxin B1	Embryo-fry static, 200 µg/l for one hour, held for 18 months	Hepatic polyps (on surface of livers) benign dysplasia
SHEEPSHEAD MINNOW <u>C. variegatus</u>	Benzidine dihydrochloride	Recirculated seawater, 1 mg/l for 166 days	*Proliferative lesions in liver; ductile prolif- eration; cholangioma; adenofibroma; adenofibrosis; oval cell hyperplasia
<u>Rivulus</u> <u>marmoratus</u>	Aflatoxin B1	Fry exposure static, 1 mg/l 2 and 8 hours	*Trabecular hepatoma, or carcinoma, a single case
<u>R. marmoratus</u>	Diethylnitros- amine	Juvenile and adult exposure, static	*Hepatocellular carcinoma
SILVERSIDE <u>Menidia</u> <u>peninsulae</u>	Aflatoxin B1	Fry exposure, static, 1 mg/l 2 and 8 hours, held 12 months	*Early altered foci in livers; enlarged cells; preneoplastic; trabecular hepatomas and carcinomas
SILVER PERCH CELL LINE	Asbestos (Crocidolite)	Asbestos in culture medium	Nodule formation in culture around asbestos fibers
BLACK BULL- HEAD CATFISH <u>Ictalurus</u> <u>melas</u>	Mixed sewage pond effluent	Introduced to pond in cages, held 600+ days	*Hyperplasia of tegument, papillomas

*Tumors or suspect neoplasms induced

Table 1. Continued

SPECIES	AGENT	EXPOSURE	RESULTS
RAINBOW TROUT <u>Salmo</u> <u>gairdneri</u>	Benzo(a)pyrene	1000 mg/l, dietary, 18 month feeding	*Hepatocellular carcinoma
RAINBOW TROUT <u>S. gairdneri</u>	Benzo(a)pyrene	IP injection (12 injections of 1 mg over 12 months)	*Hepatocellular carcinoma; fibrosarcoma of liver; swim bladder adenoma
RAINBOW TROUT <u>S. gairdneri</u>	Aroclor 1250	500 mg/l, dietary, 18 months feeding	No tumor response
RAINBOW TROUT <u>S. gairdneri</u>	Toxaphene	100 and 500 mg/l, dietary, 18 month feeding	100 mg/l toxic; No tumor response
RAINBOW TROUT <u>S. gairdneri</u>	Benzidine dihydro- chloride	Pre-exposure of brood stock to 200 mg/l Aroclor 1254; 21-day-old embryo exposure, static, 24 hours, 1000, 100, 10, 1 and 0.1 mg/l	100% mortality at 1000 mg/l; no tumor response at other doses
RAINBOW TROUT <u>S. gairdneri</u>	Benzidine dihydro- chloride	21-day-old embryo exposure, static 24 hours, 1000, 100, 10, 1 and 0.1 mg/l	100% mortality at 1000 mg/l; no tumor response at other doses
RAINBOW TROUT <u>S. gairdneri</u>	Methyl azoxy- methanol acetate	Pre-exposure brood stock to 200 mg/l Aroclor 1254 for 2 months; 21-day-old embryo exposure, static 24 hours, 1000, 100, 10, 1, and 0.1 mg/l	*100% mortality at 1000 mg/l; Hepato- cellular carcinoma, nephroblastoma, stomach, and swim bladder adenomas at 100 and 10 mg/l; no tumors at 1 and 0.1 mg/l
RAINBOW TROUT <u>S. gairdneri</u>	Methyl azoxy- methanol acetate	21-day-old embryo exposure, static 24 hours, 1000, 100, 10, 1 and 0.1 mg/l	*100% mortality at 1000 mg/l; hepatocellular carcinoma, nephroblastoma, stomach, and swim bladder adenomas at 100 and 10 mg/l; no tumors at 1 and 0.1 mg/l

Table 1. Continued

SPECIES	AGENT	EXPOSURE	RESULTS
TIGER SALAMANDER <u>Ambystoma</u> <u>tigrinum</u>	Perylene	injection i.p., 100, 50, and 10 mg/kg body weight	*Single case of hepatocellular carcinoma (possibly not related to exposure)
SALAMANDER <u>A. tigrinum</u>	Benzo(a)pyrene	injection i.p., 10 mg/kg body weight	No tumor response
SALAMANDER <u>A. tigrinum</u>	Perylene Benzo(a)pyrene	injection i.p.	No tumor response

Table 2. Regimens For Aflatoxin Exposures to Various Test Species

COMPOUND	SPECIES	CONC.	CARRIER	DURATION	LIFE STAGE
Aflatoxin B ₁	<u>Cyprinodon</u> <u>variegatus</u>	200 µg/ℓ	DMSO*	1 hr	96 hr embryo
Aflatoxin B ₁	<u>C. variegatus</u>	200 µg/ℓ	DMSO	1 hr	post-hatch fry
Aflatoxin B ₁	<u>Rivulus</u> <u>marmoratus</u>	1000 µg/ℓ	DMSO/ TEG	23.5 hr	fry
Aflatoxin B ₁	<u>C. variegatus</u>	1000 µg/ℓ	DMSO/ TEG	43.5 hr	fry
Aflatoxin B ₁	<u>R. marmoratus</u>	1000 µg/ℓ	DMSO/ TEG	2 hr	fry
Aflatoxin B ₁	<u>R. marmoratus</u>	1000 µg/ℓ	DMSO/ TEG	8 hrs	fry
Aflatoxin B ₁	<u>Menidia</u> <u>peninsulae</u>	1000 µg/ℓ	DMSO/ TEG	2 hr	fry
Aflatoxin B ₁	<u>M. peninsulae</u>	1000 mg/ℓ	DMSO TEG	8 hr	fry

*DMSO = dimethylsulfoxide

TEG = triethylene glycol

Benzidine dihydrochloride (BEN) exposures have produced no tumors at any of the tested concentrations (0.1 to 100 mg/l). Total mortality was observed at 1000 mg/l BEN. Methyl azoxymethanol acetate (MAMA) exposures proved to be highly toxic at 100 and 1000 mg/l in both Aroclor 1254 pre-exposed and non-exposed groups. Hepatocellular carcinomas were evident at 7 months in nearly all surviving fish from the 100 mg/l exposure. Tumors were present at 9 months in the 10 mg/l MAMA exposures and with greater prevalence at 12 months. Hepatocellular carcinomas were the predominant tumor type; however, nephroblastomas, stomach and swim bladder papillary adenoma were also found.

Martin (see attached report No. CR809347) has produced liver lesions, probably cholangiomatous or adenofibrotic, in C. variegatus exposed to 1, 5, and 10 mg/l Ben. Chronic (1 mg/l Ben weekly for 23 months) and single pulse exposures to near toxic concentrations (15 mg/l for 24 days; 10 mg/l for 100 days) are still being maintained in attempts to further characterize this lesion. C. variegatus cell line studies have determined chromosome number of the cell lines at various passages, revealing that soft-agar colony-forming efficiency seems to be a function of the initial cell concentration, and generated some toxicity data (LC-50 for LS cell line to 12 mM diethylnitrosamine). Completion of cell line studies in progress will yield additional data. Electrophoresis studies have revealed complex protein banding patterns. Interpretation of results from these studies is in an early stage. However, consistent patterns are emerging which indicate the involvement of the immune system following benzidine exposure. Finally, the work with the C. variegatus immune system has necessitated the development of an assay system suitable for the microliter quantities of serum available. Several assays, showing much promise, have been developed and evaluated.

Grizzle (see attached report No. CR809336) has found a reduction in both prevalence and size of oral papillomas in the black bullhead (Ictalurus melas) population present in the final oxidation pond at Tuskegee, Alabama. Prevalence has dropped from 73% (1979-1980) to 23% in 1983. No tumors have been found on other fish species present in the pond. The lesions consist of hyperplastic mucosa covering papillae of submucosa. Studies have failed to reveal any viral association with the papillomas. Similar lesions have been induced in caged black bullheads placed in the pond. Sediment extracts, administered either by injection or swab, have failed to produce tumors in fish after 8 months. Acid and XAD resin extracts of the oxidation pond water were found to be mutagenic to Salmonella typhimurium if S-9 Aroclor-induced rat liver enzymes were present. Mutagenicity is not lost during retention in the pond. Chemical analysis of the pond water and sediment revealed no significant residues of pesticides, herbicides or PCB's. Chloroform and bromodichloromethane were found in some water samples.

Rose (see attached report No. CR807740, Part I) has observed a steady decline since 1975 in the neotenic Ambystoma tigrinum population under investigation. Recent samples indicate the population to be at or near extinction. The reason for this decrease seems to be low hatching and post-hatching success; however, the cause has not as yet been determined. Transfer of Reese AFB salamander to other sewage and non-sewage treatment ponds and the introduction of non-sewage pond larvae to the Reese AFB

location have been unsuccessful. The Reese AFB neotenic salamander is specifically and narrowly adapted to its unique environment; as such, it does not manifest the desired characteristics necessary to serve as a long-term sentinel to monitor environmental pollution. Tumorigenesis studies, involving injection of perylene and/or benzo(a)pyrene, have produced a few small epidermal lesions which, however, are thought to be incidental to the treatment. One hepatocellular carcinoma was found in a perylene-injected salamander; however, its relationship to the experimental treatment is also in doubt. Melanocytoma transplants from tumorous salamanders to non-tumorous ones have been unsuccessful. Finally, epidermal papillomas on A. tigrinum from the Reese AFB location have been shown to regress when animals are maintained in a clean environment. However, if tumorous salamanders removed from the sewage pond were treated with perylene, no regression was observed.

Anderson (see Report No. CR807740, Part II) has determined that Ambystoma appears to possess the typical enzymes that can activate and/or detoxify PAH carcinogens. It has been shown that: 1) perylene (P) is an inducer of P-OHase in Ambystoma skin, 2) Ambystoma AHH mediates P-DNA binding, suggesting biological activity, and 3) Ambystoma AHH will mediate production by the 7-8 dihydrodiol, which is the precursor of the ultimate carcinogenic form of B(a)P. However, Ambystoma AHH fails to activate either P or B(a)P in Ames mutagenicity assay and direct attempts to produce neoplasia in Ambystoma with P or B(a)P have produced negative results. These data suggest that P is not a particularly potent carcinogen in Ambystoma.

The research project of Mix, at Oregon State University, (See attached report No. CR808000) has produced much data related to shellfish populations along the Oregon coast and valuable investigative methodology. Baseline measurements (mg/g, dry weight) were determined for freshwater mussels, Margaritifera margaritifera, (Cd, 0.9-6.2; and Ni, 1.6-19.4) and bay mussels, Mytilus edulis, (As, 9-15; Cd, 7.0-12.5; and Ni, 1.0-6.8). Both species were judged to be excellent monitors for evaluating concentrations of these important metals in either freshwater (M. margaritifera) or marine (M. edulis) environments. The evaluation of a long-term study (1976-1981) on a proliferative disorder in M. edulis has revealed significant differences in occurrence of the disorder with relation to geographical distribution and seasonality. The prevalence of the disorder, in the subpopulation revealing the highest level, ranged from 0.0% to 20% with a five-year mean of 9.8%. Histopathological progression of the disorder was found to occur randomly, with no seasonal patterns revealed. Extensive investigation of the disorder in M. edulis has not revealed the presence of any RNA tumor virus in either affected or control mussels. Finally, experiments testing the efficacy of an in vitro bioassay system using the gooseneck barnacle, Pollicipes polymerus, have shown great potential for testing environmental carcinogens and mutagens.

2. Biochemical Results

The kinetics of the formation of the B(a)P metabolites studied in mullet liver microsomes under conditions of different temperatures and incubation time in non-induced versus 3-MC- and PB-pretreated mullet revealed that phenols and quinones appear first and the secondary metabolites,

such as dihydrodiols of B(a)P, were either not formed or formed at very low concentrations. The formation of the most of the metabolites, except phenols, was proportional to the incubation time. But, at lower temperatures, the formation of phenols predominated. At different NADPH concentrations used, the metabolite distribution, except 4,5-epoxide and 5-OH phenol, increased disproportionately in 3-MC- and PB-induced mullet compared to non-induced mullet at 25° and 37°C. A substrate inhibition by B(a)P above 50 nmoles conc. was observed.

In order to understand the mechanism by which B(a)P is metabolized by MFO system, the effect of various inhibitors were examined. Although superoxide dismutase (SOD), a powerful scavenger of superoxide anion (O_2^-), did not inhibit the oxidation of B(a)P, the other scavengers of O_2^- , such as nitrobluetetrazolium chloride (NBT), epinephrine, cytochrome c, catechol, tiron, and ascorbic acid, inhibited the oxidation of B(a)P effectively. The spectrophotometric determination of the formation of adrenochrome from epinephrine oxidized by the MFO system was completely inhibited by SOD and thiols which compete effectively for O_2^- , preventing the oxidation of epinephrine. On the other hand, the reduction of cytochrome c and NBT by MFO was not inhibited by SOD, but thiols and tiron inhibited their reduction by 95%. These results suggest the generation of O_2^- and its involvement in the oxidation of B(a)P. The inhibition by epinephrine and catechol may be due to their ability to discharge the oxyferrous complex (an active oxygen species involved in the oxidation of B(a)P) either directly to the inactive ferric form or via other intermediate states with the concomitant release of O_2 .

MFO in non-induced mullet was inhibited differentially by ethanol and metyrapone. Formation of dihydrodiols and phenols were inhibited consistently in all the systems by sodium benzoate ($\cdot OH$ scavenger) with an increase in the production of 4,5-epoxide and quinines. The inhibition was found to be reversible by the addition of UDP-glucuronic acid, a phenomenon which is not yet understood. The B(a)P oxidation was also inhibited by the addition of p-hydroxybenzoic acid, which utilizes $\cdot OH$ for its conversion to protocatechuic acid. But the type of inhibition elicited by pHB was found to be different from that effected by sodium benzoate and the former inhibition was not found to be reversible by UDP-glucuronic acid. Catalase had no effect and H_2O_2 could not replace NADPH in the system. Sodium borohydride and ELTA replaced NADPH to a greater extent, while NADH and sodium dithionite oxidized B(a)P to a lesser degree in place of NADPH. But the horseradish peroxidase and H_2O_2 in place of MFO and NADPH could oxidize B(a)P, forming more phenols at 5,6,9,8,7,2,1 positions and less of 9,10,4,5, and 7,8-dihydrodiols and no phenol formation at 3 position. Further studies tend to suggest that one electron oxidation might be a selective mechanism of carcinogenic activation of B(a)P in mullet.

Strength (see attached report No. CR809673) found that mixed function oxidase (MFO) and UDP-glucuronosyltransferase were significantly increased in livers of rats and fish given 3-MC or phenobarbital. Sultransferase was induced in rats, mullet and channel catfish only if given large doses of 3-MC over a protracted period of time. The extent of induction of enzymes varied with compound, dosage, and period, as well as animal species.

In order to ascertain the influence of environmental conditions on

the metabolism of benzo(a)pyrene, a thorough study of channel catfish was carried out during the Winter, Spring and Summer of 1983. The induction of MFO and glucuronosyltransferase was studied in fish maintained in pools at 7-9°C or 22-25°C. These temperatures represent water temperatures at Auburn University ponds in Winter or Late Spring and Summer. Fish from cold water were transferred to the laboratory and maintained at cold temperature (79°F) or at warm temperature (22-25°C), force fed and injected with 3-MC. Fish from warm ponds were transferred to the laboratory and maintained at the two temperatures during induction. It was observed that fish at low temperatures discontinue eating and after approximately 10 days in the cold tanks were metabolically similar to fish during winter. Wintering fish after warm adaptation in the laboratory showed induction of MFO and glucuronosyl transferase. Nutritional status and size of fish (maturity), as well as temperature, markedly affected the MFO and transferase activities in fish tissues.

¹⁴C-Benzo(a)pyrene and ³H-benzo(a)pyrene metabolism was studied in vivo and in vitro in rats. Tritium is lost or exchanged by the molecule of benzo(a)pyrene. A ratio of absorbancy/radioactivity was calculated for seven major metabolites of benzo(a)pyrene. This ratio index was used to ascertain the nmoles of each major metabolite formed as a result of MFO activity. It was also used to measure the amount of each monol, quinone or diol that conjugated with glucuronic acid when UDP-glucuronic acid was added to the reaction mixture.

The monols and diones conjugated readily with glucuronic acid, if UDP-glucuronic acid was added to the reaction mixture. The diols, as a class, conjugated less rapidly with glucuronic acid. The 9, 10-diol conjugated least and 3-ol metabolite conjugated most rapidly with glucuronic acid.

In recent studies (Melius, See attached report No. CR809493), the effects of pH, temperature, protein (enzyme concentration) and incubation times have been determined on the BaP metabolite patterns obtained in the in vitro assay system. Maximum metabolite production occurred over a pH range of 6.6 to 7.5. Maximum total metabolite formation occurred at 30°C, whereas maximum B(a)P diones were formed at 25°C. Metabolite production was linear with protein concentrations of 1 to 3 mgs. in the assay medium. Metabolite production was found to be linear up to a sixteen-minute incubation time. There was some increase in MFO activity with 3-MC dose level up to 20 mg/Kg and seven day period of treatment maximum induction of MFO activity.

In the fish systems we have worked with, the latter MFO system converts B(a)P mainly to 7,8- and 9,10-diols and also 9- and 3-phenols with significant amounts of diones. The MFO activity in our fish systems was much less than that present in rat or previously reported in trout.

This study has raised more questions with regard to the biochemical fate of B(a)P than it has answered. Much previous work in the area of B(a)P metabolism in the aquatic environment lacked the interpretive quality necessary for the task at hand and had to be repeated, using more modern methods, a fact that weighed heavily on the time/manpower factor. We hope, however, that the progress we have made towards answering the questions posed has returned a reasonable yield to the inceptors of this program, and science in general.

VII. Significance to Biomedical Research and Program Needs of NCI and EPA

1. Responsiveness of a number of species to environmental carcinogens has been demonstrated. Sheepshead minnows have developed proliferative liver lesions (oval cell hyperplasia, possibly adenofibrosis) resulting from benzidine exposures and benign hepatic polyps related to aflatoxin exposure. A neoplastic condition, also from aflatoxin exposure, has been produced in Menidia livers. Finally, rainbow trout developed overt hepatocellular carcinoma and other tumor types following treatment with benzo(a)pyrene and methylazoxymethanol acetate. All three species have high potential to be used as routine carcinogen assay organisms. Additionally, the black bullhead has been successfully used to demonstrate a cause and effect relationship to an existing pollution situation. Laboratory and field responsiveness have shown it to be a good indicator organism. Finally, the tiger salamander (Abystoma tigrinum) is particularly interesting as an assay or indicator organism because of its cutaneous and liver enzyme responses to the PAH, perylene. The fact that this species has shown cutaneous tumor development in contaminated environments heightens its significance as a possible indicator-monitor species, even though perylene has not been found to be carcinogenic to the salamander in the laboratory.

Physical exposures systems that may be rigorously controlled in the laboratory are now available for either critical life-stage exposure or long-term testing.

2. Results from the field biomonitoring study (Report entitled: "A Prospective Study of Infectious and Non-infectious Diseases in Fishes and Shellfishes in relationship to Pollutant Activity in Three Gulf Coast Estuaries") indicate the following: First, a low tumor frequency in fishes suggests low risks from environmental carcinogens at locations and times sampled. Therefore, future studies should be retrospective in regard to neoplasia as an indicator of carcinogen contaminants; i.e., studies should be based on a a priori indication of tumors in certain species at certain sites. Secondly, findings indicate that oysters may be more sensitive indicators of environmental response to local contamination in coastal regions. Future studies should include careful monitoring for tissue and physiologic changes indicative of chemical-induced stress in oysters. Furthermore, several diseases of oysters and fishes were detected during the study. At least two of these, a Rickettsia found in oyster digestive gland cells and ichthyosporidiosis from several fish species, are important in terms of possible impact related to effects on biota and human health. These diseases and their role in the Gulf Coast environment should be investigated further. Finally, the overall study suggests that, while being far from pristine, the study areas were not severely unhealthy environments at the time of investigation. This study and others now provide a baseline for future comparison. Studies should be made periodically to monitor the impact of population and industrial growth along this valuable coastal region, a region predicted to be one of the fastest growing areas of the country during the next decade.
3. The biochemical investigations undertaken as part of this Interagency Agreement were based on the following questions: Can fish models

supplement biochemical information derived from mammalian models, and would they, hence, provide information useful in establishing a molecular mechanism for chemically induced, proliferative tissue changes?

The approach to answering this question was divided into three general areas, (1) enzymatic activation of pre-carcinogens, (2) carcinogen/metabolite identification, and (3) carcinogen/metabolite detoxification. The following are the major accomplishments in these areas:

- Induction of mixed-function oxygenase (MFO) systems in mullet, killifish, and catfish was shown with classic inducers, such as 3-methylcholanthrene (3-MC) and also with phenobarbital (PB).
- Induction of uridine diphosphoglucuronic acid (UDPGA) and sulfate transferases was demonstrated in rats and in mullet.
- Ames testing was used successfully for the identification of fish MFO-derived mutagenic substances.
- Separation of the twelve phenols of benzo(a)pyrene (B(a)P) and all its other metabolites was achieved. Tentative identification of some cis-diols of B(a)P was also made.
- Precise conditions for the measurements of the metabolites of B(a)P using fluorescence detection were established.
- Recommendations were published for the standardization of MFO determinations in aquatic animals.
- Effects of temperature variation on the enzymatic activity in a cold-blooded animal were shown.
- Position-specific MFO activity on B(a)P was related to MFO induction.
- Temperature-related induction of MFO was shown in a freshwater fish.

VIII. Proposed Course - Future Plans

1. The NCI/EPA collaborative project officially concluded October 1982 and no new projects were undertaken. During FY83, research projects that were already underway at Gulf Breeze and by investigators under cooperative agreements were completed. Major emphasis has been on preparing final reports (due October 1983) and collating reprints produced as a result of the research period.
2. An in-house study sponsored by the U.S. Army and USEPA (Interagency Agreement) will be conducted at Gulf Breeze on the effects of nitrosamines on fish species, using test systems developed during the NCI/EPA research program. The study will focus on assessment of diethylnitrosamine in fish.

IX. Date Contract Initiated and Period of Contract Planned

Initiated
October 1978

Expiration Date
September 30, 1982

X. Contractors Project Director

Dr. Henry F. Enos, Laboratory Director
Gulf Breeze, ERL, EPA

Project Officers for NCI and EPA

NCI:	Dr. Herman Kraybill
EPA/ORD:	Dr. Frode Ulvedal
Principal Investigator:	Dr. John A. Couch, Gulf Breeze, ERL, EPA

XI. Reports on Specific Extramural Cooperative Agreements

Reports for the complementary extramural cooperative agreements follow. These reports summarize research completed by each principal investigator, with emphasis placed on the programs final year.

Title: Teleost Carcinogen Assay Systems
Principal Investigator: B.J. Martin, University of Southern Mississippi
Cooperative Agreement Number: CR809347
Project Officer: John A. Couch

REVIEW OF PROJECT ACCOMPLISHMENTS

- 1) Closed-Recirculating Systems. These systems were devised in our laboratory in order to maintain Cyprinodon variegatus in carcinogen contaminated water in a manner that could provide minimum risk to laboratory personnel. The system has proven to be quite adequate for the maintenance of C. variegatus as a healthy laboratory species for long-term exposures to carcinogens.
- 2) Benzidine Toxicity. Routine toxicologic methods were employed to determine that the LC-50 (96 hrs) for benzidine dihydrochloride (BEN) with respect to C. variegatus was 64 ppm.
- 3) Induction of Proliferative Lesions. Proliferative lesions were produced in the livers of adult C. variegatus exposed to weekly contaminations of 1 ppm BEN. The livers of the affected individuals presented with large fibrotic regions within which a proliferation of various types of tubular profiles could be observed. Some areas had a cholangiomatous appearance and others appeared adenofibrotic. In experiments in which C. variegatus were exposed to 5 and 10 ppm BEN early manifestations of this lesion could be observed by 60 days of exposure.
- 4) Exposure of C. variegatus Embryos to BEN. These studies provided the opportunity to observe the effects of BEN exposure on the critical life stages of C. variegatus. Exposure of early C. variegatus embryos to BEN at various concentrations (5-500 ppm) indicated that significant numbers of abnormalities occurred only at exposures of 50 ppm and above. The abnormalities listed in the order of their frequency of occurrence were: tubed heart syndrome with distended pericardia, poor circulation, sparse distribution of melanophores around the yoke, inability to hatch, abnormal head morphology, scoliosis, and faint RBC pigmentation.
- 5) Studies of the SHF-1 Cell Line. A cell line (SHF-1) that had been established in Dr. Ellender's laboratory from Archosargus probatocephalus (the sheepshead) was evaluated as a potential system for studying the effects of carcinogens on teleosts at the cellular level. Existing techniques for C-band staining of mammalian cells were modified to provide C-banding of these teleost cells. C-banding was then used as a tool in the karyotyping of control preparations and preparations exposed chronically to known carcinogens. Toxicity tests were performed to determine the concentrations of benzo(a)pyrene [B(a)P], BEN and diethylnitrosamine (DNA) that were acutely toxic to this cell line. Long-term, low-level exposures of SHF-1 cells to these carcinogens provided evidence that B(a)P and BEN were capable of producing multi-layered foci in the cultures.
- 6) Early Embryogenesis of C. variegatus. Existing literature concerning the early embryogenesis of teleosts did not provide the knowledge of cellular detail needed as the background material for studies of the effects of chemical carcinogens on C. variegatus. In order to more accurately observe the cellular and sub-cellular activities that occurred during early

embryogenesis, it was necessary to devise a dechlorination technique which allows one to study excised blastoderms. By use of this technique new knowledge was obtained concerning the mechanisms that initiate polar concentration of protoplasm at fertilization. Also, it was possible to observe wedge-like structures in two of the four cells of the 4-cell stage of blastulation that seemed to function in the elongation of the blastula to produce the oval shape of the 8-cell stage. Observation of the inverted blastoderm during gastrulation provided evidence that the ectodermal cells covering the yoke travel from the superficial blastoderm via a pathway along the blastoderm floor. This observation strongly supports an existing hypothesis which states that the yoke cells originate by outward movement of blastoderm cells from deep within the blastoderm. These studies provide important background material needed if teleost critical life stages are to be used as rapid tests for detecting carcinogenic effects.

- 7) Morphological Studies of *C. variegatus* Peripheral Blood and GI Tract. Intelligent use of *C. variegatus* as a biological indicator of the effects of chemical carcinogens requires a detailed knowledge of the morphology of its blood and GI tract. Since such information was not extant in the literature, morphological studies of these two *C. variegatus* systems were accomplished. The studies provide detailed information concerning the morphology of *C. variegatus* peripheral blood and GI tract and also produced new data that are basic contributions to the literature concerning these two systems in teleosts.
- 8) In vitro Techniques for the Study of Carcinogenesis. Two innovative in vitro techniques that should be applicable to the study of carcinogenesis were the fortuitous outcome of our efforts to develop *C. variegatus* cell cultures. The first was the development of procedures for the maintenance of gnotobiotic *C. variegatus* larvae. Gnotobiotic larvae provide the opportunity to obtain data which relate to animal-pollutant interactions free of the products of microbial metabolism. Additionally, the technique provides animals that can be transferred to the septic environment that are free of parasites which are often a problem when feral fish are placed in closed laboratory systems. Our preliminary research indicates that *C. variegatus* larvae may be maintained in a good state of health for approximately 20 days, which is adequate time to conduct short-term assays using this system. With additional research, it should be possible to maintain these fish for the three to four month period required for *C. variegatus* to attain morphological and sexual maturity.

The second technique, an embryo-primary cell culture technique, resulted from our observation that, when *C. variegatus* embryos in culture dishes containing tissue culture media become attached to the surface, a mixed population of cells migrates from the embryos and forms cell monolayers. This technique is unique in that it provides the opportunity to study the effects of a toxic or carcinogenic substance on cells in monolayer and yet, for a period of time, the system contains functioning organ systems in the unattached portion of the organism. Thus, if a precarcinogen is introduced, the mixed function oxidase system in the intact liver should be able to metabolize the compound to the active molecule.

- 9) Primary Hepatocyte Cell Culture Technique. Vertebrate hepatocyte cultures have shown excellent correlation with bacterial mutagenicity assays and rat primary hepatocyte monolayers have proven to be very useful in providing a better understanding of the metabolic processes responsible for the conversion of percarcinogens to the active molecules. Furthermore, since primary hepatocyte cultures can be expected to be more genetically similar to the cells of intact organisms than established cells lines, data from a primary hepatocyte system derived from teleosts should be more directly comparable to results obtained from experiments with the whole animals. Such a system can be maintained more economically than mammalian cell cultures because teleost cells may be maintained at room temperature without the expensive incubators needed with mammalian systems to effect the required gaseous environment. We have developed procedures for producing teleost hepatocyte primary cell monolayers and are now ready to evaluate their usefulness as an in vitro carcinogen assay system.
- 10) Immunological Studies. The immune system of fish is known to be very complex involving many substances ranging from specific "acute phase" enzymes to interferon, to immunoglobulin production. Thus, it should be an excellent system to gain a greater understanding of vertebrate immune system in general and an understanding of the immune system in "normal" teleosts is a necessary prerequisite to accomplishing sophisticated studies of the effects of toxic and/or carcinogenic substances on the immuno-competence of teleosts. The primary problem with studying these systems is that only microliter quantities of blood can be obtained from such small fish. It has been necessary, therefore, to miniaturize or modify existing techniques and to develop bleeding procedures that significantly enhance blood recovery from these small animals. Using a variety of the techniques we have developed, it has been possible to demonstrate that serum from normal C. variegatus differs significantly from that of fish exposed to 1 ppm BEN for several weeks. By use of serum electrophoresis, immune rosette procedures, and immunoelectrophoresis, we are now capable of monitoring the immune system of this species in a rather sophisticated way. We can now study the relationship between stress and the incidence of diseases in fish exposed to toxic or carcinogenic chemicals. The miniaturized procedures developed in the study should be rather easily adaptable to other small organisms from which one can obtain only small quantities of blood or serum.

FINAL YEAR ACCOMPLISHMENTS

- 1) Long-term Exposures. Two experiments are in progress in which C. variegatus are being exposed to weekly 1 ppm contamination with BEN. These experiments have been in progress for approximately 23 months and we plan to continue them as long as the project period will permit. Our intent is to allow the BEN-induced lesion to progress in some of the fish to the point that it possibly could be more precisely identified as a neoplasm.
- 2) Pulse Exposures. In one experiment C. variegatus were exposed to 15 ppm BEN for 24 days. At this point the fish showed signs of stress and the BEN-contamination was removed. These fish have been maintained for ca. 7 months free of the contamination. Our plans are to maintain the fish as long as the project period will allow to see if a pulse-type exposure to an almost toxic concentration of BEN will induce the lesions observed in the chronic 1 ppm exposures.

In a similar experiment, *C. variegatus* were exposed to 10 ppm BEN for 100 days and are now being maintained in contaminant-free water.

- 3) Studies of Cyprinodon Cell Lines. We have continued to maintain and more completely characterize the four *C. variegatus* cell lines established during the previous year of this project and have now determined the chromosome number of the cell lines at various different passages. Preliminary toxicologic work has been accomplished on three of the lines; however, we have concentrated most of our efforts on one cell line (termed LS). LS cells have been exposed to 3-methylcholanthrene (3-MC), benzidine, and diethylnitrosamine (DENA). Currently, dose response work is complete only for exposures of LS to DENA and its LC-50 for this compound is ca. 12mM.

Since colony forming efficiency in soft agar seems to provide an indication of cell transformation in at least some cell lines, we have conducted preliminary tests of the colony forming efficiency of normal (non-exposed) LS cells in soft agar. Growth of LS cells was observed; however, colony forming efficiency seems to be a function of the initial concentration of cells in the agar; i.e., the higher the concentration of the cells, the higher the colony forming efficiency. We have attempted to grow LS cells exposed to 3-MC in soft agar; but, because of toxicity effects, only a very low concentration of cells has been available to place into the agar and little or no growth of colonies occurred. Because of the effect of the concentration factor, we can conduct valid tests of this type only after we have developed procedures that will allow us to place equal concentrations of non-exposed and exposed cells into the test media.

Pilot studies are being conducted using rat liver post mitochondrial supernates (PMS). PMS has been used with mammalian cell systems to metabolize precarcinogens to the active molecules and our experiments are designed to use PMS in a similar manner with the LS cell line.

- 4) Electrophoresis Studies. During the last year of the project considerable effort has been directed toward determining changes in serum protein profiles in fish exposed to benzidine. Liver tissue was prepared from these fish for subsequent histological examination, and it is our intent to establish whether significant correlations exist between observed serum protein shifts and liver changes such as those reported in previous years of the project. In most of the current studies four groups of fish were employed, specifically (1) non-benzidine-exposed, unimmunized, (2) benzidine-exposed, unimmunized, (3) non-benzidine-exposed, immunized, and (4) benzidine-exposed, immunized. In the immunized fish, the immunizing agent unless otherwise stated was the MS2 bacteriophage (in a few studies still being evaluated, a protein antigen, mouse IgG was used as the immunogen.) Altogether, five analytical techniques were applied to separate, quantitate and compare the serum protein profiles. These techniques included (1) polyacrylamide gel electrophoresis, (2) analytical isoelectric focusing, (3) Graber-Williams immunoelectrophoresis, (4) crossed immunoelectrophoresis, and (5) tandem crossed immunoelectrophoresis. As would be expected the data generated in these studies is highly complex due to the number of protein bands present, and we are in the process of interpretation at this time.

In the polyacrylamide gel electrophoresis (PEG) a consistent pattern seems

to be emerging. A total of twelve readily distinguished peaks can be found following densitometer scan of the separated proteins, although in a given fish no more than 9 peaks were observed (nor fewer than 5). In all benzidine exposed fish a very striking feature was the appearance of one or both of two slowly migrating bands. These proteins were never found in non-benzidine exposed, non-immunized fish, but interestingly, non-benzidine treated, immunized fish always had one or both of the bands. The migration pattern of these bands suggests that they represent immunoglobulins induced by the benzidine or in the case of the immunized fish by the phage immunogen. It should be noted here that in earlier studies (now being repeated) we showed that a benzidine exposure resulted in the production of a substance with (apparently) non-specific phage neutralization properties. It is planned to extract the new bands appearing in benzidine-exposed fish and determine if it is in fact these bands which are responsible for the neutralization effect. Due to its size, benzidine is haptenic, thus non-immunogenic, so if the bands do represent immunoglobulins, they were probably elicited by non-specific means. There is a possibility that the benzidine complexed with tissue of the fish, forming an immunogenic hapten-carrier complex. In either case it seems evident that benzidine exposure results in immune system involvement. We are in the process of confirming that the bands are indeed immunoglobulins by evaluating the antiphage activity of recovered protein from the bands in serum of MS2-immunized fish.

Another common feature seen in PEG studies was the disappearance of one rapidly migrating band (designated as band number 9 based on its position) in all fish exposed to benzidine. This band was found in all but one of the non-benzidine-exposed fish, regardless of their immunization status.

A final observation concerning the PEG studies, consistent with results from other studies, was that there was a general reduction of concentration of serum proteins in benzidine-exposed fish.

There were considerable fish to fish variation within any experimental group in the PEG studies, but we feel that the patterns observed provide a point from which to begin further studies.

Only a few isoelectric focusing preparations have been completed, but some differences between benzidine exposed and non-benzidine exposed fish are emerging. Benzidine-exposed fish have an average of 21 bands whereas unexposed fish showed an average of only 17 bands. As with other techniques, considerable fish to fish variation occurs.

In Graber-Williams immunoelectrophoresis studies, sera from benzidine-exposed fish were compared with sera from unexposed fish. Unexposed fish show a range of 13-17 arcs with most fish showing 16 arcs. Benzidine-exposed fish show a range of 8-17 arcs, with most fish showing 12 or 14 arcs. The Graber-Williams studies were performed chiefly to establish the efficacy of the antisera raised against whole *C. variegatus* serum (which was crucial to the success of planned two-dimensional immunoelectrophoresis studies) and to indicate the value of performing the crossed and tandem-crossed immunoelectrophoresis studies, which are much more involved and require large volumes of high-titered antisera. Based on the qualitative differences observed in the Graber-Williams studies, the decision was made to proceed with the crossed and tandem-crossed procedures.

Crossed immunoelectrophoresis is a two step, two-dimensional procedure. In the

first step, the fish serum was separated in agarose in a manner similar to that used in Graber-Williams electrophoresis. Then the separated proteins were electrophoresed at right angles to the direction of the first electrophoresis, into a gel containing a pre-determined amount of antiserum to whole C. variegatus serum. After staining, the result is a profile consisting of a series of peaks or "rockets", each of whose position is dependent on the distance traveled during the first step and whose height is dependent on the concentration of the protein represented by that peak in C. variegatus serum. Tandem crossed immunoelectrophoresis is a similar procedure, but in the first step, two sera to be compared are placed in adjacent wells prior to electrophoresis. After completion of the procedure, it was possible to directly compare the peaks produced by the two sera. The results of the crossed and tandem-crossed studies to date may be summarized as follows: In non-benzidine exposed fish, 15-19 peaks were observed. By contrast, with only one exception (which had 16 peaks), sera from benzidine-exposed fish had between 21 and 24 peaks (similar results to those obtained in isoelectric focusing studies). In benzidine exposed fish, a total of 12 peaks appeared which did not correspond (by position) to peaks in non-exposed fish (all 12 of these peaks did not of course appear in the serum of any one fish). Conversely, non-exposed fish showed 6 peaks which were never found in benzidine exposed fish. It is not yet clear which (if any) of the differences are due to the appearance or disappearance of various proteins and which represent alterations of existing proteins or perhaps fragmentation of proteins. In addition to these qualitative differences, several quantitative differences were also observed. Of special note, a peak occurred at 2.0 cm from the origin in most fish from both groups, but in the unexposed fish, the mean height of the peak was 2.7 cm, whereas it was only 1.4 cm in the exposed group. This protein is thus a potential marker for evidence of benzidine exposure. We emphasize that the interpretation of the results is in an early stage and much more work remains to be done. The fish to fish variation was expected, but complicates the interpretation of electrophoretic profiles. We are proceeding with the interpretation with all due haste.

- 5) Development of Microassays for Monitoring the C. variegatus Immune System.
In our study of the humoral immune response of C. variegatus exposed to various carcinogens (including benzidine) a continuing problem has been the availability of a standard assay system sensitive enough to use with the microliter quantities of serum available from the small fish; but simple to use, with good reproducibility. Standard serological procedures such as precipitation were unsatisfactory since they required large amounts of serum and/or very high titers of antibody in the serum.

Several types of assays have been developed and evaluated. One of the most promising is an assay based on the use of 4-methylumbelliferone (4-MUMB) as a hapten, conjugated to a protein carrier so that it would be immunogenic. The principle of this assay was as follows: The commercially available compound 4-methylumbelliferyl-B-D-galactoside is non-fluorescent. When placed in a solution with the enzyme B-D galactosidase, the galactoside bond is broken and the compound 4-MUMB is released. This compound is fluorescent and the fluorescence can be measured by means of a fluorometer. In the presence of antibody to 4-MUMB, the B-D-galactosidase is unable to cleave the galactosidase bond, thus total fluorescence is reduced, the amount of reduction being directly proportional to the level of antibody present. To raise antibodies for use in this assay, it was necessary to conjugate 4-MUMB to a protein carrier. This was accomplished via an ester bond involving the acidic amine

group of 4-MUMB using the coupling agent 1-ethyl-3 (3-dimethyl-aminopropyl) carbo diimide. The proteins chosen were bovine serum albumen (BSA) (MW 69,000) and bovine thyroglobulin (THYR) (MW 660,000). Coupling was carried out at room temperature, and the resulting conjugate was purified by a series of dialysis and gel filtration steps. For development and standardization of the assay, antibody was raised in rabbits against the two conjugates (4MUMB-BSA and 4-MUMB-THYR) using standard immunization protocols. The efficacy of the resulting antisera was tested using precipitation and passive hemagglutination procedures.

In order to carry out the developmental assay it was necessary to first standardize or measure fluorescence produced when 4-methylumbelliferyl-B-D-galactoside (the substrate) was mixed with B-D-galactosidase (the enzyme). The resulting fluorescence was then simply compared to the fluorescence seen if anti-4-MUMB was added to the substrate and allowed to bind to the hapten before the enzyme was added. Tests have to date been run using antisera raised against both 4-MUMB-BSA and 4-MUMB-THYR, and have been run at 24°C and at 37°C, with encouraging results in each case. As of yet, no anti-4-MUMB produced in C. variegatus has been listed, but a group of goldfish (Carassius auratus) and two groups of C. variegatus (one normal, one exposed to benzidine) have been immunized with 4-MUMB-carrier conjugate, and antisera will be available for application in the assay by the end of September.

Successful development of this assay provides a very useful tool for studying the humoral response to a hapten. The test is quite sensitive (in part since it uses a fluorescent indicator) and is very simple to perform. All reagents are either commercially available or simple to prepare. Importantly, the basic indicator system (substrate + enzyme) can be inhibited by antibody from any species, so long as the antibody is anti-4-MUMB, thus allowing comparisons of antibody response to 4-MUMB among a number of species. If a hapten such as 4-MUMB, can be accepted as a "model" hapten for immunization tests, in the same way as 2,4 DNP for example, the assay presented should enjoy widespread use.

Another type of assay which we have evaluated as potentially useful in measuring the immune response of benzidine exposed C. variegatus is the microwell passive hemagglutination test, which in theory has the sensitivity to be of use. In our studies, groups of normal and benzidine exposed C. variegatus were immunized with one of several protein immunogens including BSA, THYR, keyhole limpet hemocyanin, and mouse IgG. To measure the antibody level in these fish, serum collected 30-45 days following immunization was tested for ability to agglutinate tanned red blood cells to which the appropriate antigen had been previously absorbed. For this purpose, tanned gluteraldehyde stabilized human type O, or sheep erythrocytes were used. Low level antibody titers can be detected in normal C. variegatus. The benzidine treatment apparently induce a non-specific agglutinin (titers up to 1:16), found both in immunized fish and in non-immunized controls, which of course masked any specific antibodies produced. We are continuing to develop this system, as it has become evident that the presence of suitable levels of protein in the suspension buffer used reduces the non-specific agglutination. Once appropriate conditions have been established, the non-specific agglutination can be reduced to a point that will allow measurement of specific antibody in the animals. As an indicator of the effects of benzidine exposure, the non-specific agglutinin is of interest in its own right of course, and efforts

are being made to isolate it by gel electrophoresis.

A third type of assay for measurement of antibodies which was evaluated was competitive binding enzyme immunoassay. In this assay, C. variegatus (normal and benzidine exposed) were immunized with the protein keyhole limpet hemocyanin. After a suitable immunization period, serum was collected and attempts made to measure the antibody level by the following procedure. Keyhole limpet hemocyanin (a standard amount) was adsorbed to microplate wells. To each well, a mixture of alkaline phosphate labeled, rabbit anti-keyhole limpet hemocyanin (again a standardized amount) and serum from experimental C. variegatus (control and benzidine exposed, immunized and non-immunized) was added. In theory, the two antisera should compete for available binding sites on the hemocyanin, the higher the antibody level in the fish serum, the less enzyme labeled rabbit antibody which would bind. If unbound antibody is then washed away and chromogenic substrate for the enzyme added (O-nitrophenyl phosphate), and estimate of the enzyme-labeled antibody present can be made and thereby an estimate of the antibody level in the fish serum. In no case were we able to show any effect by the fish serum, although non-enzyme-labeled rabbit anti-KLH readily reduced the amount of labeled antibody which bound. One explanation could be that C. variegatus is simply unable to respond to KLH, but since pooled serum from immunized fish could precipitate KLH, once sufficient volume is attained, this can hardly be the case. We believe that the problem lies in the differential affinity of rabbit immune globulins and C. variegatus immunoglobulins. Fish immunoglobulin are of course tetrameric, and IgM-like, and the enzyme-labeled rabbit antisera used was largely IgG. We have since applied this assay to the measurement of antibodies to Mouse IgG. Using rabbit anti-mouse IgG as the enzyme labeled antisera, we can easily measure antisera mouse IgG levels in rabbit and in other mammalian sera (specifically goat, sheep, and donkey), and we currently are in the process of immunizing C. variegatus (normal and benzidine exposed) with mouse IgG. Mouse IgG, as an immunoglobulin, is a much less complicated antigen to work with than was hemocyanin (which was very large for a protein, poorly soluble, and had a large number of antigenic determinant sites). The parameters have been worked out sufficiently for this assay that, if antibodies still cannot be detected in C. variegatus serum, we are certain that the problem is one of affinity, which we can take steps to correct. This competitive enzyme immunoassay (like the 4-MUMB fluorescence inhibition assay described above) would be very useful in measuring antibody levels using small amounts of serum because it has been shown to be highly sensitive, and is applicable to a wide variety of systems.

PUBLICATIONS AND PRESENTATIONS

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Title: Causes of Papillomas On Fish Exposed To Chlorinated Sewage Effluent
Principal Investigator: John M. Grizzle, Auburn University
Cooperative Agreement Number: CR809336
Project Officer: William Davis

Introduction

A population of black bullheads Ictalurus melas in the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant had a 73% prevalence of uniformly located oral papillomas when first examined in 1979. Sewage entering this treatment plant did not include discharge from industries or other sources suspected of releasing carcinogens. The process of chlorinating the sewage effluent was considered a possible source of carcinogens.

Laboratory and field experiments were initiated to determine the cause of the papillomas. Objectives were to : (1) determine the prevalence of papillomas on fish in the final oxidation pond; (2) describe the histology and ultrastructure of the papillomas; (3) determine if the papillomas would persist on black bullheads taken from the final oxidation pond and placed in clean water; (4) chemically identify mutagenic and carcinogenic chemicals in the water; (5) determine if the water was mutagenic; (6) determine if tumors would develop in new fish placed into the final oxidation pond; (7) determine if hepatic-enzyme induction occurred in fish exposed to the water; (8) determine if viruses were associated with the papillomas by ultrastructural examination, by cell-free homogenate, and isolation of virus with cell-culture techniques; (9) determine if lesions would develop in brown bullheads exposed during embryonic development to a concentrate of the final oxidation pond water; and (10) determine if tumors would develop on black bullheads exposed to an organic concentrate of the final oxidation pond sediment injected or applied topically to the mouth.

Description of Field Study Site

The 0.8-ha final oxidation pond of the Tuskegee, Alabama, sewage treatment plant was designed for post-secondary treatment of sewage effluent. Water retention time in the pond depends on the flow rate and averages 4-5 days. Sewage treatment before the oxidation pond consists of sedimentation, activated sludge secondary treatment, and disinfection by chlorination (Fig. 1). During Jan. - Nov. 1979, monthly average residual chlorine concentration was 1.2-3.1 mg/liter in the effluent leaving the chlorine contact chamber and when entering the final oxidation pond (Fig. 2). Since Nov. 1979 this chlorine concentration has ranged from 0.3 to 1.0 mg/liter. Residual chlorine concentration of water leaving the final oxidation pond is usually 0.1 mg/liter, approximately the same as before Nov. 1979.

Description of Fish Populations in the Final Oxidation Pond

Wild, adult black bullheads in the final oxidation pond had a 73% prevalence of oral papillomas between Dec. 1979 and July 1980. The prevalence of papillomas in this population has decreased in more recent samples (Fig. 3). The latest gill-net sample on 21 Mar. 1983 showed a papilloma prevalence of 23%. Another change in the papillomas was the size; the large papillomas

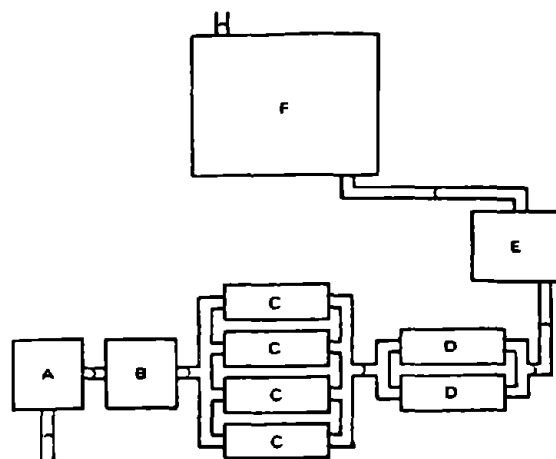


Figure 1. Schematic plan of the Tuskegee sewage treatment plant. A, pumping station; B, grit removal; C, aeration basins; D, settling basins; E, chlorine contact chamber; F, final oxidation pond.

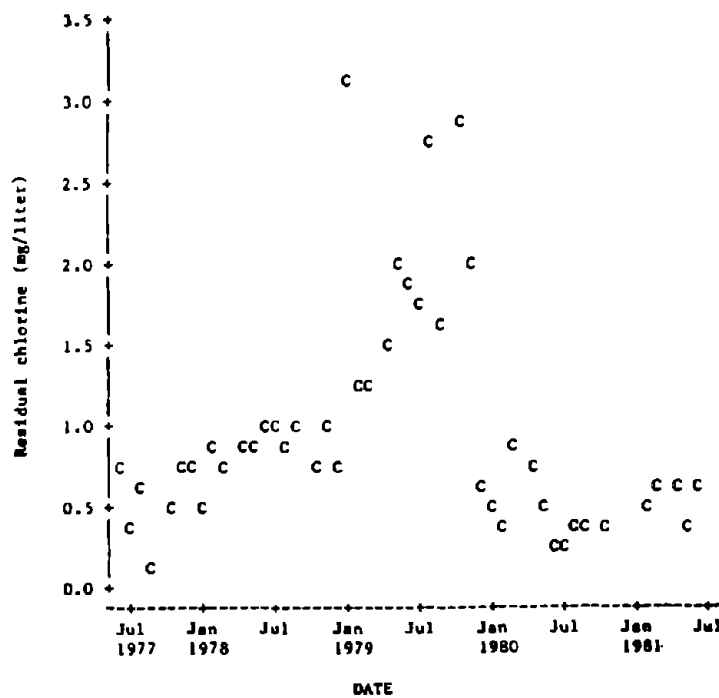


Figure 2. Total residual chlorine in water leaving the chlorine contact chamber of the Tuskegee, Alabama, sewage treatment plant.

seen protruding from the mouth of black bullheads in 1979-1980 (Grizzle et al., 1981) were not found in recent samples.

The only other species of fish originally present in the final oxidating pond were green sunfish Lepomis cyanellus and golden shiners Notemigonus crysoleucas. During May and July 1980, 2,000 silver carp Hypophthalmichthys molitrix were released in the final oxidation pond. No tumors have been found on any of these species.

Description of Papillomas on Black Bullheads in the Final Oxidation Pond

Papillomas on wild black bullheads were examined by light and electron microscopy. The papillomas consisted of hyperplastic mucosa covering papillae of submucosa. Goblet cells and alarm substance cells were absent from parts of the lesion; in other areas these cells were numerous and formed cell nests. The mid to near basal portion of the papilloma epithelium consisted of stellate cells having fewer mitochondria and less endoplasmic reticulum than epithelial cells in other portions of the lesion. The prominent feature of the cytoplasm of these cells was the numerous tonofilaments filling most of the cell. Intercellular spaces separated these cells except where connected by desmosomes, which occurred more frequently between the cells in the mid and basal area than near the surface. In the basal region of the neoplastic mucosa, intercellular spaces occurred less frequently than in the middle portion of the tumor, and numerous lymphocytes were present. Electron-dense cells containing 35-nm particles were present in the epithelial portion of some tumors examined ultrastructurally (Grizzle et al., 1981), but this cell type was not consistently present.

Histologically, the black bullhead papillomas were similar to those of brown bullheads Ictalurus nebulosus described by Lucke and Schlumberger (1941). Harshbarger (1977) considered bullhead papillomas to be a common type of fish neoplasm. However, the papillomas in the present case are distinctive because they are uniformly located in the mouth fornices rather than in variable locations.

The papillomas on 26 adult black bullheads from the final oxidation pond were measured, and the fish were placed in 3-m diameter plastic pools. Only 6 fish lived for more than a month because of infectious diseases. After 124 days, 5 fish with 8 papillomas were examined. Four of the papillomas were approximately one-half the original surface area, two decreased slightly, one disappeared, and one increased slightly. One fish that had a papilloma reduced to one-half size after 124 days retained the papilloma at this new size until necropsied after 378 days. The persistence of these lesions after removal of the fish from the final oxidation pond indicates a neoplastic rather than hyperplastic lesion.

Analytical Chemistry of Final Oxidation Pond Water

The Alamba Pesticide Residue Laboratory, Auburn, Alabama, and the Alabama Department of Public Health Laboratory, Montgomery, Alabama, used gas chromatography to determine if toxic organic chemicals were in the water or sediment of the final oxidation pond. Water collected on 16 Nov., 79, 9 Mar., 80, 22 Jan., 82 and 3 Mar., 82 indicated the presence of unidentified

nitrogen and phosphorus organic residues in the 100 ng/liter range. No significant residues of pesticides, herbicides, or PCB's were detected in the water or sediment. Water collected on 1 May, 1981 contained chloroform (9.0-13.5 µg/liter), but chlorodibromomethane and bromoform were not found with a detection limit of 1.0 µg/liter. Chloroform concentrations were similar before chlorination and in the final oxidation pond.

Mutagenicity of the Final Oxidation Pond Water

Extracts of the final oxidation pond water were tested for mutagenicity with the Ames test (Ames et al. 1975). Three types of organic extracts of the pond water were prepared. For one procedure the water was filtered through XAD resin and the organics were eluted with acetone. For the other procedures the water pH was adjusted to either 1.0 (for acid fraction) or 12-13 (for basic fraction) and then extracted three times with 200 ml of 25% ether and 75% hexane per liter of water. For all procedures, the bulk of the solvents were removed by distillation. The last 20 ml of solvent was evaporated under a stream of nitrogen.

The acid fraction and the XAD extract were mutagenic to Salmonella typhimurium tester strains TA98 and TA1000 if S-9 (aroclor-induced rat liver enzymes) were present. The mutagenicity of the organic extracts varied seasonally (Fig. 4). The basic fraction was not mutagenic. No differences have been found between extracts prepared by organic extraction of acidified water and extracts prepared by filtering the water through a resin column. Water samples collected at both the inlet and outlet of the final oxidation pond were mutagenic, indicating that the mutagenicity is not lost during retention in the pond.

Exposure of Fish to Water in the Final Oxidation Pond

Black bullheads, brown bullheads, yellow bullheads Ictalurus natalis, and channel catfish Ictalurus punctatus were placed into cages in the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. The 1-m³ cages were allowed to rest on the pond bottom (sinking cages) or suspended off the bottom by floats (floating cages). Cages were placed at three locations in the final oxidation pond: near the inlet (inlet A), approximately 60 meters from the inlet (inlet B) or near the pond outlet (outlet C). Most cages were stocked with 50 fish.

Papillomas developed on five of the black bullheads kept in cages in the final oxidation pond (Table 1). The papillomas were located in the same area of the mouth and had the same gross and histological appearance as the papillomas on the wild black bullheads in the pond. Four of the papillomas developed between 467-537 days of exposure on fish in a sinking cage near the outlet. One papilloma developed in less than 57 days on a fish in a sinking cage at the inlet B location. The small number of fish developing papillomas prevents a conclusion concerning the importance of cage location and contact with the sediment.

The most common types of lesions developing on the fish confined to cages in the final oxidation pond were oral mucosa hyperplasia and stomatitis usually located in the mouth fornices. The incidence of oral lesions on

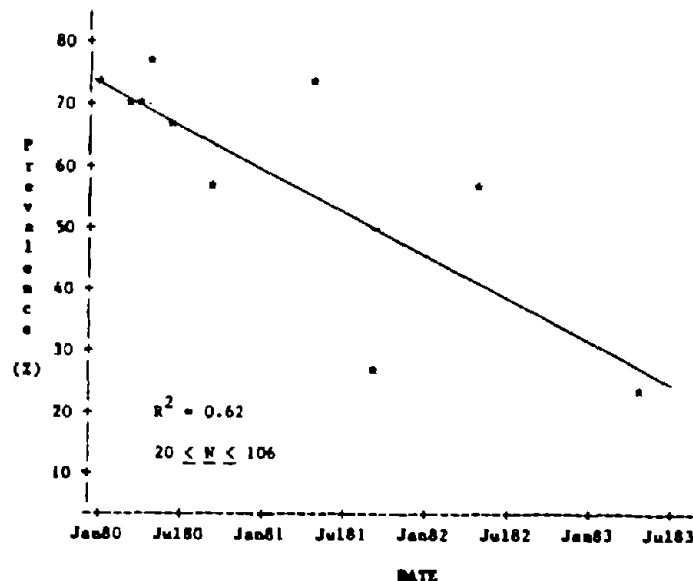


Figure 3. Prevalence of papillomas on wild black bullheads from the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant.

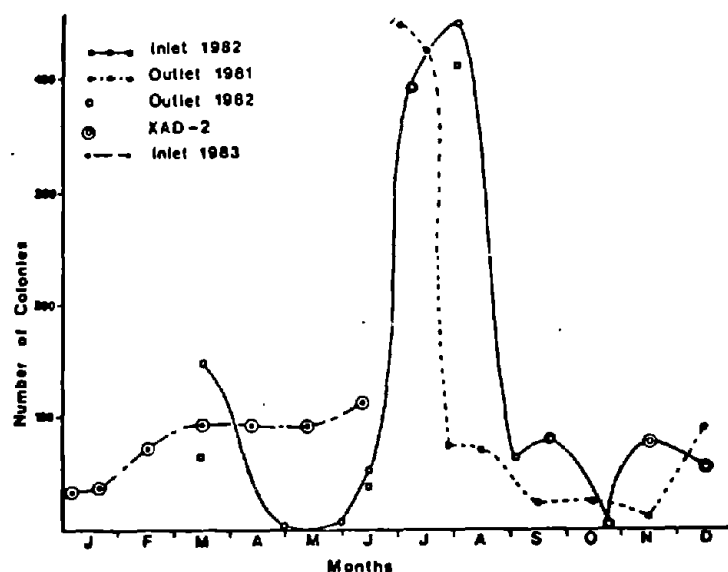


Figure 4. Response of *Salmonella typhimurium* strain TA100 in the Ames test to organic extracts of water collected at the inlet or outlet of the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant. The number of colonies is the difference between test and control plates. The extracts tested were prepared by solvent extraction of acidified water samples or by XAD-2 resin absorption.

Table 1. Maximum prevalence of oral lesions on caged fish in the final oxidation pond of the Tuskegee sewage treatment plant. Control is the maximum prevalence of hyperplastic oral lesions in caged fish kept in a control pond. Neoplasms did not occur in control fish.

species	stocking date	days of exposure	hyperplasia		papillomas			control
			%	<u>N</u>	%	<u>N</u>	<u>days</u>	
black bullhead	21Oct80	168	95	22	80	5	537	4
	23Feb81	43	50	12				
	29Oct81	407	33	6				0
	9Apr82	245	25	8				
	8Jul82	57	17	12	8	12	57	9
yellow bullhead	12May81	683	0					14
brown bullhead	29Oct81	228	0					0
channel catfish	16Jan81	436	30	10				8
	29Oct81	513	4	28				4
	8Jul82	254	0					5

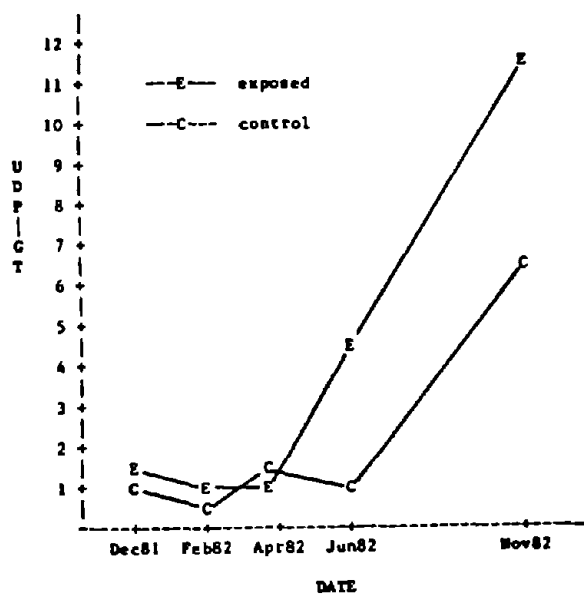


Figure 5. UDP-glucuronosyltransferase (UDP-GT) activity (nmol/min/mg protein) in liver microsomes of caged channel catfish in the final oxidation pond of the Tuskegee, Alabama, sewage treatment plant or in a control pond.

caged fish in the final oxidation pond varied depending on the fish species and the year that the fish were exposed (Table 1). The highest incidence was in black bullheads stocked during Oct. 1980 and the highest incidence of oral lesions of the four species tested. Similar oral lesions occurred on control black bullheads (Table 1) but at an incidence of 9% or less. There were no differences found between floating and sinking cages or between inlet and outlet locations.

Induction of Hepatic enzyme Systems

Sulfotransferase (ST) and UDP-glucuronosyltransferase (UDP-GT) activities (nmoles of p-nitrophenol conjugated with glucuronic acid/min/mg of microsomal protein) in livers of black bullheads, brown bullheads, and channel catfish from cages in the final oxidation pond and a control pond were determined as part of a separate EPA cooperative agreement (Strength, Auburn University, Personal communication). UDP-GT in channel catfish in the final oxidation pond was higher than in control channel catfish (Fig. 5). UDP-GT activity in other ictalurid species and ST activity in all species tested in the final oxidation pond were not consistently different than controls. The induction of UDP-GT in channel catfish confined to the final oxidation pond indicated exposure to a toxicant. Similar induction occurs in rats and fish given oral or intraperitoneal doses of carcinogens.

Virology of the Papillomas

Homogenized papilloma and supernatant from primary cell cultures were transferred to channel catfish ovary and brown bullhead cell lines, but no CPE developed. Papilloma cells growing *in vitro* appeared to be epithelial and fibroblastic and survived a maximum of five passages.

Homogenized papilloma from a final oxidation pond black bullhead was filtered through a 0.45- μ m membrane and injected subcutaneously into the mouth fornices of 19 black bullheads. Fish were observed 11-14 months without signs of papillomas or hyperplastic lesions.

Examination of papillomas with transmission electron microscopy failed to reveal virus-like structures. If a virus is associated with the black bullhead papillomas, they are unlike those previously reported to be present in papillomas.

Exposure of Brown Bullhead Embryos to Final Oxidation Pond Water

Lesions were not observed in brown bullheads 25 days after a one-hour exposure during the fifth day of embryonic development to an organic concentrate of effluent from the final oxidation pond. Lesions were also absent in brown bullheads 8 months after similar exposure to 0.5 or 1.0 mg/liter aflatoxin B₁ (AFB). The negative results with the AFB-exposed embryos indicate that further development of the brown bullhead embryo exposure model is necessary before it will be useful in carcinogenicity studies.

Laboratory Exposure of Black Bullheads to Sediment Extracts

Sediment from the inlet area was collected and allowed to dry for 96 hours.

The sediment was then further desiccated with Na_2SO_4 . Organics from the sediment were then extracted using acetone and methylene chloride. The extract obtained after distillation was dissolved in DMSO. Six fish were injected in the right mouth fornix with 0.1 ml of the extract at a concentration of 83.3 mg/ml DMSO. Six control fish received an injection of 0.1 ml DMSO. Fish were injected once every 2 months for a period of 9 months. The same extract was also dissolved with DMSO to give a concentration of 50 mg/ml DMSO. The mixture was applied to the entire oral area of 8 black bullheads using a cotton swab. Each fish was swabbed with a circular motion for 1 minute, at weekly intervals, for a period of 6 months. Eight other fish that served as controls received weekly, 1-minute swabs of DMSO only.

Black bullheads injected with 0.1 ml DMSO developed necrosis at the injection site. Bacterial infections occurred in the black bullheads being swabbed with sediment extract. No tumors developed in either the injected or swabbed fish after 8 months.

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Title: Perylene as a Possible Environmental Carcinogen (Part I).
Principle Investigator: Francis L. Rose, Texas Tech University
Cooperative Agreement Number: CR807740
Project Officer: John A. Couch

Introduction

A population of neotenic Ambystoma tigrinum inhabiting a sewage lagoon at Reese Air Force Base, Hurlwood, Texas, has been under investigation since 1969. Members of this population are able to tolerate the rigors of a sewage effluent environment with its concomitant low levels of dissolved oxygen in the summer months due to their possession of three respiratory surfaces: lungs, gills and integument. The incidence of integumental lesions in this population increased drastically until 1975 and has decreased steadily to the present. Unfortunately, the population has also decreased in numbers.

The current phase of the research has both an environmental and a laboratory component. I was to supply Dr. Robert Anderson at Sloan-Kettering Cancer Institute with salamanders for metabolite studies involving polycyclic aromatic hydrocarbons and I was to:

- a. Ascertain if neotenic A. Tigrinum might serve as a sentinel organism for the detection of toxic waste products at other localities.
- b. Ascertain whether perylene, benzo(a)pyrene, or a combination of both agents induce tumorigenesis in neotenic A. tigrinum.
- c. To ascertain if melanocytomas could be experimentally transplanted subdermally into other A. tigrinum.
- d. To ascertain whether tumors which normally regress under laboratory conditions might have accelerated growth if treated in vivo with perylene.
- e. To continue the census of the A. tigrinum population at Reese AFB and to monitor changes in the lesion frequency.

Results

- a. Development as a sentinel organism

The introduction of our groups of 20 salamanders each from Reese AFB to four other ponds, two sewage and two non-sewage was unsuccessful. In all cases the yearlings metamorphosed within two months. The introduction of over 500 larvae from non-sewage ponds into the Reese AFB lagoon resulted in the death of all the animals within one week. In essence, the Reese AFB neotene is specifically and narrowly adapted to the conditions within the lagoon. Both a genetic and an environmental component have been identified. Organisms transferred from non-sewage areas into the sewage lagoon cannot adapt to the rigors imposed by the environment. The environmental factors at Reese AFB which allow individuals to remain neotenic have not been identified; however, when these animals are transferred to other ponds these environmental parameters are eliminated, allowing metamorphosis to proceed, albeit at

a slower rate.

My conclusion is that neotenic *A. tigrinum* do not manifest the desired characteristics necessary to serve as a long-term sentinel to monitor environmental pollution.

b. Tumorigenesis

The initial objective of this aspect of the study was to ascertain if lesions could be generated in *A. tigrinum* with injections of perylene. In addition, benzo(a)pyrene was selected as a possible synergistic agent. Initially, 10, 50, and 100 mg doses of perylene per kg body weight (with olive oil carrier) were injected into groups of ten animals each. There were no differences in the weight loss or mortality of the individuals among groups and subsequent injections carried 100 mg perylene in two ml of olive oil per kg body weight. Benzo(a)pyrene was administered at 10 mg (in olive oil) per kg body weight.

Experimental groups contained 20 individuals in a schedule. Three experiments involving perylene were not completed due to water flow problems or high mortality due to bacterial infections. Combining the results, there were 60 controls, 60 experimentals injected with perylene, 40 experimentals injected with benzo(a)pyrene and 40 experimentals injected with perylene + benzo(a)pyrene. Suspect lesions were processed and sent to Dr. John Harshbarger for histological examination.

Three individuals injected with perylene exhibited 1-3 small lesions of epidermal papillomas. These growths were small, clearly delineated but never exceeded 2 mm in diameter. They decreased in size and were on the verge of complete regression when removed. One suspect melanocytoma was not verified by Dr. Harshbarger. These lesions are thought to be incidental to the treatment.

Internally, one hepatocellular carcinoma (approximately 1.3 cm in diameter) was confirmed from the perylene injection group. Although an interesting find (previously unreported in urodeles) the size of the growth and the failure to generate others causes us to doubt its relatedness to the experimental treatment.

Those individuals within the experimental groups which had died within a month after injection contained a network of cream colored anastomosing ridges within the body cavity. Lacking firmness and appearing vacuolar these growths were initially attributed to bacterial contamination although none was found in the controls. Within four months the masses were quite thick (.4 cm) and appeared to invade the liver, kidney and dorsal trunk musculature. In females the mass "encapsulated" the oviducts. Dr. Harshbarger confirmed that the masses were not invasive and attributed their presence to chemical and/or physical agitation. Perylene is a crystal and does not enter into solution in olive oil and while these crystals might cause agitation in the perylene treated group, it does not explain their presence in those treated with benzo(a)pyrene alone. Surprisingly, by the fourth month the mean weight of the mass in the perylene + benzo(a)pyrene group was double that found in the other two experimental groups (Fig. 1).

c. Melanocytoma Transplants

Fifteen yearlings were selected to receive 1 x 2 mm slivers of melanocytoma excised from a single individual. The slivers were placed in a prepared slit in the integument along the dorso-lateral aspect just anterior to the right rear leg. The sliver was positioned to where it could be seen after granulation which took about 48 hours. Within two months 12 of the experimentals showed no sign of the melanocytoma transplant externally nor could any sign be found with the aid of a dissection microscope. At the end of the third month the other two individuals showed no sign of the transplant. All of these individuals lived through the sixth month maintenance period. Autopsy revealed no abnormalities.

This experiment was to be repeated with perylene, benzo(a)pyrene and perylene + benzo(a)pyrene injected individuals but the scarcity of suitable larvae terminated this line of endeavor.

d. Epidermal papilloma treatments with perylene.

Experimentally I have shown that epidermal papillomas with a diameter of .5 to 1.5 regress when the individuals are maintained in the laboratory. The premise behind this experiment was: If perylene is an insult agent to these organisms then epidermal papillomas exposed to perylene should exhibit increased or accelerated growth.

Fifteen control and 15 experimental salamanders were selected and maintained in the laboratory for one month. At the end of one month the experimentals were injected with a mixture of 100 mg perylene in one ml of olive oil subcutaneously and immediately below the growth. That each individual did receive perylene was confirmed by the presence of the orange crystals in the injection site but the amount of perylene injected could not be ascertained. Controls were injected with carrier.

The data are variable but conclusive. There was no increase in the mean size of the growths in the experimental group; however, there was no regression. The tumors associated with the controls decreased in mean size and at the end of six months the means of the two groups were significantly different (Fig. 2). In this case it would appear that while perylene did not enhance tissue growth, it did retard regression.

e. Population census

There has been a steady decline in the number of salamanders inhabiting Reese AFB since 1975 (Fig. 3). This decrease is due, in part, to the inability of the eggs to hatch if strewn along the bottom of the pond and to a low post-hatching success. No adult salamanders were captured the last four sample times in 1983. For all practical purposes (if not absolute) the population at Reese AFB has become extinct.

In my summary I will present data to show that egg hatching success at Reese AFB and in the laboratory decreases through the egg laying season. In addition, I will present evidence to show that eggs maintained at or below .6 m water depth at Reese AFB do not hatch while those maintained

at such depths in non-polluted ponds have a high hatch success. Larvae hatched in protective screens (to eliminate predators but not food) at Reese AFB die within 72 hours while cohorts hatched in non-polluted ponds show minimal mortality. Oxygen deficiency has been ruled out as the probable cause of this low hatching success. Hydrogen sulfide may be a primary agent. An understanding of the low hatch success and the low post-hatching success is further clouded by the relatively high number of developmental anomalies exhibited in larvae from Reese AFB which were hatched in the laboratory and the recent posting of the Reese AFB lagoons as toxic dumps (with high levels of PCB's).

Recently the sewage sludge at Reese AFB was reanalyzed for polycyclic aromatic hydrocarbons by Dr. John Black at Roswell Park. Testing for six PHA's Dr. Black found all, including perylene and benzo(a)pyrene, to be minimal. These results would indicate that the PAH levels at Reese AFB were never high and that the initial tests were inaccurate or that the levels have been lowered due to degradation. The low levels are surprising in view of the large volume of diesel fuel used in the lagoon per year as an insecticide.

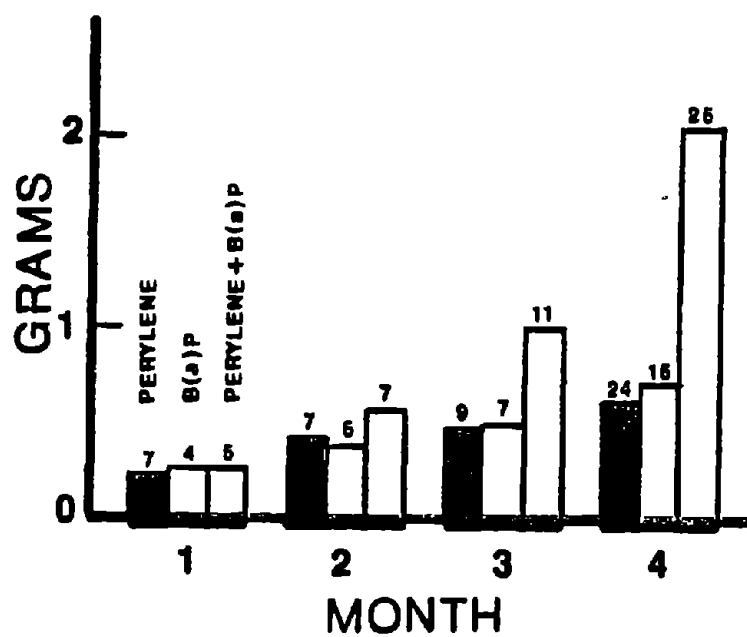


Figure 1. Weight of the reactive tissue mass in the various experimental groups of A. tigrinum. Numbers above the bars represent sample sizes.

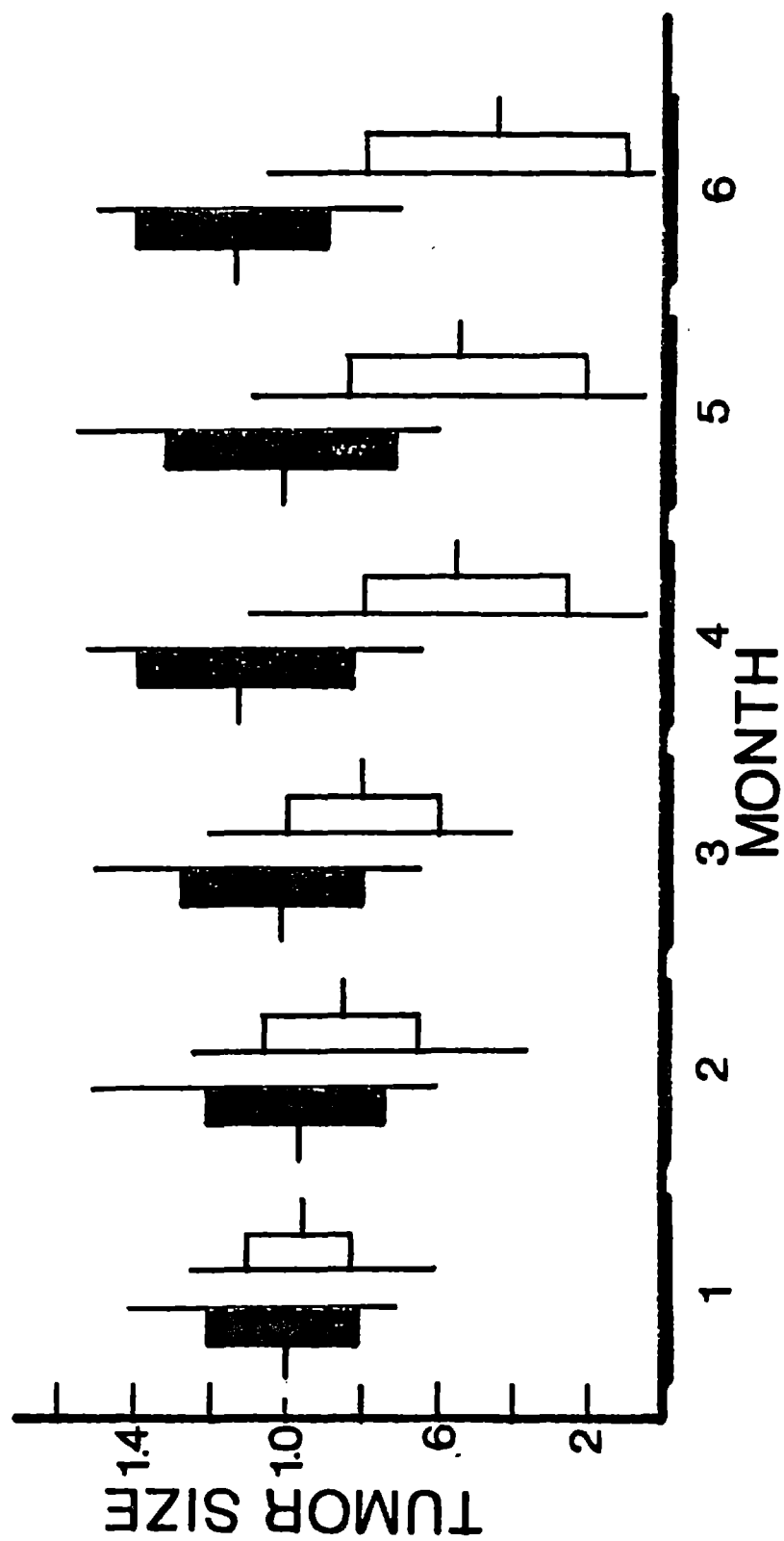


Figure 2. Mean, standard deviation and range of epidermal papilloma diameters on control (open bars) and perylene treated salamanders. The means of the two groups at six months are significantly different.

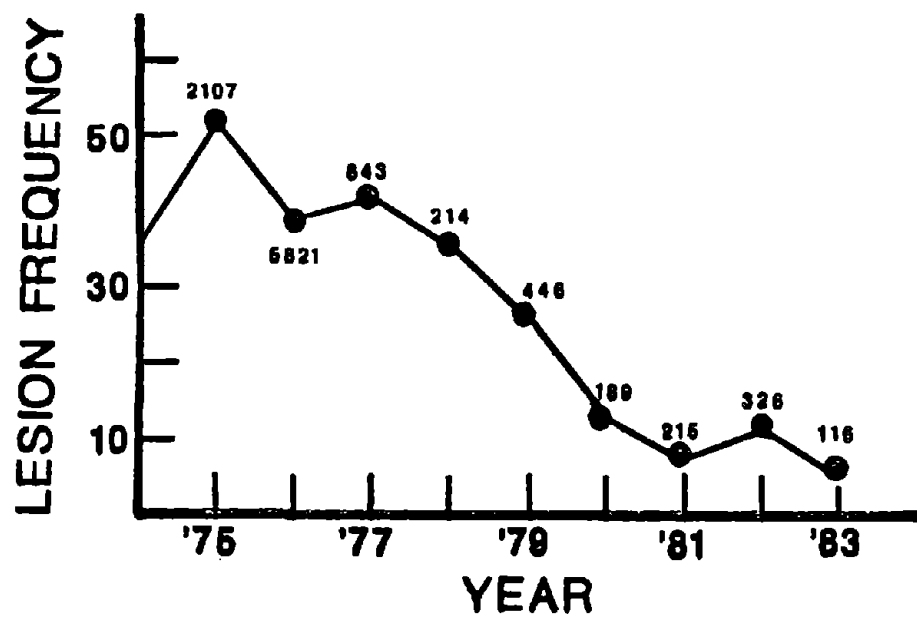


Figure 3. Lesion frequency and sample sizes of salamanders collected at Reese AFB.

Title: Perylene As A Possible Environmental Carcinogen (Part II).
Principle Investigator: Robert S. Anderson, Sloan - Kettering Institute For
Cancer Research.
Cooperative Agreement Number: CR807740
Project Officer: John A. Couch

This reports the progress made on the portion of this cooperative agreement carried out under the supervision of Dr. Robert S. Anderson; that portion directed by Dr. Francis L. Rose has been reported elsewhere.

The unusually high prevalence of various forms of dermal neoplasia in a population of tiger salamanders living in a sewage lagoon at Reese Air Force Base whose sediment was reported to contain high levels of perylene prompted this study of the possible role of perylene in the etiology of these tumors. Since perylene (P) exists in the lagoon as part of a complex mixture of hydrocarbons, its role as a cocarcinogen and/or promoter must be considered, in addition to its action as an initiator. Our major approach has been to study the metabolism of P by the microsomal monooxygenase systems of Ambystoma in the liver and in the skin, the putative target organ. Concomitantly, we looked at the metabolism of its isomer, a known environmental carcinogen, benzo(a)pyrene [B(a)P] by these enzyme systems. Also during this year we quantified P and B(a)P metabolism by another amphibian (Xenopus laevis) and evaluated P using several established mammalian testing protocols.

P and B(a)P metabolism by Ambystoma enzymes is summarized in Table 1 (liver) and Table 2 (skin) for animals collected from a "clean" lagoon not associated with Reese AFB. This site receives no sewage or other pollutants; however, a detailed analysis of the water has not been carried out. In our studies, both water-soluble metabolites (mostly conjugates and macromolecule-bound metabolites) and NaOH-soluble conjugates (some dihydrodiols and phenols) are measured; these are combined to give the total values given in these tables. The turn-over of P was more rapid than that of B(a)P in both liver and skin, this was also observed in other species reported in this paper. Apparently most of P metabolism is via conjugation in contrast to B(a)P metabolism; this is indicated by the general inability to recover NaOH-soluble P metabolites. The specific activity of rat hepatic AHH (Mutazyme) greatly exceeds that of Ambystoma AHH for both PAH substrates. In this study, Aroclor acts as a weak inducer of P metabolism and a moderate inducer of B(a)P metabolism in salamander liver. This effect was not seen at lower doses, (500mg/Kg) and shorter times (4d). Perylene itself is not an effective AHH inducer in the liver after 7d, although there is some P-OHase induction at 2d. As seen in Table 2, our samples of Reese AFB animals showed no higher AHH activity than animals from clean sites. Therefore, there is no indication that the Reese AFB environment exerts an inductive influence on monooxygenase systems in Ambystoma. It is interesting that B(a)P-OHase specific activity is comparable in liver and skin, while P-OHase specific activity is higher in skin samples. Furthermore, although P does not induce AHH activity in the liver, it is a moderately strong inducer of both P and B(a)P metabolism in the skin. Relationships between these differences in AHH characteristics in these organs and tissue sensitivity to carcinogens have yet to be determined.

Studies such as those described above provide insights about PAH metabolism, but produce little information as to the biological activity of the metabolite

produced. The ability of a PAH to bind covalently to biological macromolecules, such as proteins and nucleic acids, after metabolic activation, is thought to be directly related to its ability to exert mutagenic or carcinogenic effects. In these studies (see Table 3) ^3H -P metabolism was allowed to proceed in the presence of purified salmon sperm DNA under optimal conditions for cutaneous or hepatic salamander AHH. The DNA was subsequently extracted, extensively washed, hydrolyzed in PCA and aliquots taken for scintillation counting and DNA determination by the diphenylamine reaction. Significant P-DNA adduct formation was observed. P-DNA binding was greater in the skin than in the liver, consistent with the observations on P metabolism studies, P administration caused a marked induction of P-DNA binding in the skin, but not in the liver. Rat mutazyme, which is aroclor-induced rat hepatic AHH mediated considerable total metabolism of P (Table 1), as well as P-DNA binding. It is interesting that P-induced Ambystoma skin AHH is almost as active as mutazyme in promoting P-DNA adduct formation. We reported earlier that P and B(a)P were not converted to mutagens by salamander liver AHH, as measured by the Ames bacterial mutagenesis test. In light of the P-DNA binding studies reported above, it would be of considerable interest to try comparable tests using P-induced Ambystoma skin AHH.

One of the questions concerning P metabolism that we wanted to answer for Ambystoma was about the influences of sex and developmental stage on AHH activity. This was not possible because almost all the animals we had to work with were neotenes and most of them were female. However, we investigated these factors using Xenopus because we had access to a large number of both sexes and various developmental stages. Our findings of P and B(a)P metabolism by Xenopus hepatic AHH is summarized on Table 4. As with the salamander, both P and B(a)P were metabolized, and the turnover of P exceeded B(a)P. Among the juvenile Xenopus tested, no sex-dependent difference in AHH activity was seen in either basal or aroclor-treated animals. However, in adults AHH activity, using either P or B(a)P as substrate, was significantly lower in males than females. This result makes it potentially interesting to examine the incidence of neoplasia in adult amphibians with regard to sex.

One of the basic goals of this effort was to evaluate perylene as a potential environmental carcinogen. Since our attempts to induce neoplasia in Ambystoma were negative (as reviewed by Dr. Rose), we proceeded to assay for the effect of P in several mammalian test systems. Strain A mice have a genetic predisposition to develop lung adenomas. If these mice are exposed to even weak carcinogens, this effect is considerably magnified. We determined that the maximal tolerated dose (MTD) of P for these mice was about 1g/Kg and proceeded to set up four groups which received doses of 1xMTD, 0.5xMTD, 0.2xMTD, and no P, 3x/wk for 8wk. Sixteen weeks after the last injection the animals were sacrificed and examined for lung adenomas. The protocol and results are given in Table 5. There was no evidence that perylene treatment had any effect on the numbers of lung adenomas seen in these mice.

Since there was little evidence that P acted as tumor initiator, we carried out a study of its effectiveness as a promoting agent of B(a)P initiated skin carcinogenesis. The procedure is given in Table 6 for our study of two-stage skin carcinogenesis. Basically, we applied a dose of B(a)P which produced no tumors, without promotion, during a 14 month period. We

attempted to promote with P applied 3x/wk for one year. All necessary controls were carried out (Table 6). Perylene showed no ability to promote in this protocol as no tumors were seen in any of the two groups. Subsequent treatment with phorbol-12-myristate-13-acetate (a known promotor) produced tumors in all B(a)P-treated mice, indicating that the initiation stage had been adequately induced.

In conclusion, a brief summary of our finding on this project will be given. First, regarding Ambystoma tigrinum as a model organism for detecting environmental carcinogens: 1) It has been shown, using radiolabeled substrates, to possess rather typical mono-oxygenases that can metabolize both perylene and B(a)P. 2) The metabolites can be separated by high performance liquid chromatography to reveal many of the classical B(a)P metabolites. The identities of the P metabolites are unknown (due to lack of authentic standards), but they correspond to the dihydrodiols, quinones, and phenols of B(a)P by region. 3) Ambystoma AHH responds to classical inducers such as Aroclor 1254, under certain conditions. 4) The functional characteristics of the enzyme system vary according to their tissue localization (liver vs skin) and possibly according to the sex and developmental stage of the animal. Based on the above points it appears that Ambystoma possesses the usual enzymes that can activate and/or detoxify PAH carcinogens.

Second, regarding P or B(a)P as carcinogens for Ambystoma: 1) P is an inducer of P-OHase in the skin. 2) Ambystoma AHH mediates P-DNA binding, suggesting biological activity. 3) Ambystoma AHH fails to activate either P or B(a)P in the Ames mutagenicity assay. 4) Ambystoma AHH will mediate the production of the 7-8 dihydrodiol, which is the precursor of the ultimate carcinogenic form of B(a)P. However, direct attempts to produce neoplasia with P or B(a)P have produced negative results. Perhaps Ambystoma responds to other classes of carcinogens, such as the aromatic amines, which we have shown to be activated to potent mutagens in the Ames assay.

Third, regarding the importance of perylene as an environmental carcinogen: not only does it produce no effect on Ambystoma, but it is inactive as a promoting agent in a two-stage skin carcinogenesis assay in mice and it gives negative results in the mouse lung adenoma model system. Therefore, we have no data to suggest that P is a particularly potent carcinogen.

TABLE 1

PERYLENE AND B(a)P METABOLISM IN AMBYSTOMA LIVER

	<u>³H-PERYLENE</u>		<u>¹⁴C-BENZO(a)PYRENE</u>		
	<u>$\bar{X}$ NaOH</u>	<u>TOTAL</u>	<u>P</u>	<u>$\bar{X}$ NaOH</u>	<u>P</u>
"CLEAN SITE (FEMALES)					
BASAL	---	4.25 + 2.66		0.027	0.075 + 0.058
AROCLOR (1 g/kg 7d)	---	8.16 + 4.58	0.05	0.293	0.751 + 0.299 0.001
PERYLENE (60 mg/kg 7d)	---	2.99 + 1.19	NS	0.028	0.109 + 0.036 NS
<u>RAT</u>					
(MUTAZYME)	16.42	72.59 + 18.48		10.094	18.148 + 3.022
				n	MOLES/mg PROTEIN/30 MINUTES

TABLE 2

PERYLENE AND B(a)P METABOLISM IN AMBYSTOMA SKIN

	<u>3H-PERYLENE</u>		<u>P</u>	<u>14C-BENZO(a)PYRENE</u>		<u>P</u>
	<u>X̄ NaOH</u>	<u>TOTAL</u>		<u>X̄ NaOH</u>	<u>TOTAL</u>	
<u>"CLEAN SITE (FEMALES)</u>						
BASAL	---	10.53 + 1.47		---	0.036 + 0.023	
AROCLOR (1 g/kg 7d)	---	14.47 + 2.33	0.01	0.045	0.109 + 0.056	NS
PERYLENE (60 mg/kg 7d)	---	21.97 + 5.18	0.001	0.044	0.126 + 0.055	0.005
<u>REESE AFB</u>						
BASAL (MALES)	0.45	11.54 + 2.01		0.006	0.061 + 0.031	
BASAL (FEMALES)	---	12.37 + 2.05	NS	---	0.042 + 0.027	NS

TABLE 3

AMBYSTOMA DNA-BINDING

"CLEAN" SITE (FEMALES)

	<u>p MOLES ³H-PERYLENE/mg DNA/mg PROTEIN</u>	
	<u>(SKIN)</u>	<u>(LIVER)</u>
BASAL	13.64 + 1.17	1.99 + 1.07
PERYLENE (60 mg/kg 7d)	24.27 + 2.88	p = 0.005 1.15 + 0.24 NS
AROCLOR (1 g/kg 7d)	10.88 + 1.56	NS 3.54 + 1.14 NS
(RAT MUTAZYME $\bar{X}$ = 39.92 pMOLES ³ H-P BOUND)		

TABLE 4

P-ERYLENE AND B(a)P METABOLISM IN XENOPUS LIVER

	<u>n MOLES ³H-P/ mg PROTEIN/30 MINUTES</u>		<u>n MOLES ¹⁴C-B(a)P/ mg PROTEIN/30 MINUTES</u>	
	<u>FEMALE</u>	<u>MALE</u>	<u>FEMALE</u>	<u>MALE</u>
<u>JUVENILE</u>				
BASAL	18.13 ± 4.16	15.29 ± 2.45	0.276 ± 0.098	0.571 ± 0.296
AROCLOR (1 g/kg 7d)	19.41 ± 2.30	11.06 ± 4.11	0.477 ± 0.551	0.448 ± 0.700
<u>ADULT</u>				
BASAL	18.82 ± 3.14	5.32 ± 4.30	0.415 ± 0.026	0.174 ± 0.101
AROCLOR (1 g/kg 7d)	15.90 ± 4.68	2.41 ± 0.21	0.443 ± 0.582	0.004 ± 0.008

TABLE 5

PERYLENE - LUNG ADENOMA INDUCTION

I. MTD	1 g/kg IN 0.5 ml OLIVE OIL, 3 x/WEEK, 8 WEEKS
II. 0.5 MTD	0.5 g/kg IN 0.5 ml OLIVE OIL, 3 x/WEEK, 8 WEEKS
III. 0.2 MTD	0.2 g/kg IN 0.5 ml OLIVE OIL, 3 x WEEK, 8 WEEKS
IV. CONTROL	0.5 ml OLIVE OIL, 3 x/WEEK, 8 WEEKS
1.	EACH GROUP = 33 FEMALE STRAIN-A MICE
2.	MTD = MAXIMAL TOLERATED DOSE; DOSE TOLERATED BY 100% OF MICE, 3 x/WEEK FOR 4 WEEKS
3.	EXAMINE FOR LUNG TUMORS 16 WEEKS AFTER LAST TREATMENT
4.	METHOD OF SHIMKIN AND STONER, <u>ADV. CANCER RES.</u> , 21:1-58, 1975
5.	RESULTS

	<u>DEFINITE</u>	<u>POSSIBLE</u>	<u>TOTAL</u>
I.	13	6	19
II.	15	2	17
III.	9	4	13
IV.	10	9	19

TABLE 6

PERYLENE - SKIN TUMOR PROMOTION STUDIES

"TWO-STAGE CARCINOGENESIS"

	INITIATING DOSE (1 x ONLY)	PROMOTING DOSES (3 x/WK, 52 WKS)
I.	300 µg B(a)P IN 0.1 ml ACETONE	1% PERYLENE IN CHLOROFORM
II.	300 µg B(a)P IN 0.1 ml ACETONE	CHLOROFORM
III.	0.1 ml ACETONE	1% PERYLENE (1) CHLOROFORM
IV.	0.1 ml ACETONE	CHLOROFORM
1.	EACH GROUP = 26 FEMALES CD-1 SWISS OUTBRED MICE	
2.	ALL APPLICATIONS TO INTERSCAPULAR REGION	
3.	PHORBOL-12-MYRISTATE-13-ACETATE (STARTING ON WEEK 53)	
	PROMOTED TUMORS IN 100% OF GROUPS I and II	
	PRODUCED NO EFFECTS IN GROUPS III and IV	

DOSE = 2.5 µg IN 100 µl ACETONE

Title: Carcinogens And Neoplasia In Indigenous Populations Of Aquatic Organisms
Principle Investigator: Michael C. Mix, Oregon State University
Cooperation Agreement Number: CR808000
Project Officer: John A. Couch

Major research activities during the past year were directed toward: (1) measuring baseline levels of arsenic (As), cadmium (Cd) and nickel (Ni) in indigenous populations of bivalve molluscs; (2) developing methods for measuring trace levels of polynuclear aromatic hydrocarbons (PNAH) in seawater; (3) developing a crustacean (barnacle) bioassay system that could be utilized for testing environmental levels of carcinogens and/or mutagens; (4) determining whether or not viruses are associated with the neoplastic disorders of Mytilus edulis from Yaquina Bay; and (5) evaluating data, gathered during the past five years, on the proliferative disorders in M. edulis. Results from studies in each of these areas are summarized below.

Baseline Levels of Inorganic Carcinogens (As, Cd, Ni) in Shellfish.

Freshwater mussels (Margaritifera margaritifera) and bay mussels (Mytilus edulis) were examined monthly or bimonthly during a one-year period and tissue concentrations of As, Cd and Ni were determined by either flame atomic absorption or neutron activation analysis. For M. margaritifera, the concentration ranges of metals ($\mu\text{g/g}$, dry weight) were: Cd, 0.9-6.2; and Ni, 1.6-19.4. These levels are similar to those reported in organisms from non-industrial streams and less than, or in the lower range of, those from industrialized rivers. For M. edulis, the concentrations of As ranged from 9-15 $\mu\text{g/g}$ (dry weight), Ni, 1.0-6.8 $\mu\text{g/g}$ and Cd, 7.0-12.5 $\mu\text{g/g}$. Both species were judged to be excellent monitors for evaluating levels of these important metals in freshwater (M. margaritifera) and marine (M. edulis) environments.

Measurement of PNAH in Seawater

The U.S. EPA's Method 610 (U.S. EPA. 1979. Polynuclear Aromatic Hydrocarbons-Method 610. Federal Register, 44(223), 69514-69517) was modified for detecting and quantifying phenanthrene, fluoranthene, pyrene, benzo(a)-phenanthrene, triphenylene, benzo(c)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, divenz(a,c)anthracene, benzo(a)pyrene, dibenz(a,h)-anthracene, benzo(g,h,i)perylene, indeno (1,2,3-c,d)pyrene and coronene in seawater. This method is applicable to the determination of PNAH in water and was designed to be used to meet the monitoring requirements of the National Pollutant Discharge Elimination System.

Procedures described in EPA Method 610 and an alternative extraction process using C₁₈ mini-columns (Sep-Paks; Waters Assoc. Inc.) were investigated for application. Further evaluations of various analytical components are currently being completed. These studies were undertaken to evaluate the use of Sep-Paks for extracting PNAH from water since they seemed to offer certain procedural advantages. Experiments were designed to determine percent recoveries of individual PNAH, reproducibility and an optimum flow rate. A system to pump water through the Sep-Paks was also developed, tested and evaluated for adsorptive loss of PNAH.

Development of a Crustacean Bioassay System.

In this study, an in vitro system for culturing the eggs and larvae of the gooseneck barnacle, Pollicipes polymerus, was developed and tested in two bioassay experiments using the phthalate ester plasticizer dibutyl phthalate. The culture system employed aerated culture chambers composed of chemically inert substances and designed to be inexpensive, convenient to use, and to minimize external contamination of the cultures.

Pollicipes eggs were grown in synthetic seawater at $14^{\circ} \pm 1^{\circ}\text{C}$ under lighting conditions of alternating darkness and very low light. Water was changed every 24 hours. Antibiotics were not necessary if prescribed methods were employed. An overall mean hatching success index of 75.5 was achieved with egg masses not exposed to dibutyl phthalate (DBP). Experiments indicated that allowing 86-96 hours after the first hatch before ending the experiment resulted in sufficient stage II larvae to be able to draw conclusions regarding molting success.

One of the DBP experiments showed there was a significant decrease in molting success in larvae from eggs exposed to 1000 ppb DBP from a week prior to first hatching to the end of the experiment. Several phthalate esters were found to be persistent contaminants in synthetic sea salts.

The results of these studies indicate that the P. polymerus assay system has great potential for testing environmental carcinogens and mutagens.

Viral Studies

Because of a recent report (Oprandy et al., 1981, J. Invertebr. Pathol., 38:45-51) which indicated that hemic proliferative disorders in Mya arenaria may be caused by a B-type retrovirus, studies were undertaken to determine whether or not such a virus was associated with the neoplastic disorders in M. edulis. Extensive investigation, employing a variety of techniques and methods did not reveal the presence of any RNA tumor virus in either affected or control M. edulis.

Statistical Evaluations of Proliferative Disorders in M. edulis.

The occurrence, prevalence, seasonality and histopathological progression of a cellular disorder, thought to be a hemic neoplasm, were studied in subpopulations of Mytilus edulis inhabiting different sites in Yaquina Bay, Oregon, from 1976-1981. There were significant differences in the occurrence of the disorder that were related to geographical location. In the subpopulation with the highest levels of the disease, the prevalences ranged from 0.0% to 20% with a five-year mean of 9.8%. There was a statistically significant relationship between prevalence and season. During the five-year study period, there was a consistent pattern characterized by highest prevalences during January through March followed by a period of decline to lower levels during the summer and early fall, after which there was an increase. Data analysis revealed that there was no seasonal histopathological progression of the disorder. Numbers of stage 1 (early), 2, 3 and 4 (advanced) cases were not related to season but, rather, occurred in a random manner throughout the entire year.

Title: Oxidation and Conjugation of Carcinogenic Hydrocarbons in Marine Animals.
Principle Investigators: D.R. Strength and H.H. Daron
Cooperative Agreement Number: CR809673
Project Officer: W. Peter Schoor

Many xenobiotics, including drugs and environmental pollutants, are metabolized in two stages: (1) an initial oxidation with or without accompanying hydration followed by (2) conjugation to form the more water soluble glucuronides, sulfates, mercapturic acids, etc. Additional steps of oxidation at secondary sites may produce a variety of diols, tetrols and quinones and epoxides.

Animals exposed to drugs or polycyclic aromatic hydrocarbons (PAH) have higher than normal activities of enzymes involved in both the oxidation and the conjugation stages in extracts from their livers. Benzo(a)pyrene, [B(a)P], is a well-known carcinogenic PAH whose metabolisms has been investigated intensively in the rat. The current study is concerned with the metabolism of B(a)P in fish to examine the oxidation intermediates formed, and to ascertain which intermediates form conjugates.

Mullet, Mugil cephalus, were given injections (20mg/kg body wt.) of 3-methylcholanthrene (3-MC) in corn oil intraperitoneally. Microsomes were prepared from livers of injected and control (sham-injected with corn oil) mullet 15 days after injection and sham-injection. Oxidation products were produced in incubation mixtures (10, 20 or 30 minutes) at 37°C in Tris-HCl buffer, pH 7.5 (0.025 M), MgCl₂ (5mM), NADPH (0.1mM), BP (25µM), and microsomes equivalent to 1 mg protein in a total volume of 1 ml. The tests for conjugation of oxidation products with glucuronic acid were incubated as described above, but contained additionally an excess of UDPGA (50µM). The respective incubations were terminated by addition of 1.0 ml of acetone; the metabolites were extracted with 2, 1, na 1 ml, respectively, of ethyl acetate. The ethyl acetate extracts were dried in nitrogen and dissolved in methanol (75µl) for HPLC analysis.

Results of HPL analyses showed that injection of 3-MC increased both the mixed function oxidase and UDP-glucuronosyltransferase activities in mullet liver compared to these activities in control animals. Several experiments conducted at various seasons indicated variations in degree of induction. Late Spring and early Fall seasons were most favorable for induction. Other factors such as maturity, nutritional status and season (water temperature) appeared to affect the results of experiments with feral fish.

Animals tested were as follows:

Rattus norvegicus (laboratory)
Mugil cephalus (mullet)
Arius felis (sea catfish)
Fundulus grandis (killifish)
Ictalurus melas (black bullhead)
Ictalurus punctatus (channel catfish)
Ictalurus nebulosus (brown bullhead)
Crassostrea virginica (American oyster)
Rangia cuneate (clam)

Mixed function oxidase (MFO) and UDP-glucuronosyltransferase were significantly increased in livers of rats and fish given 3-MC or phenobarbital. Sulfotransferase was induced in rats, mullet and channel catfish only if given large doses of 3-MC over a protracted period of time. The extent of induction of enzyme varied with compound, dosage level, and period, as well as animal species.

In order to ascertain the influence of environmental conditions on the metabolism of benzo(a)pyrene, a thorough study of channel catfish was carried out during the Winter, Spring and Summer of 1983. The induction of MFO and glucuronosyltransferase was studied in fish maintained in pools 7-9°C or 22-25°C. These temperatures represent water temperatures at Auburn University ponds in Winter or late Spring and Summer. Fish from cold water were transferred to the laboratory and maintained at cold temperature (7-9°C) or at warm temperature (22-25°C), force fed and injected with 3-MC. Fish from warm ponds were transferred to the laboratory and maintained at the two temperatures during induction. It was observed that fish at low temperatures discontinue eating and after approximately 10 days in the cold tanks were metabolically similar to fish during Winter. Wintering fish after warm adaptation in the laboratory showed induction of MFO and glucuronosyltransferase. Nutritional status and size of fish (maturity), as well as temperature, markedly affected the MFO and transferase activities in fish tissues.

The induction of MFO and glucuronosyltransferase was investigated in rats and channel catfish. Animals were given 2, 4 or 7 injections of methylcholanthrene (20 mg/kg body wt. each injection) at two-day intervals and sacrificed for assay two days after the last injection (6-day, 10-day or 14-day period). Both enzyme systems showed increased induction in both animal species over the three treatment periods.

¹⁴C-Benzo(a)pyrene and ³H-benzo(a)pyrene metabolism was studied in vivo and in vitro in rats. Tritium is lost or exchanged by the molecule of benzo(a)pyrene. Since the ¹⁴C-label proved a stable entity, we developed a procedure for quantitatively measuring the major oxidation metabolites of benzo(a)pyrene. A ratio of absorbancy/radioactivity was calculated for seven major metabolites of benzo(a)pyrene. This ratio index was used to ascertain the nmoles of each major metabolite formed as a result of MFO activity. It was also used to measure the amount of each monol, quinone or diol that conjugated with glucuronic acid when UDP-glucuronic acid was added to the reaction mixture.

The monols and ionenes conjugated readily with glucuronic acid, if UDP-glucuronic acid was added to the reaction mixture. The diols as a class conjugated less rapidly with glucuronic acid. The 9, 10-diol conjugated least and the 3-ol metabolic conjugated most rapidly with glucuronic acid.

A kinetic analysis of rate of appearance, disappearance and conjugation of metabolites was conducted with microsomes of control and induced rats and channel catfish. Analyses were conducted on various reaction mixtures at the following incubation periods: 0, 1, 2, 3, 5, 10, 20, 30 and 60 minutes. We were able to measure quantitatively the rate of and metabolism of the major metabolites of benzo(a)pyrene: 3-ol, 9-ol, 1,6-dione, 4,5-diol, 7,8-

diol and 9,10-diol. Although these were the major metabolites, many other metabolites were observed at certain times and under certain conditions.

The metabolism of PAH compounds such as BP is highly complex, involving many enzymes of the MFO system. These enzymes are affected profoundly by environmental and experimental variables.

Title: Metabolism of Polyaromatic Hydrocarbons in Marine Organisms.
Principle Investigator: Paul Melius
Cooperative Agreement Number: CR809493
Project Officer: W. Peter Schoor

The objective of this work has been to trace the metabolism of polynuclear aromatic hydrocarbons (PAH) in marine organisms. Benzo(a)pyrene [B(a)P] was selected as the model PAH substance to be used as many oxygenated derivatives are available and many studies had already been done in the rat. HPLC has been used to analyze for the wide variety of metabolites produced from B(a)P and the Ames test has been used for the detection of mutagenic metabolites which may also be tumorigenic substances. This assay is particularly useful where the B(a)P metabolites are labile and difficult to detect with HPLC or other analytical chemical methods.

Unless the fish, like rats, are exposed to an inducing agent for the mixed function oxidases, there is very little oxidation of polyaromatic hydrocarbons by liver enzymes. Thus we have used polychlorinated biphenyl (PCB) aroclor 1254 and 3-methylcholanthrene (3-MC) to induce high levels of the mixed function oxidases (MFO) in various fish liver preparations. We have always used well-defined rat liver systems to compare with our fish systems in order to be certain that the biochemical, microbiological and chemical assay systems were working properly.

We have worked with mullet (Mugil cephalus), sea catfish (Arius felis), gulf killifish (Fundulus grandis), channel catfish (Ictalurus punctatus) black bullhead (Ictalurus melas), and gold fish (Carassium auratus).

In our earliest work we were able to demonstrate that MFO activity was minimal in non-induced mullet, sea catfish and killifish whereas the aroclor induced mullet, and 3-MC induced sea catfish and killifish all had significant increases in MFO activity in an in vitro system.

In more recent studies the effects of pH, temperature, protein (enzyme concentration) and incubation times have been determined on the B(a)P metabolite patterns obtained in the in vitro assay system. Maximum metabolite production occurred over a range of pH of 6.6 to 7.5. Maximum total metabolite formation occurred at 30°C whereas maximum B(a)P diones were formed at 25°C. Metabolite production was linear with protein concentrations of 1 to 3 mgs. in the assay medium. Metabolite production was found to be linear up to a sixteen minute incubation time. There was some increase in MFO activity with 3-MC dose level up to 20 mg/Kg and a seven day period of treatment gave maximum induction of MFO activity.

The 20 mg/Kg 3-MC dose level gave maximum revertancy rate of TA 98 strain of Salmonella typhimurium while the seven day treatment period at the 20 mg/Kg 3-MC level also produced the maximum revertancy rate. Thus, it appears that the 20 mg/Kg 3-MC dose level gives maximum B(a)P metabolite formation and maximum mutagenic effect in the Ames test.

In the fish systems we have worked with, the liver MFO system converts B(a)P mainly to 7,8- and 9,10-diols and also 9- and 3-phenols with significant amounts of diones. The MFO activity in our fish systems was much less than that present in rat or previously reported in trout.

Final report for cooperative agreement number CR809344 (Dr. Jerry Hendricks, Oregon State University) will be added when received.

XII. Publications and Reports Resulting From Program
Cumulative List of Publications and Reports (1978-1983)

- Anderson, Robert S. 1978. Benzo(a)pyrene metabolism in the American oyster Crassostrea virginica. EPA-600/3-78-009, U.S. Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, FL. 19p.
- Anderson, Robert S., Jessica E. Ocos, and Francis L. Rose. 1982. Differential ability of Ambystoma tigrinum hepatic microsomes from polycyclic aromatic hydrocarbons and aromatic amines. *Cancer Lett.* 16(1):33-42. (ERL,GB X460).¹
- Biba, Diane Mary. 1983. Effects of aflatoxin on the brown bullhead Ictalurus nebulosus. M.S. Thesis. Auburn University, Auburn, AL. 53p. (ERL,GB X357).
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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Registry of Tumors in Lower Animals

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REGISTRY OF TUMORS IN LOWER ANIMALS

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ABSTRACT

Specimens with presumptive neoplasms were accessioned into the Registry's collection at an annual rate of 324, increasing the total number of accessions to 3,064. Fifty-two percent were contributed independently and 48% were field collected as part of collaborative projects. Tissue was processed as needed for diagnosis and the information computerized by key word. Preliminary results show that approximately 63% of the lesions are neoplastic.

Papers for the reprint library increased at an annual rate of 220 to a total of 4,250. These are being abstracted and computerized by the same key words used for the specimens.

Computerization and retrieval efficiency is being significantly upgraded by implementing an inhouse multiprocessor, multiuser microcomputer with direct cable access to the Smithsonian's mainframe computer.

Collaborative studies included: electron microscopy of Sydney rock oyster mantle papilloma with Peter H. Wolf (New South Wales State Fisheries, Sydney, Australia); pathology and epizootiology of neoplasms of liver and other organs in sauger from Torch Lake, Michigan polluted by copper mine tailings with John J. Black (Roswell Park Memorial Institute, Buffalo, New York); epizootiology and pathology of liver and skin neoplasms in bullheads, from industrially polluted Black River, Ohio with Paul C. Baumann (U.S. Fish and Wildlife Service, Columbus, Ohio) and from industrially polluted Buffalo River and a waste dump site in western New York with Dr. Black.

The Registry of Tumors in Lower Animals played a central role in a surge of public concern about the relationship between environmental carcinogens and epizootic fish cancer by giving the lead testimony in a Congressional hearing and by providing information or participation in extensive media coverage.

PURPOSE

The general purpose of the Registry of Tumors in Lower Animals, AKA RTLA, is to facilitate the study of neoplasms in invertebrates and cold-blooded vertebrates.

ACTIVITIES

The RTLA has seven main activities:

1. Provide a diagnostic service.
2. Maintain a specimen depository.
3. Maintain a collection of pertinent literature.
4. Abstract and computerize these specimen and literature files.
5. Act as a clearinghouse for information.
6. Collaborate on field and experimental studies primarily by providing histopathology and diagnoses, but may also assist in collecting, do electron microscopy and do cell culture. We do not initiate independent experiments.
7. Publicize the Registry by published papers, lectures, personal contacts and reports.

Nineteen eighty-three was a banner year in some ways. The specimen collection grew faster than ever with 324 new accessions. Two-thirds of which had neoplasms.

The literature file increased by 220 references.

Abstracted data from both specimen and reprint collections are being computerized.

The computer system was enormously improved and conversion to the new system will soon be complete.

As an information clearinghouse we handle multiple requests daily. Many requests are from non-scientists which considerably reduces our time for science, but we answer most serious requests. Requests from scientists have high priority and this year a record amount of material was sent out on loan.

We do our best to accommodate serious visitors and have been most fortunate to have had Dr. Ikuo Kimura, Japan's foremost fish tumor pathologist, visit the Registry for one month. Dr. Kimura is the only person in Japan currently doing both field and laboratory studies. He brought many examples of carcinogen induced tumors to the Registry, including tumors induced by Nifurpirinol whose original carcinogenicity he discovered in fish and confirmed in rodents.

FISH TUMOR MEDIA COVERAGE

Nineteen eighty-three was the year fish tumors were discovered by television. Thirteen specifically filmed TV stories have been shown. These were complemented by TV news spots and scores of newspaper, magazine and radio pieces. In response, the U.S. House of Representatives' Subcommittee on Fisheries and Wildlife Conservation and the Environment held a hearing on the relationship between fish tumors and environmental pollutants on September 21, 1983. I assisted in the preparation for the hearing and gave the lead testimony. Since the hearing, media coverage has continued and intensified. Another hearing is set for about April 16, 1984 in Michigan. Legislation to emphasize the use of fish in environmental monitoring is expected to be introduced.

The media and the Committee focused on fish tumor epizootics in five locations where the relationship between neoplasms and pollution seems strong:

1. Puget Sound, Washington
2. Torch Lake, Michigan
3. Black River, Ohio
4. Buffalo River, New York
5. Hudson River, New York

These and 21 other lower animal tumor epizootics in North America are detailed on the text map.

CARCINOGENESIS BIOASSAYS

Experimentally, some fish have ideal features for broad carcinogenesis bioassays including:

1. Cheap rearing.
2. Efficient processing and evaluation (one longitudinal section of small species and fry of larger species fit a 1 x 3 slide and reveal all organs).
3. Low genetic bias (some are natural clones).
4. Highly sensitive (tumors appear within weeks).
5. Many systems respond (digestive, integumentary, nervous, and musculature).

Most exciting, recent work shows fish embryos can be directly exposed inside the chorion to a precisely measured minute amount of chemical, thus maximizing safety and minimizing disposal material.

OBSERVATIONS AND APPLICATIONS

I would like to close with philosophy.

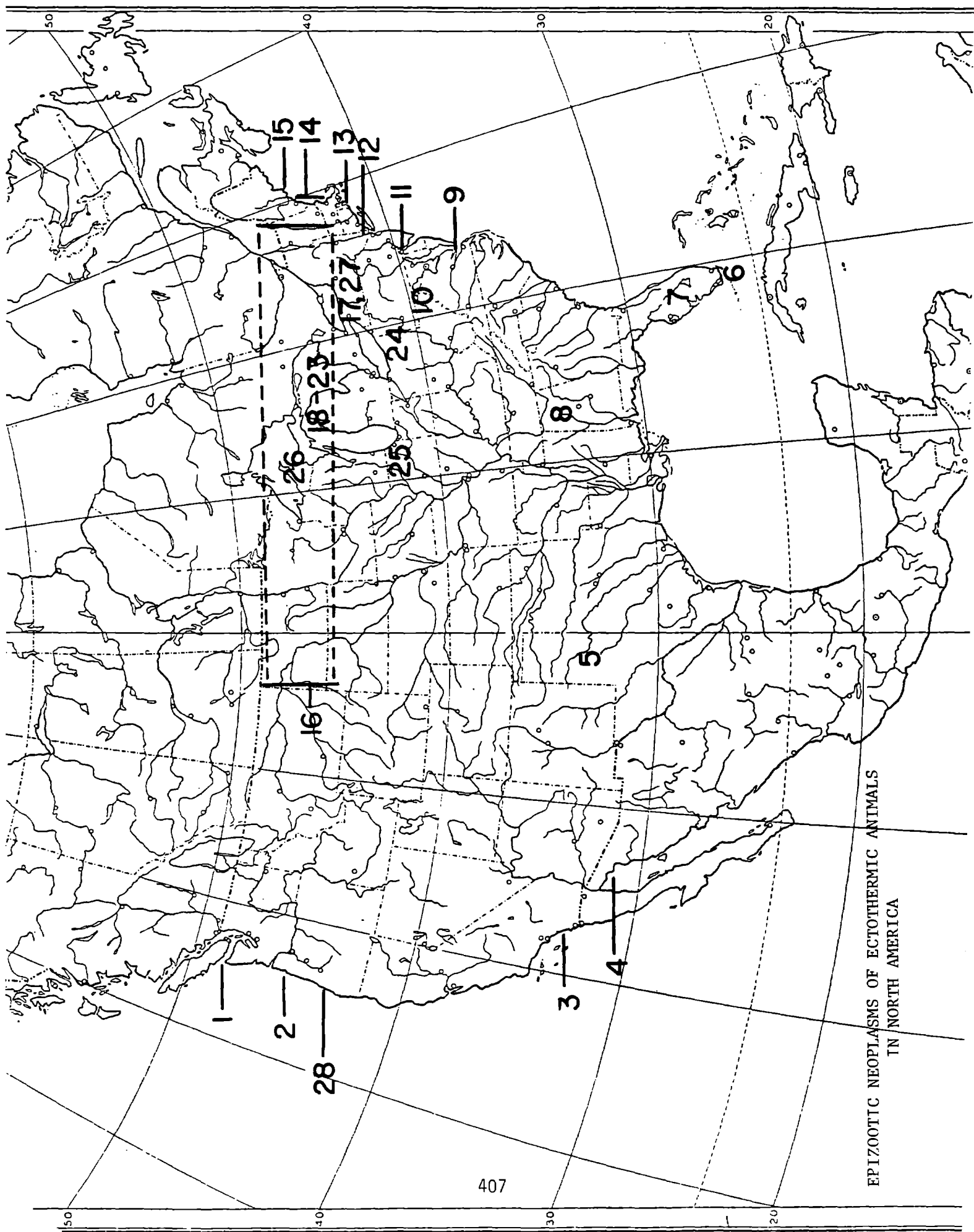
1. The mapped tumor epizootics suggest that fish and other ectotherms could be useful for environmental carcinogen surveys.
2. Recent success with embryo bioassay suggests that fish could be imminently useful in broad chemical bioassays to funnel suspect carcinogens to rodent bioassays.
3. Fish embryo bioassays could also virtually eliminate aqueous pollution of waterways by carcinogens if they were used to test all effluent. Every effluent pipe could be identified and regular bioassay of effluent at termini could be mandatory. When carcinogens are found at termini, bioassay at prior points could identify their source to facilitate elimination. Spot checks by regulatory agencies could prove compliance. Non-compliance would mandate penalties. Bioassay data showing no carcinogens in effluent would be legal verification of non-pollution.

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EPIZOOTIC NEOPLASMS OF ECTOTHERMIC ANIMALS
IN NORTH AMERICA

KEY TO MAP OF EPIZOOTIC NEOPLASMS OF ECTOTHERMIC ANIMALS
IN NORTH AMERICA

1. Puget Sound, Washington. Hepatocarcinoma in English sole. (Clustered at sites high in organic chemicals.)
2. Yaquina Bay, Oregon. Hemic neoplasms in blue mussel and Olympic oyster. Prevalence of blue mussel neoplasms correlated with aromatic hydrocarbons in environment, although in England similar tumors found in blue mussels from areas not believed to be high in industrial pollutants.
3. Santa Ana, California. Sewage outfall in Pacific Ocean. Oral papillomas in white croaker. None reported since about 1970 when sewage treatment system was improved and outfall was extended into deeper water out of white croaker range.
4. Sea of Cortez near San Felipe, Mexico. Pigmented neurilemmoma versus melanoma.
5. Lubbock, Texas. Reese Air Force Base sewage settling pond. Epidermal papillomas, melanoma, and fibrosarcoma in tiger salamanders.
6. Florida Keys from Miami to Dry Tortugas. Neurilemmoma or pigmented neurilemmoma versus melanoma in various fish species -- bicolor damselfish, gray snapper, dog snapper, slippery dick and schoolmaster.
7. Central Florida. Various lakes in the citrus growing region. Integumentary papillomas and oral squamous carcinoma.
8. Tuskegee, Alabama. Final oxidation pond of municipal sewage treatment plant. Oral papilloma in black bullhead. Rate declining following chlorine reduction.
9. Chesapeake Bay. Cholangiocarcinoma in white perch. Hemic neoplasms in American oysters and soft clams. Gill carcinomas of deposit feeding clam Macoma balthica.
10. Deep Creek and Pleasant Valley Lakes in Western Maryland. Hepatocarcinoma and cholangiocarcinoma in white sucker.
11. Delaware and Schuylkill Rivers, Philadelphia. Oral and integumentary papillomas. (Reported 1941 -- no more recent survey.)
12. Hudson River, New York. Hepatocarcinoma and pancreatic carcinoma in tomcod.
13. Sakonnet River, Rhode Island. Various odontogenic neoplasms in cunners.

14. Estuaries of Rhode Island, Massachusetts and Maine. Hemic neoplasms in soft clams.
15. Estuaries of Maine (Searsport and others). Germinomas in soft clams.
16. Northern United States from New England to North Dakota. Herpesvirus induced renal adenocarcinoma of leopard frogs.
17. Buffalo and Niagara Rivers and eastern Lake Erie. Neoplasms of liver, pigment cell, integumentary GI tract, gonadal and connective tissue origin in brown bullheads, goldfish, white sucker, redhorse sucker, drum, walleye, carp x goldfish hybrids.
18. All Great Lakes. Gonadal neoplasms of carp, goldfish and goldfish x carp hybrids.
19. All Great Lakes, rivers and lakes of northern United States and Canada. Lymphosarcoma in northern pike and muskellunge (also in northern pike of Ireland and Baltic Sea).
21. Great Lakes Huron, Ontario and Superior. Gonadal and other neoplasms of yellow perch.
22. All Great Lakes plus Canadian lakes from Alberta to Quebec. Oral and integumentary papillomas of white suckers (c-type virus particles have been reported in tumors).
23. Lakes Oneida, Champlain, Erie, Huron and elsewhere. Dermal fibrosarcoma in walleye (c-type particles reported in tumors).
24. Black River, Ohio. Oral squamous carcinoma, cholangiocarcinoma, hepatocarcinoma in brown bullhead.
25. Fox and Des Plaines Rivers, Illinois. Hepatocarcinoma of brown bullhead and occasional neoplasms in 15 other species.
26. Torch Lake (upper peninsula), Michigan. Hepatocarcinoma of sauger and walleye.
27. Buffalo, New York. Nature preserve on site of old steel industry waste dump site. Carcinomas of liver and oral mucosa in brown bullhead.
28. Springfield, Oregon. Hatchery supplied with chlorinated-dechlorinated McKenzie River water downstream from a pulp mill. Neuroblastoma in coho salmon.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Biochemical, Pharmacological and Tumorigenic Studies on a
Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic
Animals for Bioassays

Robin M. Overstreet
William E. Hawkins
William W. Walker

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BIOCHEMICAL, PHARMACOLOGICAL AND TUMORIGENIC STUDIES ON A
COMPOSITE OF DRINKING WATER CARCINOGENS AND MUTAGENS UTILIZING AQUATIC
ANIMALS FOR BIOASSAYS

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Small species of fish appear to offer some advantages as models for carcinogenicity testing including economy, rapid tumorigenic response, and the opportunity to study the activities of environmental carcinogens in which the experimental animals can be totally immersed (Dawe and Couch, In Press). These advantages could be realized in testing mixtures of carcinogens where levels of individual compounds in the mixtures could be easily monitored and administered to the experimental organisms. Drinking water supplies contain numerous compounds, many of which are proven or suspected carcinogens (Kraybill, 1980). The purpose of this study is to test the carcinogenic properties of mixtures of drinking water contaminants, mainly halogenated organics, using small fish species as experimental subjects. Compared with large fish species such as rainbow trout, relatively few studies have used small fish species as experimental organisms (Hendricks, 1982). The choice of species to be used for carcinogenesis studies depends on several factors including availability, heartiness, reproductive capacities, and propensity to develop tumors when challenged with a known carcinogen.

In this presentation we report results of some studies conducted from the date of initiation of the contract, October 1, 1982, through February, 1984. The report focuses on induction of neoplasms in various fish species by pulse exposures of methylazoxymethanol acetate (MAM-Ac) and does not include data on microsomal enzyme studies, flow-through system development, range-finding toxicological tests, analytical chemistry, or pulse exposures using other carcinogens and drinking water contaminants.

Materials and Methods

Species that were tested for tumor induction with MAM-Ac included Japanese medaka (Oryzias latipes), Gulf killifish (Fundulus grandis), sheepshead minnow (Cyprinodon variegatus), guppy (Poecilia reticulata), fathead minnow (Pimephales promelas), inland silverside (Menidia beryllina), and rivulus (Rivulus marmoratus). Specimens approximately 10 days old were dosed with up to 100 ppm MAM-Ac for 2 hours, then placed in carcinogen-free water and sampled periodically, usually monthly. Either whole fish or visceral masses were embedded in paraffin blocks.

Results

MAM-Ac induced neoplastic lesions in livers of some specimens of all species tested. About one month after exposure to 50 ppm or more MAM-Ac, livers contained extremely hypertrophied eosinophilic cells often concurrently with biliary ductule hyperplasia. This was probably a cytotoxic response but may be useful as an indicator of subsequent neoplastic change.

Preliminary electron microscopical examination showed that the cells contained large amounts of rough endoplasmic reticulum scattered throughout the cytoplasm and mitochondria with unusual cristae. The fate of these cells has not been determined but we suspect that they degenerate.

In sections stained with hematoxylin and eosin, neoplastic nodules consisted of hyperbasophilic cells arranged in cords that were discontinuous with those of surrounding normal liver parenchyma. Often, a single large neoplastic nodule occupied most of a liver. Trabecular carcinoma consisted of large irregular neoplastic cells with frequently occurring, sometimes bizarre, mitotic figures. Lesions resembling adenocarcinoma consisted of stacked neoplastic cells arranged in glandular patterns. Neoplastic nodules and carcinomas appeared to progress to lesions that consisted of undifferentiated to spindle-shaped cells with numerous atypical mitotic figures. These cells sometimes totally replaced normal liver parenchyma. The lesions described above were often accompanied by bile duct hyperplasia that was characterized by nodules of proliferated bile ducts with numerous mitoses in the epithelium. Large biliary cysts were often associated with bile duct proliferation. Adenofibrosis which closely resembled the lesion seen in rats occurred in some specimens. The time of onset and the prevalence of hepatic lesions varied among the species tested. The time of onset of the lesions appeared to be dose dependent. The prevalence of neoplastic and related lesions, which included bile duct proliferation and adenofibrosis, for each species tested are presented in Tables 1-7. The data are incomplete and some studies are ongoing. Neoplastic lesions were not observed in control specimens. The guppy and medaka developed hepatic lesions one to two months after exposure whereas a substantial percentage of fathead minnows did not develop lesions until 10 months post-exposure. The degree of malignancy of hepatic neoplasms and whether or not they metastasize have not been determined. One medaka had a well-advanced liver neoplasm and large neoplastic masses in the septum transversum and kidney, and emboli of tumor cells in the heart. The non-hepatic neoplasms possibly were metastases of primary liver neoplasm or neoplasms of some other tissue cell origin.

Fishes exposed to MAM-Ac developed neoplasms in other organ-systems besides the liver. These lesions were tentatively identified as undifferentiated sarcomas, retinal dysplasias, and retinal neoplasms. Eight undifferentiated sarcomas were seen in medaka and one in a guppy. Some undifferentiated sarcomas were grossly visible nodules usually on the lateral or ventral surfaces of the fishes. Histologically, the tumors were unencapsulated and highly cellular with a variety of cell sizes and shapes. Mitoses occurred frequently and were often bizarre. Other sarcomas were not grossly visible but histologically appeared to be highly invasive with numerous mitotic figures. One of these resembled a rhabdomyosarcoma.

Retinal lesions were observed in medaka several months after MAM-Ac exposure. To date, we have seen 21 retinal lesions of which 14 were dysplastic lesions and 7 appeared to be neoplastic. Dysplastic changes involved rearrangement of the layers of the retina and the occurrence of oval or elongated rosettes composed of visual cells. A variety of types of retinal neoplasms occurred. They appeared to arise from the ganglion cell layer, the inner nuclear layer, or the visual cell layer. The tumors often burst through the cartilagenous sclera and invaded the periorbital tissues.

Discussion

These studies demonstrate that several species of small fishes develop hepatic neoplasms when exposed to a known carcinogen. The rate of neoplastic development and the prevalence of neoplasms vary among species and appear to be dose and possibly time dependent. The response of the guppy and medaka was investigated more thoroughly than that of other fishes. Following a 2 hour exposure to levels up to 100 ppm MAM-Ac, about 50% of individuals of these species develop hepatic neoplasms usually within 2 months. Although the histogenesis of the liver lesions has not been analyzed in detail, a cytotoxic response characterized by giant, possibly degenerating hepatocytes is followed by the development of neoplastic nodules and carcinoma patterns. Lesions with anaplastic and sarcomatous features followed. Bile duct proliferation with cyst formation and adenofibrosis often accompanied the neoplastic lesions. The neoplastic and related lesions in the fishes in this study shared many features with the better defined and more thoroughly studied lesions chemically induced in rats (Stewart et al., 1980).

Undifferentiated sarcomas of the body wall and retinal lesions were unexpected findings in this study and served to underscore one of the advantages of using small fish in carcinogenesis testing; i.e., that a whole specimen including most major tissues can be examined on a single microscope slide. Central nervous system tumors of fish are encountered rarely. Fewer than 10 cases have been submitted to the Registry of Tumors of Lower Animals (Harshbarger, 1965-1981). The retinal lesions in medaka may prove to be the first induced retinal neoplasms reported in fishes. The lesions include both dysplasias that are possibly pre-neoplastic and neoplastic lesions that appear to be in different stages of development and highly invasive.

Concurrent with these studies, we have developed an enclosed, environmentally-safe exposure system for administering consistent levels of highly volatile compounds for prolonged periods to test organisms. We will use guppies and medaka to test the carcinogenicity of a mixture of drinking water contaminants that includes carbon tetrachloride, chloroform, bromoform, bromodichloromethane, chlorodibromomethane, and trichloroethylene. Some specimens will be exposed to several concentrations of this mixture for 28 days then sampled at 6 week intervals for the onset of neoplastic change. Other specimens will be pre-exposed to various levels of MAM-Ac then to the halomethane mixture in order to define tumor promoting properties of the drinking water contaminants. Later studies will involve longer (6-12 month) exposures to the above mixture and to a mixture of halogenated benzenes and phenols.

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Table 1. Neoplastic and related lesions in livers of the guppy (Poecilia reticulata) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish								
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Control	0/3	0/5	0/6	0/5	0/1	0/0	0/1	0/1	0/1
10 ppm	0/4	1/6	1/6	0/6	3/8	4/8	3/4	7/9	4/7
50 ppm	2/7	0/3	3/3	2/3	3/3	4/4			
100 ppm	5/24	6/9	2/4	2/5	1/3	2/2	1/3	6/7	5/5

Table 2. Neoplastic and related lesions in livers of the Japanese medaka (Oryzias latipes) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish								
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Control	0/12	0/15	0/12	0/11	0/13	0/8	0/18	0/3	0/8
10 ppm	0/8	3/7	3/6	3/6	4/11	4/8	1/9	3/4	3/6
30 ppm	0/3	2/3	1/3	0/3	5/7	2/3	3/6	2/3	1/1
50 ppm	6/23	7/18	14/21	15/24	13/20	13/20	4/9	2/3	7/9
80 ppm	0/3	1/3	2/3						
100 ppm	4/13	3/5	5/5	1/4	3/4	1/1			

Table 3. Neoplastic and related lesions in livers of the sheepshead minnow (Cyprinodon variegatus) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish								
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Control	0/3	0/2	0/0	0/1	0/3	0/3	0/1		
10 ppm	0/4	0/3	3/3	2/2	1/4	1/3	1/3	3/3	1/3
50 ppm	0/3	1/1	1/2	3/4	2/2	1/3			
100 ppm	0/3	3/3	0/1						

Table 4. Neoplastic and related lesions in livers of fathead minnows (Pimephales promelas) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish									
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo	10 mo
Control	0/3	0/6	0/6	0/2	0/6	0/2	0/3	0/3	0/3	0/3
10 ppm	0/0	0/3	0/0	0/2	0/3	0/4	0/1	0/3	0/3	0/6
50 ppm	0/6	0/3	0/3	0/4	0/4	0/6	0/1			
100 ppm	0/0	0/3	0/5	0/2	0/3	0/2	1/8	0/4	0/6	3/5

Table 5. Neoplastic and related lesions in livers of the Gulf killifish (Fundulus grandis) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish								
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Control	0/3	0/8	0/6	0/9					
10 ppm	0/1	0/3	0/3	0/5					
50 ppm	0/3	0/2	2/2	1/2	2/5	2/2			
100 ppm	0/2	0/3	0/1	0/3	0/1				

Table 6. Neoplastic and related lesions in livers of the Atlantic silverside (Menidia beryllina) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish								
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Control	0/0	0/3	0/3	0/9					
50 ppm	2/3	0/2	2/7	6/14					
100 ppm	1/3	3/6	0/3	3/7	0/2				

Table 7. Neoplastic and related lesions in rivulus (Rivulus marmoratus) following a brief exposure to MAM-Ac.

Exposure conditions	Prevalence of lesion-bearing fish								
	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo	7 mo	8 mo	9 mo
Control	0/3								
50 ppm	0/5	1/3	0/5	2/4	0/2	0/2			

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
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PRESENTATIONS:

Metabolism of Nine Benzidine-Congener-Based Azo Dyes
in Rats Based on Gas Chromatographic Assays
of the Urine for Potentially Carcinogenic Metabolites

Larry K. Lowry
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Charles R. Nony
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and

Metabolism and Distribution of Two
¹⁴C-Benzidine-Congener-Based Dyes in Rats as
Determined by GC, HPLC, and Radioassays

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Metabolism of Nine Benzidine-Congener-Based Azo Dyes in Rats Based on Gas Chromatographic Assays of the Urine for Potentially Carcinogenic Metabolites

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Abstract

Metabolism experiments were conducted with rats dosed with nine azo dyes based on dimethyl-, dimethoxy-, or dichlorobenzidine to determine whether the free amine congeners, their monoacetyl or diacetyl metabolites, or alkaline hydrolyzable conjugates were excreted in the urine. After preliminary tests of the dyes, 2-mg doses were administered to each animal and urine samples were collected at intervals up to 96 hours. EC/GC procedures were based on the analysis of heptafluorobutyl derivatives of the free amine congener moieties or their monoacetyl metabolites. Peak levels of metabolites were excreted either 0-12 or 12-24 hours after administration and, in seven of nine instances, no metabolites persisted in the urine after 48 hours. Minimum detectable levels of all metabolites were 12 ppb or less. All nine dyes were shown to be converted to measurable levels of their benzidine-congener-based metabolites in rats.

Introduction

Azo dyes based on benzidine (Bzd) and benzidine-congeners [*i.e.*, 3,3'-dimethylbenzidine (DiMeBzd) and 3,3'-dimethoxybenzidine (DiMxBzd)] constitute a group of some 92 discrete chemicals that are widely used in the United States. For example; during 1978, domestic production and imports amounted to about 4.1 million pounds (1). Exposure of the general public to these materials is through contact with products such as paper, fabrics and leather to which the dyes have been applied; more importantly, packaged dyes for home use and paints contain these chemicals. The Dyes Environmental and Toxicology Organization (DETO) has estimated that about 1,000 workers are exposed to dyes during the manufacturing process and that as many as 15,000 others are exposed in the various dye application industries (1).

Bzd is a known human carcinogen that causes cancer of the urinary bladder (2) and since DiMeBzd and DiMxBzd were reported to induce tumors of the Zymbal's gland and at other sites in rats, the International Agency for Research on Cancer (IARC) classified these compounds as animal carcinogens (3,4). Results from several experiments indicated that several azo dyes based on Bzd, DiMeBzd, and DiMxBzd are metabolized to their parent amines by laboratory animals (5-8) suggesting that the dyes may also be animal carcinogens. Three dyes (Direct Blue 6, Direct Black 38 and Direct Brown 95) known to be metabolized to Bzd, were shown to be carcinogens in rats (9). The National Institute for Occupational Safety and Health (NIOSH) evaluated the evidence and issued a special hazard review concluding that benzidine-based azo dyes are potential human carcinogens, and that exposure to these dyes should be eliminated (10).

As part of the National Toxicology Program (NTP) and in response to the need for scientific information for regulatory decisions in the Environmental Protection Agency (EPA), Consumer Product Safety Commission (CPSC) and Occupational Safety and Health Administration (OSHA), a strategy program was designed to test prototypical dyes to establish basic principles which could be applied to the entire group of dyes. This approach was sought to circumvent the high cost of conducting long-term bioassays of each dye. The scientific objectives and approaches were developed by scientists from the EPA, CPSC, OSHA, National Institutes of Health (NIH), NIOSH, and the Food and Drug Administration (FDA).

The chemical phase of the work related to metabolism of DiMeBzd- and DiMxBzd-based azo dyes to their potentially carcinogenic free amines, as determined by analysis of urine from dosed rats, was conducted at the FDA's National Center for Toxicological Research (NCTR). Results of these tests with four representative dyes from each of the two benzidine-congener bases are presented in this paper. Results from tests with Direct Red 46 [a 3,3'-dichlorobenzidine (DiClBzd)-based dye], a dye of particular interest to the EPA, are also presented.

Experimental

Test Materials

General information concerning the benzidine-congener-based dyes selected for testing in rats is presented in Table I with their structures depicted in Figure 1 (exception: the structure of Black 114 is not known). Samples of these dyes were obtained from

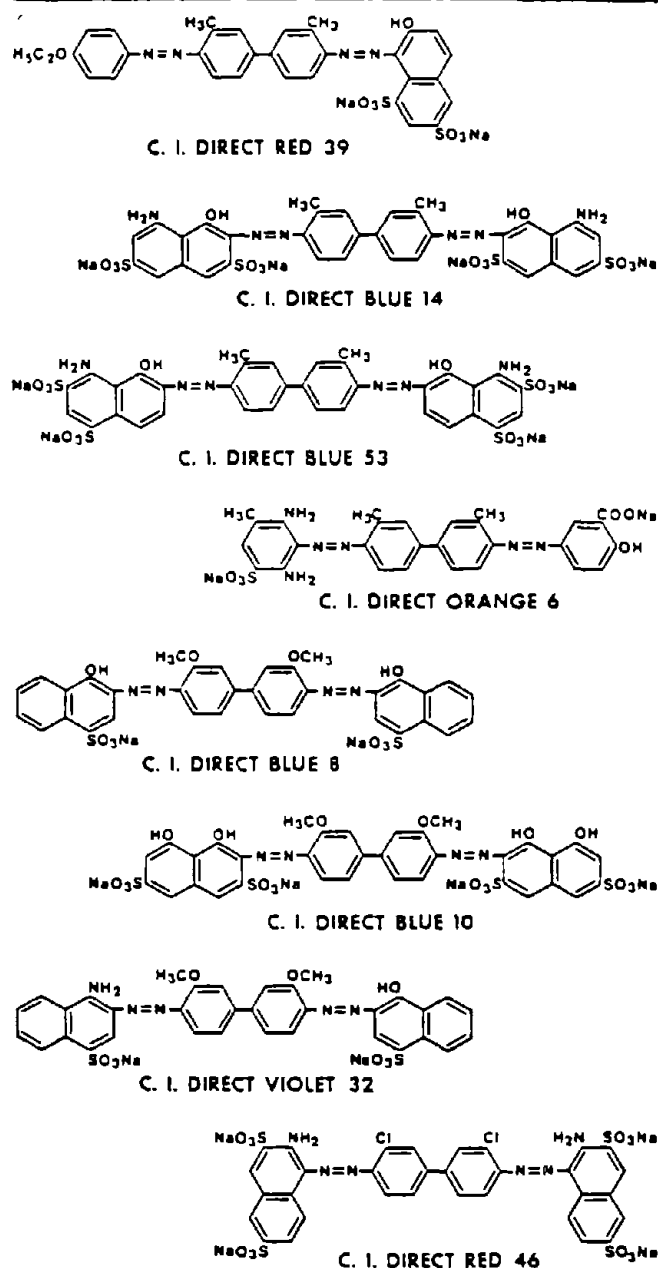


Figure 1. Formulas of eight benzidine-congener-based azo dyes.

the DETO and/or the Midwest Research Institute (MRI) and are described in Table II. A chemical reduction test (11) was used to verify the presence of the correct benzidine-congener base in the dye molecule and to calculate the purity of the dye. In this test, an aqueous solution of the dye was reduced with 0.2 M stannous chloride in 12 N HCl at 40 °C for about 3 hours (to complete decolorization), made strongly alkaline, and the benzidine-congener free amine was extracted with chloroform and analyzed by gas chromatography employing a flame ionization detector (GC/FID). The purity of the dye was calculated on the basis of the amount of benzidine-congener amine obtained from a known amount of dye as compared to the amount theoretically obtainable (Tables I and II). In designing the metabolism tests, it was decided that all dyes should be at least 50% pure and contain no more than 50 ppm of congener amine impurities; five of the nine dyes required purification prior to animal dosing. For purification, the dye was mixed with about twice its weight of cold water (3 °C), filtered on a Buchner funnel and washed with three additional portions of cold water to remove water soluble impurities. The residue on the filter was dissolved in 1 L of water (25 °C), and extracted four times with benzene to remove impurities of aromatic amines. The aqueous phase was freeze dried to recover the purified product. In all cases, this regimen provided the necessary improvement in purity to meet the established criterion; the range of purity for eight

Table II. Purity and Stability of Benzidine-Congener-Based Dyes Administered to Rats

Direct Dye	Purity of Dye (%) ^a		Congener Amine Impurities (ppm) ^b	Stability at 25° in Urine	
	as Received	as Dosed		Rat	Human
Red 39	70.7	70.7	0.58	U	S
Blue 14	61.9	76.1	21.	U	S
Blue 53	67.4	67.4	1.4	U	S
Orange 6	31.9	61.8	0.31	U	S
Blue 8	29.9	63.8	0.22	U	U
Black 114	8.25†	18.6†	0.39	U	S
Blue 10	47.9	71.6	0.060	S	U
Violet 32	83.4	83.4	0.13	S	S
Red 46	39.7	78.6	7.0	U	U

^a Determined by chemical reduction and GC assays.

^b Determined by EC/GC of HFB derivatives. Direct Blue 53 and Direct Orange 6 also contained DiMeBzd impurities of 1.6 and 5.9 ppm, respectively.

† Purity not determined; MW of dye not known; values are percentages (w/w) of DiMeBzd in the dye.

S = stable, U = unstable.

Table I. General Information for the Benzidine-Congener-Based Azo Dyes Administered to Rats

Direct Dye	Benzidine Congener-base ^a	Color Index Number	CAS Number	Molecular Formula	Molecular Weight	Bzd-Congener in Pure Dye (%)
Red 39	DiMeBzd	23630	6358-29-8	C ₃₂ H ₂₆ O ₆ N ₄ S ₂ Na ₂	704.65	30.12
Blue 14	DiMeBzd	23850	72-52-1	C ₃₄ H ₂₄ O ₆ N ₄ S ₂ Na ₄	960.81	22.09
Blue 53	DiMeBzd	23860	314-13-6	C ₃₄ H ₂₄ O ₆ N ₄ S ₂ Na ₄	960.81	22.09
Orange 6	DiMeBzd	23375	6637-88-3	C ₂₈ H ₂₂ O ₆ N ₄ SNa ₂	618.55	34.32
Blue 8	DiMeBzd	24140	2429-71-2	C ₃₄ H ₂₄ O ₁₀ N ₄ S ₂ Na ₂	758.65	32.20
Black 114	DiMeBzd	NK	NK	NK	NK	NK
Blue 10	DiMeBzd	24340	4198-19-0	C ₃₄ H ₂₂ O ₁₀ N ₄ S ₄ Na ₄	994.77	24.56
Violet 32	DiMeBzd	24105	6428-94-0	C ₃₄ H ₂₂ O ₉ N ₄ S ₂ Na ₂	757.71	32.24
Red 46	DiClBzd	23050	6548-29-4	C ₃₂ H ₁₈ O ₁₂ N ₄ Cl ₂ S ₂ Na ₄	969.61	26.11

^a DiMeBzd is 3,3'-Dimethylbenzidine (MW 212.28); DiMeBzd is 3,3'-Dimethoxybenzidine (MW 244.28); DiClBzd is 3,3'-Dichlorobenzidine (MW 253.13).

NK = Not known.

of the dyes was 61.8 to 83.4% (Table II). The purity of Direct Black 114 could not be calculated without knowing its molecular weight (MW); however, the material was purified prior to use as described and the DiMxBzd content was increased from 8.25 to 18.6% (w/w).

Nuclear magnetic resonance (NMR) spectra of the dyes were obtained and found to be consistent with their structures except for Direct Blue 10; however, the presence of the DiMxBzd moiety in Direct Blue 10 was confirmed by NMR.

All dyes that met the criterion for purity, either as received or after purification, were subjected to analysis for free aromatic amine impurities (12,13). Briefly, an aqueous solution of dye (1 mg/mL) was extracted with benzene and any extracted congener amine residues were derivatized using heptafluorobutyric anhydride (HFBA) and the derivative was assayed by electron-capture gas chromatography (EC/GC). All dosed dyes met the established criterion for impurities in the metabolism experiments (*i.e.*, ≤ 50 ppm of congener amine impurities as reported in Table II).

Stability tests were conducted on all dyes that met the criteria for purity, impurity and confirmation of structure to determine any *in vitro* contribution by a dye to the metabolite content of urine resulting from bacterial and/or enzyme action in the event that the urine might become contaminated by intact dye. The assumption was made that if a dye was stable in urine at 5 °C, it would also be stable under the conditions employed for collecting urine samples from animals (under dry ice) and in storage prior to assay (-20°C). The stability tests were conducted as reported (12); 1000 ppm solutions of the dye in human urine or control urine from the test animals were sampled at 0-, 24-, 48-, 72- and 96-hour intervals and analyzed for the appropriate benzidine congener.

Test Animals and Housing Conditions

Male Fischer 344 rats, reared at the NCTR, were housed (2 rats/cage) in s.s. cages (Wahmann Model No. LC-177). An additional 16 mesh s.s. screen was placed on the animal support screen to prevent fecal pellets from contaminating the urine and to allow for facile collection of feces in the event that assays were later required. The rats were seven weeks old, weighed 180 to 220 g at the beginning of the experiments and were kept on a 12-hour light cycle (6 am to 6 pm) for the entire experiment. The animals were allowed free access to water and feed except for a 12-hour fasting period prior to dosing when the feed was withheld and then restored 1 hour after the dose was administered. Urine was collected in a 50-mL graduated glass tube submerged in solid dry ice and positioned under the drain tube of the cage.

Metabolism Tests

Four groups of 4 rats were used in tests with each dye: 3 groups were dosed with the dye and one group, dosed with water, served as a control. Pretreatment samples of urine were collected from all groups during the 36-hour period prior to administration by gavage of a single dose (1 mL) of an aqueous dye solution containing the equivalent of 2 mg of 100% dye. Because of the limited solubility of some dyes in water at ambient temperature, all dyes were weighed and dissolved in water heated to 39 °C (body temperature of the rat) and administered at that temperature. Samples of urine from each group (2 cages) were collected for intervals of 0-12, 12-24, 24-48 and 48-96 hours after the dose was administered and stored at -20°C until analysis.

Analytical Chemical Methodology

Samples of urine from the metabolism tests with dyes were assayed by EC/GC (13,14). Separate portions of the same sample were assayed for the benzidine-congener free amines and their monoacetyl (MoAc) metabolites, alkaline hydrolyzable conjugates (AHC) or for diacetyl (DiAc) metabolites as shown in Figure 2 (13). The benzidine-congener free amine and its MoAc and DiAc metabolites were extracted from urine under alkaline conditions with benzene; the aqueous phase was subjected to alkaline hydrolysis and again extracted with benzene to remove AHC metabolites as the benzidine-congener amine. The two benzene extracts were then subjected to derivatization with HFBA with trimethylamine as a catalyst and analyzed by EC/GC; any DiAc metabolites present in these extracts were not derivatized or detected. A second sample was made strongly acid with HCl and extracted with chloroform to remove and separate the DiAc metabolite from any free benzidine-congener or MoAc metabolite. The extracted DiAc metabolite was then hydrolyzed to the free amine, derivatized, and analyzed as described. Minimum detectable levels for the three benzidine-congener free amines and their MoAc and DiAc metabolites in rat urine were 12 ppb or less.

The results of assays for benzidine congeners, their MoAc or DiAc derivatives, or congeners excreted as AHC were corrected for pretreatment sample backgrounds and recoveries. Since AHC have not been characterized, recoveries of the individual congener bases were determined through the AHC procedure for use in correcting the analytical results.

The concentrations of the metabolites (ppb or ng/mL) for each of the three groups of test animals were multiplied by their respective sample volumes to determine the amount of each metabolite excreted during each collection interval of the post-treatment period; then the mean and standard deviation (SD) were calculated. These results were converted mathematically to total μg -equivalents of benzidine congener excreted as the free amine, MoAc, DiAc and/or AHC metabolites as follows: $\text{DiMeBzd} = \text{MoAcDiMeBzd} \times 0.835$, $\text{DiAcDiMeBzd} \times 0.716$; $\text{DiMxBzd} = \text{MoAcDiMxBzd} \times 0.853$, $\text{DiAcDiMxBzd} \times 0.744$; $\text{DiClBzd} = \text{MoAcDiClBzd} \times 0.858$, $\text{DiAcDiClBzd} \times 0.751$. No conversion factor was required for the free amines or AHC.

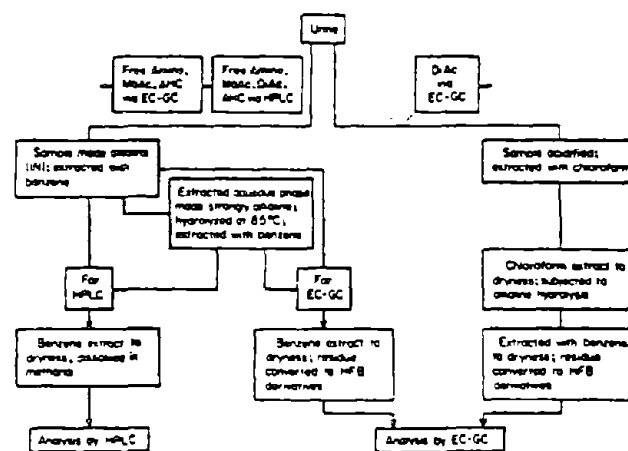


Figure 2. General scheme for extraction, separation, and analysis of benzidine-congener free amines and their monoacetylated (MoAc), diacetylated (DiAc), and conjugated (AHC) products in urine (8).

Results and Discussion

The nine dyes described in Tables I and II were administered to rats as described. None of the animals showed overt symptoms of shock or discomfort except those dosed with Direct Red 39; all 12 rats became lethargic and several indicated signs of shock immediately after the dosing. These rats showed no interest in eating or drinking for several hours after the fasting period; however, they later recovered and began to eat and drink normally. All of the animals survived to the end of the experiment. The weight gained by each rat averaged 18 g during the 96-hour test period; this was comparable to gains observed with control rats.

All dyes were found to be stable in both rat and human urine at 5°C for 96 hours. However, six of the dyes were shown to liberate high ppm levels of benzidine-congener amines when held in either rat or human urine at 25°C for 96 hours. This degradation may be due to microbial or enzymatic action and points out the need to collect and store samples of urine at 5°C or less to avoid erroneous results in the event that the sample is contaminated with dyes. All samples of urine from the test animals were collected under dry ice and stored at -20°C until analysis; therefore, any amounts of free amines in the urine in excess of the small amount dosed as impurities must be attributed to breakdown of the dye in the animals.

Urine samples from the test and control animals were thawed and assayed in groups of eight using the EC/GC methods

described. The data from these assays (ppb or ng/mL) were calculated and the mean quantities of the three metabolites and AHC excreted during each interval of the post-treatment period are presented in Table III. Values for the pretreatment interval (-36 to 0 hour) represent background levels and were used to correct the post-treatment values prior to corrections for recovery. A cursory examination of the excretion values obtained during the post-treatment intervals indicated that all nine dyes were metabolized to yield appreciable levels of the corresponding free amine, MoAc, DiAc and AHC products. Metabolites of six of the nine dyes were excreted at peak levels during the first 12 hours after the dyes were administered. Only Direct Red 39, Direct Orange 6 and Direct Red 46 showed peak excretion levels during the 12- to 24-hour interval. All metabolite levels for all dyes decreased markedly from 24 to 48 hours after dosing and no residues of metabolites were detected above pretreatment levels at the 48- to 96-hour interval, except for Direct Red 39 and Direct Blue 14. Excretion of metabolites derived from the DiMxBzd-based dyes tended to peak and dissipate faster than for those based on DiMeBzd or DiClBzd. Nevertheless, consistent and measurable metabolism of all nine dyes was observed in the rats.

Generally, DiAcDiMeBzd was the major metabolite of those measured from the DiMeBzd-based dyes, followed by MoAc-DiMeBzd and smaller quantities of DiMeBzd and AHC. In contrast, AHC were the major metabolites of those from the DiMxBzd-based dyes; appreciable quantities of DiAcDiMxBzd

Table III. EC/GC Assays of Three Metabolites and AHC in Urine from Rats Given a Single Dose (2 mg) of Dye Indicated

Metabolite	Metabolites (μ g) Excreted ($\bar{x} \pm SD$) ^a					Congener Excreted as Metabolite ^{b,c} (μ g-equiv.)
	Pretreatment (hr)	Post-treatment Interval (hr)				
	-36 to 0	0 to 12	12 to 24	24 to 48	48 to 96	
DIRECT RED 39						
DiMeBzd	0.12 $\pm$ 0.02	0.98 $\pm$ 0.30	0.70 $\pm$ 0.06	0.51 $\pm$ 0.16	1.4 $\pm$ 0.87	3.6
MoAcDiMeBzd	0.46 $\pm$ 0.26	0.43 $\pm$ 0.09	1.1 $\pm$ 0.30	0.62 $\pm$ 0.05	ND	1.8
DiAcDiMeBzd	0.02 $\pm$ 0.01	3.3 $\pm$ 1.5	4.4 $\pm$ 1.1	2.2 $\pm$ 0.30	0.58 $\pm$ 0.19	7.6
AHC as DiMeBzd	0.13 $\pm$ 0.01	0.68 $\pm$ 0.23	0.68 $\pm$ 0.27	0.30 $\pm$ 0.29	ND	1.7
Total DiMeBzd μ g-equiv.	Nil	4.4	5.5	2.9	1.8	15.
DIRECT BLUE 14						
DiMeBzd	0.31 $\pm$ 0.03	1.7 $\pm$ 0.10	0.66 $\pm$ 0.07	0.76 $\pm$ 0.28	3.4 $\pm$ 1.6	6.5
MoAcDiMeBzd	0.17 $\pm$ 0.06	4.5 $\pm$ 0.29	3.4 $\pm$ 0.68	1.6 $\pm$ 0.00	ND	7.9
DiAcDiMeBzd	0.06 $\pm$ 0.02	6.6 $\pm$ 1.4	5.1 $\pm$ 1.0	0.23 $\pm$ 0.30	ND	8.6
AHC as DiMeBzd	0.07 $\pm$ 0.02	1.2 $\pm$ 0.29	0.74 $\pm$ 0.04	ND	ND	1.9
Total DiMeBzd μ g-equiv.	Nil	11.	7.9	2.2	3.4	25.
DIRECT BLUE 53						
DiMeBzd	0.60 $\pm$ 0.14	2.9 $\pm$ 0.25	1.3 $\pm$ 0.72	0.58 $\pm$ 0.60	ND	4.8
MoAcDiMeBzd	0.23 $\pm$ 0.15	4.5 $\pm$ 0.32	1.2 $\pm$ 0.44	1.1 $\pm$ 0.55	ND	5.7
DiAcDiMeBzd	0.04 $\pm$ 0.01	9.6 $\pm$ 0.80	3.4 $\pm$ 0.78	0.50 $\pm$ 0.45	ND	9.7
AHC as DiMeBzd	0.03 $\pm$ 0.00	2.6 $\pm$ 0.89	0.62 $\pm$ 0.11	0.08 $\pm$ 0.13	ND	3.3
Total DiMeBzd μ g-equiv.	Nil	16.	5.3	1.9	Nil	24.
DIRECT ORANGE 6						
DiMeBzd	0.58 $\pm$ 0.13	3.1 $\pm$ 0.40	1.1 $\pm$ 0.06	ND	ND	4.2
MoAcDiMeBzd	0.10 $\pm$ 0.03	4.1 $\pm$ 0.29	3.8 $\pm$ 0.86	0.94 $\pm$ 0.04	ND	7.4
DiAcDiMeBzd	0.10 $\pm$ 0.02	3.3 $\pm$ 1.4	10. $\pm$ 4.8	ND	ND	9.6
AHC as DiMeBzd	0.06 $\pm$ 0.01	2.5 $\pm$ 0.19	2.5 $\pm$ 0.19	ND	ND	5.0
Total DiMeBzd μ g-equiv.	Nil	11.	14.	0.78	Nil	26.
DIRECT BLUE 8						
DiMxBzd	0.08 $\pm$ 0.01	9.3 $\pm$ 1.7	0.46 $\pm$ 0.07	ND	ND	9.8
MoAcDiMxBzd	0.01 $\pm$ 0.00	3.0 $\pm$ 0.4	0.07 $\pm$ 0.01	ND	ND	2.7
DiAcDiMxBzd	0.13 $\pm$ 0.03	10. $\pm$ 1.	2.5 $\pm$ 0.4	1.7 $\pm$ 1.2	ND	10.
AHC as DiMxBzd	0.07 $\pm$ 0.01	11. $\pm$ 1.	2.5 $\pm$ 0.2	0.86 $\pm$ 0.30	ND	14.
Total DiMxBzd μ g-equiv.	Nil	30.	4.9	2.2	Nil	50.

Table III. Continued

Metabolite	Metabolites (μg) Excreted ($\bar{x} \pm \text{SD}$)*					Congener Excreted as Metabolite** ($\mu\text{g-equiv.}$)
	Pretreatment (hr)	Post-treatment Interval (hr)				
	-36 to 0	0 to 12	12 to 24	24 to 48	48 to 96	
DIRECT BLACK 114						
DiMxBzd	0.10 $\pm$ 0.02	6.1 $\pm$ 1.7	1.0 $\pm$ 0.2	ND	ND	7.1
MoAcDiMxBzd	0.03 $\pm$ 0.00	8.1 $\pm$ 2.4	2.1 $\pm$ 0.3	ND	ND	8.7
DiAcDiMxBzd	0.03 $\pm$ 0.01	14. $\pm$ 3.	4.8 $\pm$ 0.5	0.86 $\pm$ 0.20	ND	14.0
AHC as DiMxBzd	0.05 $\pm$ 0.01	14. $\pm$ 2.	5.4 $\pm$ 0.6	0.70 $\pm$ 0.24	ND	20.
Total DiMxBzd $\mu\text{g-equiv.}$	Nil	37.	12.	1.3	Nil	50.
DIRECT BLUE 10						
DiMxBzd	0.19 $\pm$ 0.03	10. $\pm$ 6.	0.68 $\pm$ 0.25	ND	ND	11.
MoAcDiMxBzd	0.07 $\pm$ 0.02	4.7 $\pm$ 1.1	0.96 $\pm$ 0.22	ND	ND	4.8
DiAcDiMxBzd	0.06 $\pm$ 0.02	7.5 $\pm$ 1.5	5.6 $\pm$ 1.3	ND	ND	9.8
AHC as DiMxBzd	0.01 $\pm$ 0.00	20. $\pm$ 5.	4.8 $\pm$ 1.3	ND	ND	25.
Total DiMxBzd $\mu\text{g-equiv.}$	Nil	40.	11.	Nil	Nil	51.
DIRECT VIOLET 32						
DiMxBzd	0.09 $\pm$ 0.02	8.6 $\pm$ 3.2	4.5 $\pm$ 1.6	0.33 $\pm$ 0.05	ND	13.
MoAcDiMxBzd	0.25 $\pm$ 0.02	4.2 $\pm$ 1.4	3.3 $\pm$ 1.6	ND	ND	6.4
DiAcDiMxBzd	0.08 $\pm$ 0.05	7.3 $\pm$ 2.3	5.8 $\pm$ 1.6	ND	ND	10.
AHC as DiMxBzd	0.02 $\pm$ 0.00	23. $\pm$ 10.	15. $\pm$ 5.	ND	ND	38.
Total DiMxBzd $\mu\text{g-equiv.}$	Nil	41.	27.	0.7	Nil	68.
DIRECT RED 46						
DiCiBzd	0.06 $\pm$ 0.00	0.65 $\pm$ 0.07	1.5 $\pm$ 0.4	1.2 $\pm$ 0.1	ND	3.4
MoAcDiCiBzd	0.11 $\pm$ 0.02	1.0 $\pm$ 0.2	3.3 $\pm$ 0.9	1.6 $\pm$ 0.3	ND	5.1
DiAcDiCiBzd	0.04 $\pm$ 0.01	1.3 $\pm$ 0.3	3.6 $\pm$ 1.2	ND	ND	3.7
AHC as DiCiBzd	0.03 $\pm$ 0.01	0.87 $\pm$ 0.33	2.3 $\pm$ 0.7	1.9 $\pm$ 0.4	ND	5.1
Total DiCiBzd $\mu\text{g-equiv.}$	Nil	3.4	9.3	4.5	Nil	17.

* Mean and SD from three groups of four rats. Results were corrected for background and recovery.

** Mean total amount of metabolites from three groups of four rats expressed as the corresponding amine congener.

ND = No residues detected above pretreatment levels.

and DiMxBzd were observed followed by lesser amounts of MoAcDiMxBzd. These results are similar to those reported for ^{14}C -Direct Red 2 (DiMeBzd-based) and ^{14}C -Direct Blue 15 (DiMxBzd-based) (8).

The data from Table III were used to calculate the total amount of congener (μg) found in the urine of 12 rats for each dye and the results are reported in Table IV. These values were then related to the amounts of the corresponding congeners (μg) theoretically available from the doses of dye (24 mg/12 rats) administered and the results were expressed as the percentage of dose converted to the congener (Table IV). An examination of these values, which ranged from 0.62% for Direct Red 39 to 2.6% for Direct Violet 32, indicated that only small quantities of the congeners potentially available from their respective dyes could be found in the urine by the methods employed. These data provide conclusive evidence that all of the dyes were metabolized; small contributions of congeners dosed as impurities in the dyes (0.002-0.50 μg) are also reported in Table IV. It should also be pointed out that the dyes whose metabolites peaked 12 to 24 hours after dosing (*i.e.* Direct Red 39, Direct Orange 6 and Direct Red 46) were least converted to their respective congeners (*i.e.*, 0.62%, 0.95% and 0.81%, respectively) in contrast with the other six dyes that ranged from 1.4 to 2.6%.

This experiment was specifically designed to determine whether the nine dyes were sufficiently metabolized in rats to yield detectable levels of benzidine-congener metabolites in the urine. Although this objective was met, the methodology employed provided little information concerning the extent of absorption, metabolism and distribution of the dyes. However, it has been reported (8,11) that rats dosed with ^{14}C -Direct Red

Table IV. Benzidine Congeners in Urine From Twelve Rats Each Given a Single Oral Dose (2 mg) of Dye Indicated

Direct Dye	Base	Congener (μg):			Dose Converted to Congener (%)
		Available from Dose*	Dosed as Impurity	Found in Urine	
Red 39	DiMeBzd	7,200.	0.014	45.	0.62
Blue 14	DiMeBzd	5,300.	0.50	75.	1.4
Blue 53	DiMeBzd	5,300.	0.051	72.	1.4
Orange 6	DiMeBzd	8,200.	0.012	78.	0.95
Blue 8	DiMxBzd	7,700.	0.005	110.	1.4
Black 114	DiMxBzd	8,700.	0.018	150.	1.7
Blue 10	DiMxBzd	5,900.	0.002	150.	2.5
Violet 32	DiMxBzd	7,700.	0.004	200.	2.6
Red 46	DiCiBzd	6,300.	0.210	51.	0.81

*Based on complete reduction of dye to its corresponding free amine.

2 (DiMeBzd-based) or ^{14}C -Direct Blue 15 (DiMxBzd-based) excreted 19 to 21% of the administered dose (2 mg) in the urine and 73 to 74% in the feces; 10 to 12% of the dose in feces was intact dye. In these experiments with ^{14}C -dyes, assays for the free congener amine, MoAc, DiAc and AHC metabolites were also performed as described in the present study. Treatment of data from the ^{14}C -dyes in the manner presented in Table IV indicated that a total of 0.6 and 1.3% of the benzidine-congener moieties available from the dosed dyes were found as the specified metabolites with ^{14}C -Direct Red 2 and ^{14}C -Direct Blue 15, respectively. These results are comparable to those found for the eight DiMeBzd- and DiMxBzd- dyes reported in the pre-

sent study, where DiMeBzd-based dyes generally yielded larger amounts of metabolites measurable by the EC/GC procedures than did the DiMeBzd-based compounds. Although unlabeled dyes were used in the present experiment and only a small percentage of the metabolites were detected, their complete metabolic profiles may be expected to parallel those of ¹⁴C-Direct Red 2 and ¹⁴C-Direct Blue 15 (8).

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Metabolism and Distribution of Two ¹⁴C-Benzidine-Congener-Based Dyes in Rats as Determined by GC, HPLC, and Radioassays

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Abstract

Absorption, metabolism, and tissue distribution studies were conducted in the rat with ¹⁴C-biphenyl ring-labeled Direct Blue 15, a 3,3'-dimethoxybenzidine (DiMeBzd) based azo dye; Direct Red 2, based on 3,3'-dimethylbenzidine (DiMeBzd) and the corresponding benzidine congener amines. Single oral doses of the ¹⁴C-labeled dyes (12 mg/kg, 62 μ Ci/kg) and molar equivalent doses of the respective amines were administered and urine and fecal samples collected at intervals up to 192 hours. Urine specimens were analyzed for ¹⁴C content and further characterized by EC/GC for free amines, acetylated metabolites, and conjugates. Feces were assayed for ¹⁴C content and for unchanged dosed dyes or amines by HPLC. A comparison of the metabolism of Direct Blue 15 with its base DiMeBzd, indicated that the base was more extensively metabolized and that most of the ¹⁴C in various extracts was identified as known metabolites. The metabolism of Direct Red 2 compared with its base, DiMeBzd, indicated that the base was more extensively metabolized, yet only a small percentage of the ¹⁴C in extracts was identified as known metabolites. Most of the ¹⁴C present in the urine could not be extracted with benzene nor chloroform, indicating high polarity. Distribution studies conducted with both dyes showed that liver, kidney, and lung accumulated and retained higher levels of ¹⁴C than other tissues (at 72 hrs). Peak levels of ¹⁴C, which occurred 8-12 hours after dosing, were significantly higher with Direct Red 2 than Direct Blue 15. Tissue distribution data (72 hr) for rats dosed with the free amines compared with the dyes showed a generally lower but similar distribution pattern.

Introduction

Benzidine- and benzidine-congener-based azo compounds have been widely used in the dye industry for many years.

However, benzidine is known to be a human carcinogen (1) and the benzidine-based azo dyes Direct Black 38 (CAS 1937-37-7), Direct Brown 95 (CAS 10300-74-0), and Direct Blue 6 (CAS 2002-46-2) were found to be carcinogenic in rats (2). Furthermore, Direct Black 38 was reported to be converted to benzidine and its metabolites in the hamster (3) and other work has demonstrated that a variety of benzidine-based azo dyes are metabolized to benzidine in the dog (4). The National Institute for Occupational Safety and Health (NIOSH) has therefore evaluated the evidence and issued a special hazard review, which concluded that benzidine-based azo dyes are potential human carcinogens, and that exposure to these dyes should be eliminated (5).

Dyes based on the congeners of benzidine, *o*-tolidine (3,3'-dimethylbenzidine, DiMeBzd) and *o*-dianisidine (3,3'-dimethoxybenzidine, DiMeBzd) possess structures analogous to the benzidine-based dyes and may also be metabolized to the corresponding free amines (4,6,7). Although these congeners are not regulated human carcinogens, they have been shown to be mutagenic (8-10) and to produce tumors in experimental animals (11-18). However, only limited experiments have been conducted to determine whether azo dyes, based on these congeners, are converted to the potentially carcinogenic products in test animals. Lynn *et al.* (4) employed gas chromatography with nitrogen-phosphorus detection (GC/NPD) and gas chromatography/mass spectrometry (GC/MS) to analyze urine from dogs and rats dosed with several DiMeBzd- and DiMeBzd-based azo dyes; the corresponding free amines were detected. Nony *et al.* (19), employing electron-capture/gas chromatography (EC/GC), reported the presence of DiMeBzd and metabolites in the urine of rats and hamsters dosed with Direct Red 2 (DiMeBzd-based); the levels of these metabolites in the rat were about three times more than in the hamster.

An interagency agreement between NIOSH and the Food and Drug Administration (FDA), with the work to be conducted at the National Center for Toxicological Research (NCTR), was therefore initiated to obtain additional data concerning the absorption, distribution, and metabolism of a DiMeBzd- and DiMeBzd-based dye in rats. Direct Red 2 and Direct Blue 15,

each labeled with ^{14}C , were selected for use in the experiment as representatives of the two classes of dyes; their structures are shown in Figure 1.

In this study, rats were given a single dose (2 mg, 10 μCi) of the dye or the corresponding free amine. The urine was collected at several intervals (up to 192 hr) and assayed for the free amine, its monoacetyl (MoAc) and diacetyl (DiAc) metabolites, and alkaline hydrolyzable conjugates (AHC) by EC/GC, as described by Nony, *et al.* (20). Assays of the MoAc and DiAc metabolites of the congener amines were sought since Morton *et al.* (21) reported that they were present in the metabolism sequence for benzidine. A general scheme for the analytical procedure is presented in Figure 2 (20). Samples of feces collected during the 192-hour study were also analyzed by HPLC to determine the amounts of intact dye or free amines excreted (22). Radiochemical (RC) assays were performed on the same solutions assayed by EC/GC, the discarded fractions, and feces collected at corresponding intervals. Tissues, organs, fluids and excreta from animals sacrificed at various intervals after receiving a single dose of the labeled dye or free amine were assayed radiochemically to determine the extent of absorption and distribution of the ^{14}C .

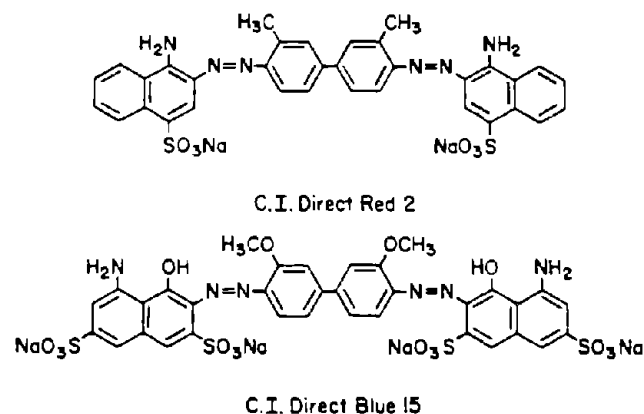


Figure 1. Structures of Direct Red 2 and Direct Blue 15.

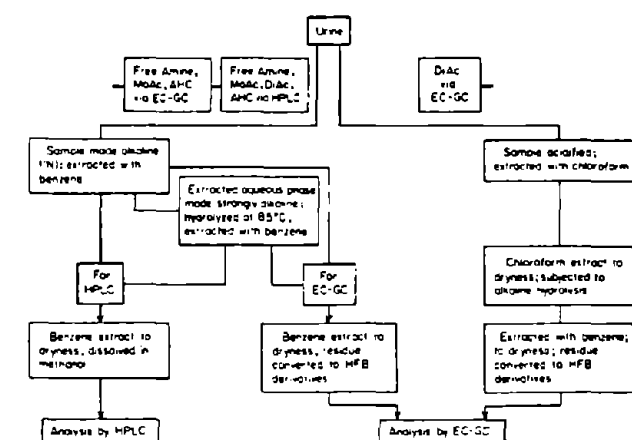


Figure 2. General scheme for extraction, separation, and analysis of benzidine-congener free amines and their monoacetylated (MoAc), diacetylated (DiAc), and conjugated (AHC) products in urine (20).

Experimental

Test Materials

The ^{14}C -Direct Red 2 (CAS 992-59-6) [3,3'-{[(3,3'-Dimethyl(1,1'-biphenyl)-4,4'-diyl)bis(azo)]bis-(4-amino-1-naphthalenesulfonic acid) disodium salt] and ^{14}C -Direct Blue 15, (CAS 2429-74-5) [3,3'-{[(3,3'-Dimethoxy(1,1'-biphenyl)-4,4'-diyl)bis(azo)]bis-(5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid) tetrasodium salt] each dye was uniformly ring labeled ^{14}C (biphenyl moiety only) with specific activities of 0.176 and 0.180 $\mu\text{Ci}/\mu\text{g}$ (i.e. 128 and 179 $\mu\text{Ci}/\mu\text{mole}$), respectively, were synthesized by the Dynapol Co. Reduction of the dyes with stannous chloride and subsequent analysis by GC/FID (22) confirmed the presence of the correct benzidine-congener base. Radiometric scans of Direct Red 2 and Direct Blue 15, or their corresponding free amines recovered from reduction of the dyes, developed with several solvent systems on thin layers of silica gel or C_{18} indicated that purities were 99% and 96%, respectively. Aqueous solutions of the ^{14}C -labeled dyes, extracted three times with equal volumes of benzene, yielded 25 and 517 ppm of radiochemical impurities for Direct Red 2 and Direct Blue 15, respectively. Radiometric scans of the extracts developed on thin layers of silica gel indicated that about 150 ppm of the radiochemical impurities from Direct Blue 15 were ^{14}C -DiMeBzd; however, the low level of impurities from Direct Red 2 could not be characterized by the procedure. Based on these assays, and because the ^{14}C -dyes were to be diluted about 33-fold with unlabeled dyes, the ^{14}C -dyes were used as received without further purification. The corresponding ^{14}C -labeled free amines of the Bzd congeners were prepared by reducing an appropriate amount of the ^{14}C -dyes (22).

The unlabeled Direct Blue 15 was obtained from the Atlantic Chemical Corp. via the Dyes Environmental and Toxicology Organization (DETO), and the unlabeled Direct Red 2 was from DETO. Both dyes were purified and analyzed for purity prior to use as described later. The Direct Blue 15 was found to be 65.5% pure and contained 15 ppm of DiMeBzd; the Direct Red 2 was 79.0% pure and contained 0.19 ppm of DiMeBzd.

Aqueous doses of the dyes (1 mL) were prepared to contain 2 mg of dye (based on 100% purity) and 10 μCi of ^{14}C . Therefore, each dose of Direct Blue 15 contained 2.96 mg of the unlabeled dye (65.5% pure) and 0.055 mg of ^{14}C -Direct Blue 15; each dose of Direct Red 2 contained 2.458 mg of the unlabeled dye (79.0% pure) and 0.058 mg of ^{14}C -Direct Red 2.

Doses of the corresponding amine congeners (1 mL) were prepared in aqueous HCl (pH 4) to contain the amount of amine (as the dihydrochloride) available from complete cleavage of the 2-mg dose of dyes and 10 μCi of ^{14}C . Therefore, each dose of DiMeBzd contained 621 μg of unlabeled DiMeBzd-2 HCl and 14 μg of ^{14}C -DiMeBzd; each dose of DiMeBzd contained 765 μg of DiMeBzd-2 HCl (Pfaltz and Bauer, Inc.) and 17 μg of ^{14}C -DiMeBzd.

Animal Experiments

The animals were housed in stainless steel metabolism cages (No. LC-177) from Wahmann Manufacturing Co. An additional 16 mesh stainless steel screen was placed over the animal support screen to retain the smaller fecal pellets and thus separate them from the urine. Urine was collected in a graduated glass tube submerged in solid dry ice and positioned under the drain tube of the cage.

Samples of control rat urine separately spiked with each dye (1 mg/mL) were stored at 5° and 25 °C and assayed at various intervals up to 96 hours, to determine whether the compounds were stable under the experimental conditions.

The male Fischer 344 rats, reared at the NCTR, were six weeks old and weighed 150 to 175 g. The rats were kept on a 12-hour light cycle (6 am to 6 pm) and were allowed free access to water. Control samples of urine and feces were collected during the 36 hours preceding the administration of the single dose (1 mL) of dye or amine by intubation into the stomach. Feed was removed from the cages 12 hours prior to dosing and restored 1 hour after the dose was administered.

Metabolism Tests. Six groups of 4 rats (2 rats/cage) were used in tests with each dye; 5 groups were dosed with the dye and one group, dosed with water, served as a control. Samples of urine and feces from each group (2 cages) were collected for intervals of 0-8, 8-16, 16-24, 24-48, 48-96, 96-144, and 144-192 hours after dosing and stored at -20 °C. In tests with the amines, 3 rats which represented one group were used for each amine. The animals were dosed and the urine and feces collected at various intervals and stored in the manner described for dyes.

Distribution Tests. Six groups of 3 rats (1 rat/cage) were dosed with each dye. At intervals of 2, 4, 8, 12, 24, and 72 hours after the dose was administered, three rats were removed, and anesthetized with carbon dioxide; a blood sample was taken from each animal. Each animal was then killed with carbon dioxide and the various tissues, organs, fluids, carcass, and the urine and feces from the cage were separately collected and stored at -20 °C until analysis. In tests with the amines, 3 rats in separate cages were used for each amine. The animals were removed from the cages 72 hours after the dose was administered, anesthetized, and the various samples were separately collected and stored as described for the dyes.

Preparation of Samples for Analysis

Portions of the urine were taken from the composited samples for extraction and preparation for analysis by EC/GC and for direct analysis of ¹⁴C content. The feces from each sampling interval were weighed and ground to a uniform consistency with a mortar and pestle. Weighed portions of the mixture were extracted and prepared for analysis by HPLC, EC/GC or combusted to determine ¹⁴C content.

Stomach, small intestine, and large intestine (including the cecum) were partially opened at each end with a razor blade to permit complete extrusion of the contents; the stomach and cecum were also washed with about 3 mL of water after the contents were extruded. The contents and any washings for each tissue were combined and separately analyzed for each animal. The bulk of the urine present in the urinary bladder was removed with a syringe and needle and added to the sample of urine. The organs, tissues, and blood were weighed, then macerated with a razor blade until a homogenous sample was obtained. (Exception: the entire urinary bladder and spleen were separately combusted to determine ¹⁴C content.) The carcass was weighed and macerated with 100 mL of water in a Waring blender operated at high speed for 10 minutes. Appropriate amounts (0.5 g or less) of the prepared samples were then combusted to determine ¹⁴C content.

Analytical Chemical Methodology

EC/GC Assays. Samples of urine and selected samples of feces from the metabolism tests with dyes and amines were

assayed as described by Nony *et al.* (20). Separate portions of the same sample were assayed for the benzidine-congener free amines and their MoAc and AHC metabolites or for the DiAc metabolite as shown in Figure 2. The following is a brief description of the procedure. The benzidine-congener free amine and its MoAc and DiAc metabolites were extracted from urine under alkaline conditions with benzene; the aqueous phase was subjected to alkaline hydrolysis and again extracted with benzene to remove AHC metabolites as the benzidine-congener amine. The two benzene extracts were then subjected to derivatization with heptafluorobutyric anhydride with trimethylamine as a catalyst and analysis by EC/GC. Any DiAc metabolites present in the initial benzene extract were not derivatized or detected. A second sample, made strongly acid with HCl, was extracted with chloroform to remove the DiAc metabolite and thus separate it from any free benzidine-congener or MoAc metabolite. The extracted DiAc metabolite was then hydrolyzed to the free amine, derivatized, and analyzed as described. Assays of several samples of feces from the metabolism studies with dyes were also performed by the EC/GC procedure for urine to determine the levels of benzidine-congener amines and MoAc, DiAc and AHC metabolites excreted via the feces. Minimum detectable levels for the two benzidine-congener free amines and their MoAc and DiAc metabolites in rat urine were at least 12 ppb. Parallel RC assays were performed on each extract as well as discarded fractions and plugs of sodium sulfate used to dry the extracts.

HPLC Assays. The amounts of intact dyes excreted in fecal samples from the metabolism tests were determined as described previously (22). Briefly, the fecal samples from the Direct Blue 15 tests were extracted with dimethylformamide-50% water, cleaned up on a guard column of octadecylsilane (ODS) and analyzed on an ODS column using a mobile phase of 45% acetonitrile: 55% phosphate buffered tetrabutylammonium hydrogen sulfate (0.01 M, pH 7.3). Direct Red 2 was assayed in the same manner, except the mobile phase contained 50% acetonitrile. The detector was set at 600 and 500 nm for Direct Blue 15 and Direct Red 2, respectively. The minimum detectable level (based on twice background) for both dyes in feces was about 0.2 ppm.

Fecal samples (0.5 g) from the metabolism tests with amines were analyzed as described previously (20) according to the scheme illustrated in Figure 2. The minimum detectable level (based on twice background) for the amine, MoAc and DiAc metabolites of each dye in feces, was about 0.2 ppm.

RC Assays. The amount of ¹⁴C in samples from the metabolism and distribution tests was determined with a Searle Analytic Model 92 liquid scintillation analyzer. Samples of raw urine (100 µL) were analyzed in 20-mL vials containing 15 mL of Aquasol™ (New England Nuclear). Parallel RC assays were also performed on all extracts analyzed by the EC/GC procedure as well as the fractions discarded. The volumes of these solutions added to the Aquasol™ cocktail for analysis ranged from 0.1 to 2.0 mL depending upon the volume and nature of the liquid, and the amount of ¹⁴C present. Chloroform extracts which were not compatible with the liquid scintillation procedure were evaporated to dryness and the residue dissolved in benzene prior to analysis; the acidic and basic solutions (1 mL) were adjusted to pH 7 before addition to the cocktail. The liquid scintillation procedure was monitored for chemical and color quench by using the channels ratio technique; however, quench corrections were made by using the internal standard procedure. The efficiency of the RC procedure was about 93%. Recovery

factors used to correct the EC/GC data were also applied to corresponding RC assays.

All fecal samples (0.5 g) were oxidized to carbon dioxide by a Packard Model C306 sample oxidizer. Samples were placed in the furnace burner where oxidation products were quantitatively transferred by the fully automated instrument to a column that was flushed with 7 mL of Carbo-Sorb™ (Packard Instrument Co.), a base-containing absorber fluid that traps the carbon dioxide, and delivered into a scintillation vial along with 10 mL of Permafluor™ (Packard). The vials containing the oxidized samples were then subjected to RC assays by liquid scintillation, which yielded an efficiency of 78% which includes the 99% recovery from sample oxidation.

Samples of tissues, organs, blood, and carcass were assayed exactly as described for feces except that the amounts combusted varied from 0.1 g for fatty samples such as adipose and brain to 0.5 g for muscle, liver, blood and other non-fatty samples.

Purification and Analysis of the Unlabeled Dyes

The unlabeled Direct Blue 15 and Direct Red 2 were purified and analyzed for purity as described previously (22) and for amine impurities as described by Nony *et al.* (20,23). For purification, the dye was mixed with about twice its weight with cold water (3°C), filtered on a Buchner funnel and washed with three additional portions of cold water to remove water soluble impurities. The residue on the filter was dissolved in water, and extracted four times with benzene to remove impurities of aromatic amines. The aqueous phase was freeze-dried to recover the purified product. Assays for purity were conducted as follows: an aqueous solution of the dye was reduced with 0.2 M stannous chloride in 12 N HCl at 40°C for about 3 hours (to complete decolorization), made strongly alkaline and the benzidine-congener free amine was extracted with chloroform and analyzed by GC/FID. The purity of the dye was calculated on the basis of the amount of benzidine-congener amine obtained from a known amount of dye as compared to the amount theoretically obtainable by using the following factors: DiMeBzd × 4.06 = Direct Blue 15; DiMeBzd × 3.41 = Direct Red 2. Amine impurities were extracted from an aqueous solution of the dye with benzene, converted to heptafluorobutyric anhydride (HFB) derivative and assayed by EC/GC.

Results and Discussion

The rats, after being dosed with the dyes or amines, showed no outward signs of shock or discomfort and all animals survived to the end of the experiment. The weight gained by treated rats was similar to that of controls and normal intake of water and feed was observed with all animals. The coloration of urine and feces samples was normal except that fecal pellets from dosed rats were colored red or blue during intervals of peak excretion of the dyes.

In experiments concerning the stability of the dyes in rat urine, both compounds were found to be stable at 5°C for 96 hours; however, significant amounts of the corresponding free amines were detected after 48 hours at 25°C. This degradation was probably due to microbial action. Since all samples of urine were collected under dry ice and stored at -20°C until analysis, any amounts of free amines in the urine, in excess of the small amount dosed as impurities, must result from breakdown in the animals.

Absorption and Excretion

Previous studies conducted at the NCTR (23) and elsewhere (4) on the metabolism of benzidine and benzidine-congener-based azo dyes did not determine the degree of absorption of the dyes. Table I shows excretion profiles for Direct Blue 15 when given as a single oral dose (2 mg, 10 µCi) to each 150-175 g rat (ca. 12 mg/kg and 62 µCi/kg). Most of the dose (*i.e.*, 74.4% as ¹⁴C) was excreted in the feces with about 18.8% found in the urine. Peak fecal and urinary excretion of ¹⁴C occurred during the 8-16 hour interval, although detectable amounts of radioactivity were still being excreted 144-192 hours after dosing.

HPLC assays of the feces showed that about 12% of the dose was excreted as the unchanged dye. The period of peak excretion was 8-16 hours, with no intact dye detected after 48 hours. The percentage of dose in the feces as ¹⁴C (74.4%) compared with the percentage of intact dye (12%) indicated that 84% of the fecal radioactivity was unidentified metabolic products, possibly from bile or some form of azo reduction. The high levels of radioactivity found in the urine (18.8%) indicated extensive metabolism.

Comparable excretion profiles from Direct Red 2 dosed in a manner identical to Direct Blue 15 are presented in Table II.

Table I. Excretion Profiles of ¹⁴C-Direct Blue 15 as Determined by RC or HPLC*

Sampling Interval (hr)	Urine, Feces and Dose Excreted**				
	Urine Excreted (mL)	% Dose in Urine	Feces Excreted (g)	% Dose in Feces	% Dose as Intact Dye in Feces (HPLC)
Pretreatment (36)	33 ± 5	<0.002 ± 0.001	3.36 ± 1.46	0.0002 ± 0.0000	<0.004 ± 0.002
0 - 8	4.0 ± 0.7	2.22 ± 0.26	2.73 ± 0.78	2.10 ± 1.30	0.871 ± 0.589
8 - 16	8.8 ± 2.0	6.59 ± 0.78	4.70 ± 0.46	43.9 ± 6.5	9.91 ± 2.11
16 - 24	8.4 ± 1.3	4.34 ± 0.39	4.23 ± 0.64	15.0 ± 2.4	1.02 ± 0.15
24 - 48	31. ± 5.	4.40 ± 0.64	9.58 ± 1.45	7.70 ± 4.34	0.235 ± 0.104
48 - 96	76 ± 10	1.08 ± 0.15	30.8 ± 4.0	5.21 ± 2.14	Nil
94 - 144	80 ± 5	0.103 ± 0.007	31.2 ± 4.2	0.348 ± 0.066	Nil
144 - 192	83 ± 8.	0.055 ± 0.008	34.6 ± 1.5	0.141 ± 0.028	ND
Total	-	18.79	-	74.40	12.04

*Results for post-treatment samples are corrected for background of pretreatment samples

**Mean and standard deviation of 5 groups of 4 rats each. Nil denotes results that do not exceed background. ND indicates that the assay was not determined

Most of the dose (i.e., 73.5% as ^{14}C) was excreted in the feces with about 20.6% found in the urine. Peak fecal and urinary excretion intervals were the same as those of Direct Blue 15. HPLC assays of the feces showed 10.1% of the dose excreted as unchanged dye. The excretion profiles of the dye in feces were identical to those of Direct Blue 15, with more than 86% of the ^{14}C excreted in the feces as unidentified metabolites. A comparison of the data in Tables I and II illustrate that excretion profiles of the two dyes are essentially the same.

Attempts were made to determine the levels of free amines and their acetylated metabolites via EC/GC in feces from rats dosed with both dyes. Samples of the 8-16 hour interval from rats dosed with Direct Blue 15 or Direct Red 2 were found to contain about 2.5 and 13 ppm of the free amines, respectively. Further assays were abandoned because of low recoveries and the presence of excessive amounts of interfering substances in the extracts caused a rapid deterioration of the GC system.

Since the two dyes were reduced to their respective benzidine congener bases, excretion profiles with molar equivalent doses of DiMBzd and DiMeBzd were determined in rats (Table III) for comparison with data presented for the dyes in Tables I and II. Data in Table III indicate that about 52-59% of the doses

were excreted in the feces as ^{14}C and about 35-40% in the urine from rats dosed with the two benzidine-congener bases. Although the intervals for peak urinary and fecal excretion of ^{14}C were identical to those for the dyes, fecal excretion of only 1-6% of the dose was observed for the free amines including acetylated metabolites; whereas, 10-12% of the dosed dyes were excreted as intact compounds. Unidentified metabolites comprised about 97-89% of the ^{14}C excreted via the feces from rats dosed with DiMBzd and DiMeBzd. These data indicate that biliary metabolites, unextractable by the described procedures, were probably excreted via the feces (24). The free benzidine-congener amines showed significantly more metabolism than the dyes (19-21%) since higher levels of the amines were excreted in the urine (35-40%). The amines also showed significantly more urinary excretion than the dyes since less fecal excretion of ^{14}C occurred with the amines (52-59% of the dose) than with the dyes (73-74%).

Metabolism

Studies recently completed at the NCTR with nine benzidine-congener dyes consistently showed metabolic breakdown to free

Table II. Excretion Profiles of ^{14}C -Direct Red 2 as Determined by RC or HPLC*

Sampling Interval (hr)	Urine, Feces and Dose Excreted**				
	Urine Excreted (mL)	% Dose in Urine	Feces Excreted (g)	% Dose in Feces	% Dose as Intact Dye in Feces (HPLC)
Pretreatment (36)	44 ± 4	<0.001 ± 0.002	4.15 ± 1.70	0.0002 ± 0.0000	<0.005 ± 0.002
0 - 8	4.4 ± 1.6	0.98 ± 0.46	4.00 ± 0.78	1.82 ± 2.04	0.519 ± 0.594
8 - 16	10.3 ± 1.2	8.29 ± 1.06	5.53 ± 0.51	34.7 ± 8.7	8.26 ± 2.76
16 - 24	12.6 ± 0.6	6.38 ± 1.51	5.45 ± 0.77	17.6 ± 3.5	1.10 ± 0.40
24 - 48	42. ± 7.	3.52 ± 0.84	13.5 ± 2.2	12.4 ± 4.1	0.244 ± 0.132
48 - 96	104. ± 30.	1.32 ± 0.29	34.7 ± 2.7	6.06 ± 1.70	Nil
96 - 144	119. ± 41.	0.110 ± 0.029	33.1 ± 4.0	0.650 ± 0.165	Nil
144 - 192	107. ± 35.	0.050 ± 0.018	32.5 ± 3.4	0.284 ± 0.080	ND
Total	—	20.65	—	73.51	10.12

*Results for post-treatment samples are corrected for background of pretreatment samples.

**Mean and standard deviation of 5 groups of 4 rats each. Nil denotes results that do not exceed background. ND indicates that the assay was not determined.

Table III. Excretion Profiles of ^{14}C -Dimethoxybenzidine or ^{14}C -Dimethylbenzidine by RC or HPLC*

Sampling Interval (hr)	Urine, Feces and Dose Excreted**									
	Dimethoxybenzidine			Dimethylbenzidine						
	Urine Excreted (mL)	% Dose in Urine	Feces Excreted (g)	% Dose in Feces	% Dose in Feces as DiAc Metabolites†	Urine Excreted (mL)	% Dose in Urine	Feces Excreted (g)	% Dose in Feces	% Dose in Feces as DiAc Metabolites†
Pretreatment (36)	37	<0.03	8.92	<0.003	<0.012	58	<0.005	1.58	<0.001	<0.002
0 - 8	12	17.5	5.13	9.69	0.69	8	11.1	4.62	8.51	2.60
8 - 16	28	10.7	5.76	23.6	0.70	21	17.3	5.57	26.9	3.37
16 - 24	17	2.92	5.42	9.89	0.07	19	4.43	2.81	8.38	0.25
24 - 48	55.	3.14	11.9	5.67	Nil	70.	4.62	10.9	9.60	Nil
48 - 96	102.	0.84	23.1	2.40	Nil	123.	1.89	21.6	4.04	Nil
96 - 144	80	0.12	24.5	0.52	ND	89	0.20	15.8	0.77	ND
144 - 192	109.	0.09	24.9	0.28	ND	132.	0.12	18.0	0.44	ND
Total	—	35.31	—	52.05	1.46	—	39.66	—	58.64	6.22

*Results for post-treatment samples are corrected for background of pre-treatment samples.

**Samples are composites from 3 rats. Nil denotes results that do not exceed background. ND indicates that the assay was not determined.

†Determined by HPLC. The main constituent was the amine and acetylated metabolite.

amines and their acetylated products in rats (25). The experiments described in this paper were performed concurrently with ^{14}C -labeled dyes to gain additional information on the metabolism of these classes of compounds.

Urinary excretion profiles of DiMxBzd, MoAcDiMxBzd, and AHC from rats given a single oral dose (2 mg μCi) of ^{14}C -Direct Blue 15 are shown in Table IV. Excretion profiles of the DiAcDiMxBzd metabolites obtained from assays of separate aliquots of the urine samples are presented in Table V. Results reported in Tables IV and V are from parallel GC and RC assays of fractions outlined in Figure 2. Metabolites assayed via GC were: AHC (0.48% of the dose) MoAcDiMxBzd (0.27%), free DiMxBzd (0.22%) and DiAcDiMxBzd (0.22%) (Table IV). Peak excretion occurred during the 8-16 hour interval, with no metabolites detected after 25 hours by GC. The free amine fraction assayed via RC accounted for 0.68% of the dose; however, 90% of the DiAcDiMxBzd (Table V) was also present in the free amine fraction and detected by RC but not by GC. Therefore, after corrections for this discrepancy between the

two methods, essentially all of the ^{14}C in the free amine fraction was ascribed to known metabolites as determined by GC analysis. Also, all of the ^{14}C in the AHC fraction was attributed to DiMxBzd by GC analysis. However, it should be noted that 94% of the total ^{14}C found in raw urine was not extracted by benzene at alkaline pH (Table IV). In Table V, all of the ^{14}C in the raw urine remained unextractable by chloroform. The nature of these unextractable water soluble metabolites is not known.

Urinary excretion profiles of DiMeBzd, MoAcDiMeBzd, AHC, and DiAcDiMeBzd from rats given a single oral dose (2 mg μCi) of ^{14}C -Direct Red 2 determined as described for Direct Blue 15 are presented in Tables V and VI. The metabolites assayed via GC were: DiAcDiMeBzd (0.21% of the dose), DiMeBzd (0.15%), AHC (0.12%) and MoAcDiMeBzd (0.08%) (Table VI). The interval of peak excretion was 8-16 hours, and no metabolites were detected by GC after 48 hours. The free amine fraction assayed by RC represented 1.8% of the dose and, after correction for the DiAcDiMeBzd present, only 13.2%

Table IV. Excretion Profiles of DiMxBzd, MoAcDiMxBzd and AHC (as DiMxBzd) of ^{14}C -Direct Blue 15 by (GC) and (RC) Assays*

Sampling Interval (hr)	μg -Equivalents of Dye Indicated**					
	Free Amine Fraction†			AHC Fraction†		Unextractable Metabolites† (RC)
	DiMxBzd (GC)	MoAcDiMxBzd (GC)	Total (RC)	DiMxBzd (GC)	Total (RC)	
Pretreatment (36)	$<0.71 \pm 0.07$	$<0.12 \pm 0.06$	$<0.01 \pm 0.01$	$<0.22 \pm 0.04$	$<0.02 \pm 0.03$	$<0.01 \pm 0.01$
0 - 8	2.59 ± 0.78	7.19 ± 3.54	12.3 ± 2.3	12.8 ± 2.1	11.7 ± 2.1	153 ± 18
8 - 16	13.8 ± 5.6	10.9 ± 2.9	32.7 ± 9.0	21.3 ± 5.6	20.6 ± 4.4	473 ± 56
16 - 24	1.39 ± 0.51	3.52 ± 1.19	7.43 ± 1.11	3.99 ± 0.36	4.42 ± 1.07	335 ± 30
24 - 48	Nil	Nil	1.56 ± 0.27	Nil	1.35 ± 0.39	349 ± 51
48 - 96	Nil	Nil	0.459 ± 0.125	Nil	0.329 ± 0.087	85 ± 12
96 - 144	Nil	Nil	0.103 ± 0.056	Nil	0.144 ± 0.091	7.60 ± 0.52
144 - 192	Nil	Nil	0.064 ± 0.038	Nil	0.108 ± 0.043	4.36 ± 0.64
% ^{14}C in Raw Urine	1.26	1.53	3.88	2.70	2.74	93.63
% Dose	0.22	0.27	0.68	0.48	0.48	17.59

*Results for post-treatment samples are corrected for background of pretreatment samples

**Mean and standard deviation from 5 groups of 4 rats each. Nil denotes results that do not exceed background. ND denotes assays not determined

†Extracted with benzene under strongly alkaline conditions

Table V. Excretion Profiles of DiAcDiMxBzd and DiAcDiMeBzd in Urine by GC and RC Assays*

Sampling Interval (hr)	μg -Equivalents of Dye Indicated**					
	Direct Blue 15†			Direct Red 2†		
	DiAcDiMxBzd (GC)	Total (RC)	Unextractable Metabolites (RC)	DiAcDiMeBzd (GC)	Total (RC)	Unextractable Metabolites (RC)
Pretreatment (36)	$<0.07 \pm 0.10$	$<0.02 \pm 0.10$	$<0.01 \pm 0.01$	$<0.17 \pm 0.03$	$<0.02 \pm 0.01$	$<0.01 \pm 0.01$
0 - 8	4.09 ± 0.87	4.23 ± 0.67	168 ± 20	0.848 ± 0.254	2.68 ± 1.17	68 ± 31
8 - 16	11.3 ± 2.8	11.4 ± 2.5	503 ± 59	9.49 ± 1.65	55.1 ± 13.6	476 ± 61
16 - 24	2.34 ± 1.14	2.81 ± 0.64	343 ± 31	$6.53n \pm 1.27$	32.9 ± 17.5	381 ± 90
24 - 48	Nil	0.689 ± 0.211	351 ± 51	Nil	7.99 ± 3.23	265 ± 62
48 - 96	Nil	0.232 ± 0.072	86 ± 12	Nil	1.94 ± 0.85	90 ± 20
96 - 144	Nil	0.176 ± 0.361	8.22 ± 0.56	Nil	0.217 ± 0.111	7.27 ± 1.93
144 - 192	Nil	0.087 ± 0.047	4.42 ± 0.65	ND	ND	3.96 ± 1.41
% ^{14}C in Raw Urine	1.18	1.31	97.4	1.02	6.10	78.2
% Dose	0.22	0.24	18.3	0.21	1.25	16.1

*Results for post-treatment samples are corrected for background of pretreatment samples

**Mean and standard deviation from 5 groups of 4 rats. Nil denotes results that do not exceed background. ND denotes assays not determined

†Extracted with chloroform under strongly acid conditions

of the ^{14}C compounds in the fraction responded to GC analysis. The AHC fraction (RC) contained 1.7% of the dose; however, GC assays accounted for only 7.4% of the ^{14}C metabolites. Excretion profiles for DiAcDiMeBzd in Table V indicate that 16.7% of the ^{14}C compounds in that fraction were detected by GC. Therefore, GC assays of the specified metabolites of Direct Red 2 account for only a small percentage of the ^{14}C present in the various fractions. Excessive amounts of unidentified ^{14}C compounds in the fractions from Direct Red 2 was in sharp contrast to the total accountability found with Direct Blue 15. However, most of the ^{14}C in raw urine from both dyes remained unextracted with benzene or chloroform.

Results from metabolism of the two benzidine-congener amines in rats were compared with those from their respective dyes by administering molar equivalent doses of the free amines. Urinary excretion profiles of DiMxBzd, MoAcDiMxBzd, AHC, and DiAcDiMxBzd from rats dosed with DiMxBzd are shown in Tables VII and VIII. The metabolites determined by GC were: AHC (1.56% of dose), DiMxBzd (1.18%), DiAcDiMxBzd

(0.93%), and MoAcDiMxBzd (0.35%). The peak excretion intervals and the duration of excretion for each metabolite detected by GC showed some variation, but generally peaked earlier and was eliminated sooner than the same metabolites from rats dosed with Direct Blue 15. GC analysis accounted for all of the ^{14}C metabolites in the free amine fraction from rats dosed with Direct Blue 15, while 78% of the AHC (RC) could be attributed to known metabolites by GC. However, in contrast to Direct Blue 15, only 35% of the ^{14}C products in the DiAcDiMxBzd fraction responded to GC analysis. Most of the ^{14}C in raw urine was not extracted with benzene or chloroform as observed with dosed dyes.

A comparison of the metabolism of Direct Blue 15 with its base (DiMxBzd) shows: A) the base was more extensively metabolized and excreted; AHC was the major product, B) the dye was metabolized more slowly producing DiAcDiMxBzd as its major product, C) GC assays accounted for all of the ^{14}C products in the free amines and AHC fractions from both the base and the dye, and D) while GC accounted for all of the

Table VI. Excretion Profiles of DiMeBzd, MoAcDiMeBzd and AHC (as DiMeBzd) of ^{14}C -Direct Red 2 by GC and RC Assays*

Sampling Interval (hr)	μg -Equivalents of Dye Indicated**					
	Free Amine Fraction			AHC Fraction		Unextractable Metabolites† (RC)
	DiMeBzd (GC)	MoAcDiMeBzd (GC)	Total (RC)	DiMeBzd (GC)	Total (RC)	
Pretreatment (36)	<3.13 ± 0.5	<0.87 ± 0.27	<0.01 ± 0.01	<0.41 ± 0.13	<0.02 ± 0.03	<0.10 ± 0.10
0 - 8	1.33 ± 0.62	0.71 ± 0.32	6.45 ± 3.03	1.31 ± 0.49	5.98 ± 2.69	66 ± 32
8 - 16	5.16 ± 1.01	4.01 ± 0.73	77.0 ± 11.1	6.34 ± 0.22	5.88 ± 12.9	509 ± 65
16 - 24	2.09 ± 0.47	2.00 ± 0.33	46.3 ± 18.6	2.31 ± 1.00	47.2 ± 15.9	399 ± 95
24 - 48	3.13 ± 0.56	Nil	13.8 ± 3.3	Nil	15.2 ± 4.4	251 ± 60
48 - 96	Nil	Nil	3.33 ± 0.83	Nil	5.75 ± 2.32	96 ± 21
96 - 144	Nil	Nil	0.317 ± 0.106	Nil	0.602 ± 0.294	7.9 ± 2.1
144 - 192	ND	ND	ND	ND	ND	-
% ^{14}C in Raw Urine	0.71	0.41	9.48	0.60	8.08	80.4
% Dose	0.15	0.08	1.84	0.12	1.67	16.6

*Results for post-treatment samples are corrected for background of pretreatment samples.

**Mean and standard deviation from five groups of 4 rats each. Nil denotes results that do not exceed background. ND denotes assays not determined.

†Extracted with benzene under strongly alkaline conditions.

Table VII. Excretion Profiles of DiMxBzd, MoAcDiMxBzd and AHC (as DiMxBzd) of ^{14}C -Direct Blue 15 by (GC) or (RC) Assays*

Sampling Interval (hr)	μg -Equivalents of Compound Indicated**					
	Free Amine Fraction†			AHC Fraction †		Unextractable Metabolites† (RC)
	DiMxBzd (GC)	MoAcDiMxBzd (GC)	Total (RC)	DiMxBzd (GC)	Total (RC)	
Pretreatment (36)	<0.09	<0.12	<0.01	<0.13	<0.02	<0.01
0 - 8	16.8	4.71	28.0	19.2	22.5	209
8 - 16	0.644	0.406	2.75	2.32	3.45	151
16 - 24	Nil	Nil	0.374	0.544	0.922	42.6
24 - 48	Nil	Nil	0.495	0.990	2.59	42.3
48 - 96	Nil	Nil	Nil	Nil	Nil	12.4
96 - 144	Nil	Nil	Nil	Nil	Nil	1.75
144 - 192	Nil	Nil	Nil	Nil	Nil	1.30
% ^{14}C in Raw Urine	3.34	0.98	6.06	4.42	5.65	88.22
% Dose	1.18	0.35	2.14	1.56	2.00	31.19

*Results for post-treatment samples are corrected for background of pretreatment samples.

**Samples are composites from 3 rats.

†Extracted with benzene under strongly alkaline conditions.

¹⁴C products in the DiAcDiMxBzd fraction from the dye, only 35% of the radioactivity from the base could be attributed to DiAcDiMxBzd in that fraction.

Urinary excretion profiles of DiMeBzd, DiAcDiMeBzd, AHC, and MoAcDiMeBzd from rats dosed with DiMeBzd are shown in Tables VIII and IX. The metabolites determined by GC were: DiAcDiMeBzd (2.1% of the dose), AHC (0.4%), DiMeBzd (0.4%) and MoAcDiMeBzd (0.2%) with peak excretion intervals similar to DiMxBzd. Only 13.9% of the ¹⁴C products in the free amine fraction, 11.0% in the AHC fraction and 34.4% in the DiAcDiMeBzd fraction were attributed to known metabolites by GC. These results were similar to those observed with the parent dye. Again, the bulk of the ¹⁴C in raw urine was not extracted with the two solvents employed.

A comparison of the metabolism of Direct Red 2 with its base (DiMeBzd) shows: A) the base was more extensively metabolized and excreted; DiAcDiMeBzd was the major metabolite for both

the dye and base, and B) only a small percentage of the ¹⁴C in the extracted fractions from both base and dye responded to GC assays for specific metabolites.

The metabolism patterns for the dyes correlated well with their corresponding bases. As expected, the bases were metabolized more extensively than the dyes.

Preliminary results recently reported by Ilias *et al.* (26) on the metabolic fate of ¹⁴C-benzidine in the rat showed that the major portions of the radioactivity appeared in the feces, with about 20% found in the urine. In bile exteriorized animals, up to 70% of the dose was eliminated via the bile. A variety of N-acetylated and N-glucuronide metabolites were reported. The results are consistent with those reported for DiMxBzd and DiMeBzd in the present study.

Tissue Distribution

Results from tissue distribution tests with the two dyes deter-

Table VIII. Excretion Profiles of DiAcDiMxBzd of ¹⁴C-DiMxBzd and DiAcMeBzd of ¹⁴C-DiMeBzd by GC and RC Assays*

Sampling Interval (hr)	μg-Equivalents of Compound Indicated**					
	Dimethoxybenzidine†			Dimethylbenzidine†		
	DiAcDiMxBzd Fraction		Unextractable Metabolites (RC)	DiAcDiMeBzd Fraction		Unextractable Metabolites (RC)
	DiAcDiMxBzd (GC)	Total (RC)		DiAcDiMeBzd (GC)	Total (RC)	
Pretreatment (36)	<0.06	<0.02	<0.01	<0.15	<0.02	<0.01
0 - 8	2.59	7.80	250.	6.30	32.3	116.
8 - 16	10.9	30.2	129.	10.6	53.4	157.
16 - 24	0.164	1.07	41.7	0.385	8.90	49.8
24 - 48	Nil	0.476	43.3	2.26	10.8	57.5
48 - 96	Nil	Nil	12.4	Nil	1.48	25.3
96 - 144	Nil	Nil	1.75	Nil	Nil	3.56
144 - 192	Nil	Nil	1.30	Nil	Nil	2.11
% ¹⁴ C in Raw Urine	2.62	7.58	91.88	5.28	15.31	58.92
% Dose	0.93	2.68	32.48	2.09	6.08	23.29

*Results for post-treatment samples are corrected for background of pretreatment samples.

**Samples are composites from 3 rats.

†Extracted with chloroform under strongly acid conditions.

Table IX. Excretion Profiles of DiMeBzd, MoAcDiMeBzd and AHC (as DiMeBzd) of ¹⁴C-Dimethylbenzidine by GC or RC Assays*

Sampling Interval (hr)	μg-Equivalents of Compound Indicated**					
	Free Amine Fraction†			AHC Fraction†		Unextractable Metabolites† (RC)
	DiMeBzd (GC)	MoAcDiMeBzd (GC)	Total (RC)	DiMeBzd (GC)	Total (RC)	
	DiMeBzd (GC)	MoAcDiMeBzd (GC)	Total (RC)	DiMeBzd (GC)	Total (RC)	
Pretreatment (36)	<0.99	<0.95	<0.01	<0.12	<0.02	<0.01
0 - 8	4.14	1.52	41.5	3.94	21.1	124.
8 - 16	0.792	1.44	43.2	3.07	34.5	113.
16 - 24	0.961	Nil	6.16	0.414	6.06	219.
24 - 48	0.192	Nil	8.76	0.430	7.13	62.5
48 - 96	Nil	Nil	1.84	Nil	2.92	26.9
96 - 144	Nil	Nil	Nil	Nil	Nil	3.56
144 - 192	Nil	Nil	Nil	Nil	Nil	2.11
% ¹⁴ C in Raw Urine	0.87	0.42	14.54	1.13	10.27	78.95
% Dose	0.35	0.17	5.77	0.45	4.08	31.35

*Results for post-treatment samples are corrected for background of pretreatment samples.

**Samples are composites from 3 rats.

†Extracted with benzene under strongly alkaline conditions.

mined at six intervals (up to 72 hr) by assays of twenty different tissues, fluids, organs, and contents are presented in Tables X and XI. Additionally, similar test results with the corresponding amine bases determined at 72 hours are shown in Table VII.

Tissue distribution data for rats dosed with Direct Blue 15 are shown in Table X and, as expected, the oral dose was easily traced from the stomach contents through the GI tract. At 2 hours, in addition to expected accumulation in the GI tract, significant ^{14}C was found in urinary bladder, liver, kidney, carcass, and lung. At 8 hours, all tissues showed peak ^{14}C content except brain, heart, lung, and small intestine which all peaked at 4 hours. Excluding the GI tract, the highest content of ^{14}C was in liver, kidney, lung, and carcass. At 72 hours, liver kidney, lung, and carcass contained highest levels of ^{14}C . These results

are similar to those cited for benzidine distribution in tissues (24).

Tissue distribution data for rats dosed with Direct Red 2 are shown in Table XI. High concentrations of ^{14}C were observed in the GI tract as with Direct Blue 15. At 2 hours, in addition to the GI tract, significant accumulations were found in liver, lung, kidney, and carcass. At 12 hours, most tissues showed peak ^{14}C accumulation with liver, lung, and kidney containing the highest levels. At 72 hours, highest accumulations were in liver, urinary bladder, kidney, lung, and carcass. The peak levels of ^{14}C in rats dosed with Direct Red 2 were consistently higher than Direct Blue 15.

Tissue distribution data determined at 72 hours from rats dosed with DiMxBzd and DiMeBzd are shown in Table XII. The

Table X. Distribution of ^{14}C -Direct Blue 15 in Tissues at Several Intervals*

Tissue	μg -Equivalents of ^{14}C -DiMxBzd in Tissue at Sampling Interval Indicated											
	2 hr		4 hr		8 hr		12 hr		24 hr		72 hr	
Adipose	0.043 $\pm$ 0.025		0.130 $\pm$ 0.072		0.164 $\pm$ 0.139		0.103 $\pm$ 0.046		0.058 $\pm$ 0.030		0.021 $\pm$ 0.001	
Blood	0.125 $\pm$ 0.077		0.200 $\pm$ 0.081		0.291 $\pm$ 0.153		0.149 $\pm$ 0.022		0.121 $\pm$ 0.060		0.041 $\pm$ 0.010	
Brain	0.024 $\pm$ 0.005		0.075 $\pm$ 0.038		0.046 $\pm$ 0.017		0.030 $\pm$ 0.011		0.019 $\pm$ 0.007		0.003 $\pm$ 0.004	
Carcass	0.333 $\pm$ 0.247		0.515 $\pm$ 0.309		1.03 $\pm$ 0.84		0.421 $\pm$ 0.496		0.206 $\pm$ 0.046		0.039 $\pm$ 0.012	
Feces	0.015 $\pm$ 0.023		0.870 $\pm$ 1.42		90.4 $\pm$ 79.3		565 $\pm$ 391		452 $\pm$ 182		144 $\pm$ 29	
Heart	0.115 $\pm$ 0.072		0.241 $\pm$ 0.105		0.219 $\pm$ 0.100		0.159 $\pm$ 0.033		0.120 $\pm$ 0.041		0.060 $\pm$ 0.016	
Kidney	0.452 $\pm$ 0.297		0.777 $\pm$ 0.235		1.14 $\pm$ 0.53		0.919 $\pm$ 0.210		0.860 $\pm$ 0.352		0.395 $\pm$ 0.173	
Large Intestine and Contents	5.10 $\pm$ 4.06		14.9 $\pm$ 11.3		15.0 $\pm$ 3.3		12.2 $\pm$ 2.1		9.66 $\pm$ 6.23		0.244 $\pm$ 0.199	
Liver	57.3 $\pm$ 44.9		838 $\pm$ 501		668 $\pm$ 249		271 $\pm$ 92		378 $\pm$ 326		0.713 $\pm$ 0.905	
Lung	0.867 $\pm$ 0.574		3.52 $\pm$ 2.05		4.06 $\pm$ 1.67		2.99 $\pm$ 0.33		3.14 $\pm$ 1.74		1.14 $\pm$ 0.37	
Muscle	0.232 $\pm$ 0.102		0.864 $\pm$ 0.543		0.672 $\pm$ 0.377		0.698 $\pm$ 0.239		0.366 $\pm$ 0.196		0.043 $\pm$ 0.004	
Spleen	0.040 $\pm$ 0.018		0.330 $\pm$ 0.163		0.437 $\pm$ 0.291		0.279 $\pm$ 0.096		0.047 $\pm$ 0.014		0.016 $\pm$ 0.003	
Small Intestine and Contents	0.156 $\pm$ 0.130		0.295 $\pm$ 0.093		0.362 $\pm$ 0.299		0.227 $\pm$ 0.028		0.124 $\pm$ 0.019		0.016 $\pm$ 0.024	
Urinary Bladder	2.13 $\pm$ 0.54		7.14 $\pm$ 3.58		5.51 $\pm$ 2.42		3.76 $\pm$ 0.50		3.78 $\pm$ 2.48		0.106 $\pm$ 0.044	
Stomach and Contents	826 $\pm$ 546		398 $\pm$ 196		146 $\pm$ 96		70.6 $\pm$ 9.5		44.3 $\pm$ 22.9		0.714 $\pm$ 0.486	
Testes	1.59 $\pm$ 0.31		4.04 $\pm$ 2.41		9.62 $\pm$ 7.88		3.99 $\pm$ 0.71		8.10 $\pm$ 6.70		0.404 $\pm$ 0.471	
Urine	22.3 $\pm$ 6.4		5.37 $\pm$ 4.26		0.588 $\pm$ 0.089		0.298 $\pm$ 0.047		0.193 $\pm$ 0.031		0.053 $\pm$ 0.027	
	149 $\pm$ 42		54.7 $\pm$ 74.6		1.34 $\pm$ 1.87		0.264 $\pm$ 0.267		0.790 $\pm$ 0.750		0.028 $\pm$ 0.021	
	0.033 $\pm$ 0.022		0.116 $\pm$ 0.062		0.136 $\pm$ 0.071		0.031 $\pm$ 0.041		0.050 $\pm$ 0.018		0.022 $\pm$ 0.006	
	2.8 $\pm$ 0.15		9.0 $\pm$ 4.75		26.7 $\pm$ 16.4		24.6 $\pm$ 7.0		26.3 $\pm$ 10.3		17.0 $\pm$ 12.0	

*Mean and standard deviation from 3 rats

Table XI. Distribution of ^{14}C -Direct Red 2 in Tissues at Several Intervals*

Tissue	μg -Equivalents of ^{14}C -DiMeBzd in Tissue at Sampling Interval Indicated											
	2 hr		4 hr		8 hr		12 hr		24 hr		72 hr	
Adipose	0.157 $\pm$ 0.016		0.112 $\pm$ 0.010		0.661 $\pm$ 0.106		1.43 $\pm$ 0.54		0.122 $\pm$ 0.036		0.048 $\pm$ 0.025	
Blood	0.121 $\pm$ 0.063		0.284 $\pm$ 0.090		2.01 $\pm$ 0.36		4.50 $\pm$ 1.44		0.437 $\pm$ 0.154		0.108 $\pm$ 0.053	
Brain	0.045 $\pm$ 0.023		0.066 $\pm$ 0.033		0.688 $\pm$ 0.147		1.40 $\pm$ 0.40		0.067 $\pm$ 0.033		<0.002 $\pm$ 0.001	
Carcass	0.136 $\pm$ 0.013		0.444 $\pm$ 0.415		1.34 $\pm$ 0.37		3.02 $\pm$ 0.90		0.388 $\pm$ 0.027		0.104 $\pm$ 0.051	
Feces	0.006 $\pm$ 0.008		0.048 $\pm$ 0.046		0.581 $\pm$ 0.540		82.2 $\pm$ 140		408 $\pm$ 15		169 $\pm$ 35	
Heart	0.143 $\pm$ 0.057		0.264 $\pm$ 0.092		3.96 $\pm$ 2.07		5.00 $\pm$ 0.96		0.386 $\pm$ 0.178		0.066 $\pm$ 0.032	
Kidney	0.231 $\pm$ 0.087		0.563 $\pm$ 0.128		2.91 $\pm$ 0.59		5.39 $\pm$ 1.29		1.08 $\pm$ 0.24		0.459 $\pm$ 0.160	
Large Intestine and Contents	13.6 $\pm$ 4.8		12.3 $\pm$ 6.2		32.2 $\pm$ 9.0		24.2 $\pm$ 6.2		8.30 $\pm$ 1.80		0.462 $\pm$ 0.394	
Liver	265 $\pm$ 149		338 $\pm$ 55		562 $\pm$ 25		447 $\pm$ 117		81.1 $\pm$ 16.5		6.20 $\pm$ 3.89	
Lung	0.963 $\pm$ 0.429		1.85 $\pm$ 0.42		8.60 $\pm$ 1.33		15.9 $\pm$ 5.8		8.50 $\pm$ 2.53		3.64 $\pm$ 1.04	
Muscle	1.43 $\pm$ 0.35		2.38 $\pm$ 0.90		6.39 $\pm$ 1.32		11.7 $\pm$ 1.5		8.35 $\pm$ 1.54		2.08 $\pm$ 0.79	
Spleen	0.130 $\pm$ 0.074		0.328 $\pm$ 0.175		1.76 $\pm$ 0.54		3.58 $\pm$ 1.03		0.234 $\pm$ 0.072		0.030 $\pm$ 0.011	
Small Intestine and Contents	0.153 $\pm$ 0.056		0.342 $\pm$ 0.121		1.68 $\pm$ 0.36		3.63 $\pm$ 1.26		0.403 $\pm$ 0.111		0.135 $\pm$ 0.041	
Urinary Bladder	7.63 $\pm$ 4.62		17.1 $\pm$ 3.6		6.89 $\pm$ 2.37		8.43 $\pm$ 2.65		1.78 $\pm$ 0.67		0.208 $\pm$ 0.104	
Stomach and Contents	657 $\pm$ 496		559 $\pm$ 332		79.7 $\pm$ 29.1		77.4 $\pm$ 39.5		32.2 $\pm$ 6.0		2.26 $\pm$ 2.15	
Testes	2.14 $\pm$ 2.09		3.37 $\pm$ 2.93		15.5 $\pm$ 0.5		18.1 $\pm$ 9.7		12.4 $\pm$ 11.6		0.560 $\pm$ 0.364	
Urine	13.6 $\pm$ 15.8		45.8 $\pm$ 40.9		2.90 $\pm$ 1.05		4.29 $\pm$ 1.52		1.05 $\pm$ 0.72		0.121 $\pm$ 0.072	
	454 $\pm$ 448		781 $\pm$ 594		5.7 $\pm$ 4.9		2.17 $\pm$ 0.86		1.66 $\pm$ 1.23		0.310 $\pm$ 0.481	
	0.049 $\pm$ 0.037		0.185 $\pm$ 0.033		1.11 $\pm$ 0.17		2.44 $\pm$ 0.55		0.323 $\pm$ 0.135		0.049 $\pm$ 0.010	
	**		6.8 $\pm$ 10.8		33.0 $\pm$ 3.2		42.6 $\pm$ 20.2		73.0 $\pm$ 29.0		34.4 $\pm$ 12.9	

*Mean and standard deviation from 3 rats

**None excreted.

Table XII. Distribution of ¹⁴C-DiMxBzd and ¹⁴C-DiMeBzd in Tissue Samples at 72 Hours*

Sample	DiMxBzd μg Equiv/g	DiMeBzd μg Equiv/g
Adipose	<0.014 ± 0.000	<0.014 ± 0.000
Blood	0.027 ± 0.002	0.039 ± 0.011
Brain	0.006 ± 0.001	0.004 ± 0.002
Carcass	0.015 ± 0.003	0.026 ± 0.011
Feces	22.6 ± 1.6	26.8 ± 2.5
Heart	0.036 ± 0.011	0.021 ± 0.006
Kidney	0.127 ± 0.029	0.130 ± 0.032
Large Intestine	0.034 ± 0.006	0.058 ± 0.019
and Contents	0.302 ± 0.134	0.551 ± 0.219
Liver	0.568 ± 0.065	0.907 ± 0.252
Lung	0.049 ± 0.006	0.459 ± 0.063
Muscle	0.008 ± 0.002	0.009 ± 0.003
Spleen	0.030 ± 0.006	0.036 ± 0.010
Small Intestine	0.035 ± 0.003	0.101 ± 0.054
and Contents	0.395 ± 0.169	1.13 ± 0.70
Urinary Bladder	0.147 ± 0.029	0.048 ± 0.016
Stomach	0.061 ± 0.056	0.145 ± 0.099
and Contents	0.104 ± 0.080	0.768 ± 0.665
Testes	0.023 ± 0.006	0.019 ± 0.008
Urine	4.85 ± 0.66	6.61 ± 0.82

*Mean and standard deviation from 3 rats

highest accumulation of ¹⁴C from rats dosed with DiMeBzd was in the liver, urinary bladder, and kidney. The highest accumulated levels of ¹⁴C in rats dosed with DiMeBzd occurred in the liver, lung, stomach, and kidney. The levels of ¹⁴C found in animals dosed with DiMxBzd did not differ appreciably from those dosed with DiMeBzd. The levels of ¹⁴C at 72 hours in tissues from rats dosed with amines were generally lower than comparable tissues from rats dosed with dyes; however, the general distribution of ¹⁴C in rats dosed with both amines and dyes was similar. It should be noted that both dyes significantly accumulate in the liver, a target organ for rats dosed with benzidine (24) and the site of tumors in rats dosed with benzidine-based azo dyes (2).

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION IV - EXPERIMENTAL STUDIES
Chairman: Dr. Herman F. Kraybill
National Cancer Institute

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. KRAYBILL: Thank you, Dr. Lowry. I think, as we have done before, we will have the four speakers please come up front to the table over here where we can direct questions to you; and if the fish pathologists want to get to debating or arguing among themselves, why, that might be fine for the rest of us to listen in because there is a lot of debate in that area.

Dr. Weisburger has the first question.

DR. WEISBURGER: I would like to ask Dr. Lowry whether he really thinks that those dyes are extensively metabolized? I like the emphasis you put on that and I just question that.

DR. LOWRY: Well, if you find non-colored urine that contains a great deal of radioactivity, like 80 or 90 percent of radioactivity that's dosed, what other conclusions can you come to?

DR. WEISBURGER: But you identify only, say, a small percentage of the metabolites.

DR. LOWRY: That's true. I did not say extensively metabolized.

DR. WEISBURGER: Oh! All right. I guess I missed that subtle difference.

DR. BRESNICK: Bresnick, at Eppley. Two questions to two of the fish men, Dr. Couch and Dr. Hawkins. First of all, parenthetically I might mention that I see nothing funny about dealing with 3-centimeter fish. I spent 7 years of my life catching them.

DR. BRESNICK: The question I have is: Dr. Couch, you seem to have cast your lot, in terms of activation, upon the P450 mixed function oxygenases and what I would ask is, is there a flavin oxygenase, the Ziegler enzyme, in your fish and is there a prostaglandin synthesizing system so that you could have an arachidonic acid-dependent co-oxygenation pathway?

DR. COUCH: I can't answer your question. I am not a biochemist. I am a pathologist. But Dr. Schoor at our laboratory, who did most of the biochemistry, could answer it.

There are P450 systems and P449 systems in the fish. The prostaglandin question -- I don't think anyone has looked into it with the fish.

DR. BRESNICK: That might be worthwhile doing.

DR. COUCH: Right.

DR. BRESNICK: The question I have for Dr. Hawkins, just again as a nonfish person interested in carcinogenesis, when I was a graduate student I was always taught that you want to try to keep your experiments clean in terms of animals. Yet, here you have a batch of creatures that you are putting together in a medium, they're taking in the substance you're proffering them, they are excreting metabolites which may be more and more active or more

proximal, and those metabolites are now going into the neighbors that are also in there. Does that pose any sort of problem?

DR. HAWKINS: Well, with the studies that I was talking about, it was only a 2-hour exposure. The studies that we will be doing later will involve mainly flow-through systems to compounds that are less carcinogenic than MAM or MNNG, and over the course of a day the water will completely change about five to seven times.

DR. BRESNICK: So, practically, it's not a problem?

DR. HAWKINS: If it is, I don't know how to get around it.

DR. LONGFELLOW: Longfellow, NCI. I'll address my question to John and perhaps some of the "fish" people can kick in as well.

I am wondering whether or not you can learn something from the types of fish from the various areas where you've found tumors, as to the type of environmental behavior they have. Some of these fish are bottom feeders, some of them are free swimmers, some of them tend to have different habitats within the areas that they live.

I am wondering, from that can we postulate as to the possible origin of the carcinogen that is responsible? In some cases, you were noting that in fact you had a pretty good clue as to the types of chemicals they were exposed to that one might expect are, or already knows are, carcinogenic.

And then a second question, for the rest of you that are working in the laboratory: which is coming first, the tumor or the fish? Are you selecting fish because they're 1 or 2 inches long and they work well in a tank? Or, are you selecting fish that are already known to be susceptible to certain types of tumors and therefore try to find one that's adaptable to go into a tank?

DR. HARSHBARGER: Bottom feeding fish get most of the presumed pollution-related tumors (e.g., flatfish, tomcod, croaker and cunner in the marine environment; catfish, drum, sucker and sauger in the freshwater environment). Where fish tumors are prevalent, pollution is usually severe. However, surveys probably understate tumor prevalences because fish compromised by cancer are more likely to be consumed by predators.

I think wild fish get most of their carcinogens through the food chain rather than absorbing them through the skin because: (1) most suspect carcinogens are water-insoluble and do not maintain high concentrations in the water column except under turbid conditions; (2) flow-through experimental studies by John Couch using realistic environmental levels did not induce tumors in Sheepshead minnows, whereas much higher levels did, and; (3) the invertebrate benthic fauna that bottom feeders eat accumulate enormous tissue levels of PAHs and other chemicals. However, we need more data on this.

Pollution can also alter the mix of species. Data from surveys of the Ohio River reputedly show that some species in abundance today were not abundant years ago, whereas some species that were abundant years ago are no longer found or are found in smaller numbers. These changes suggest a

relative influence of accumulated chemicals from agriculture, industry, mining and transportation on the health, fecundity and competitive behavior of the various species.

DR. COUCH: Let me say a couple of things about why we select certain species to work on in our experimental systems.

When we started our program we were interested in coastal species of fish because eventually we hope to be able to use them in the field and in the laboratory to screen and test pollutants that might be generated in coastal locales -- harbors, bays, waterways.

We were interested also in fresh water fishes. The Rainbow trout had been worked on (that is, the Shasta strain of Rainbow trout at Oregon State) for many years and had been shown to be sensitive as a laboratory fish to a variety of different carcinogens.

I would say that we are still pioneering in this area with other species. We are doing what was done many years ago with rodents in one sense; that is, we are looking at the potential to respond to carcinogenesis as a phenomenon by a spectrum of fishes. These fishes are all genetically heterogeneous -- that is, they are not cloned or selected or genetically inbred to provide an enhanced susceptibility to carcinogenesis. We do have those techniques with fish and that is being worked on to produce inherently more sensitive fish for test systems. But I think, in terms of environmental testing and ecologic monitoring for these tumors or neoplasms, they are without a doubt found in fish with a heterogenetic background, or wild fish so to speak.

Controls in these experiments usually will have zero incidence of tumors, so it is very significant, striking, and dramatic evidence of the risk or the carcinogenicity of the agent you're testing when significant tumor incidences are induced in the experimentals.

While we want to develop laboratory fish assay systems, we also want to use fishes as environmental monitors and screening tools, as well as models for other organisms and other species. As we go into using fish more as models, experimental models in the laboratory, then we definitely need to get more and more into the question of sensitivity, relative sensitivity based on genetic background of the organism.

DR. HAWKINS: Just to say a little bit more about that, the choice of the species for laboratory studies depends on a number of different things, one of which is the carcinogenic response, but it also has to do with ease of breeding, relative hardiness to different environmental conditions.

One aspect that is sort of ironic is that a fish like the Medaka would probably not be a good specimen to look at the progression of carcinogenesis because it takes place so rapidly and there is a superimposition of different kinds of tumors within the fish. So, if we want to look at the progression of hepatic tumors, by six or eight months we've got tumors of other origins that are hard to distinguish from the ones that may be from the liver.

DR. COUCH: In that regard, I think the Rainbow trout and Cyprinodon, the Sheepshead minnow, are fish in which tumor progression, at least in the liver, occurs more slowly and one may study tumor progression more discretely. That is something we are involved in right now, attempting to abbreviate time to the end point in the assays by inductively going backward from the tumor (the general) to the earliest altered foci (specific steps), which is being done with mammals.

DR. HARSHBARGER: And lest there be some misunderstanding of what was said, I do not know of any species or strain of fish, no matter how inbred, that has a natural base of tumor incidence. It is all or none, even in the inbred ones. However, certain hybrid crosses do have a background tumor rate.

DR. KRAYBILL: Another question?

DR. JOHNSON: Johnson, from NIOSH. I would like to begin by complimenting each of the four presenters for the quality of their presentations.

Secondly, I have a question of Dr. Couch. You alluded to some work that pertained to the identification of teratogens. Could you expand on that a little bit, please?

DR. COUCH: After this program began over five or six years ago at Gulf Breeze, we had a person (Dr. Davis) join our staff who is interested in using fish as models for teratogenesis studies and about two and a half or three years ago Dr. Davis began to look at dibutylphthalates as possible environmental teratogens and he used the fish, Rivulus marmoratus, which I mentioned earlier.

Rivulus is a small fish that can be cultured individually in little containers in the laboratory very neatly. It requires a minimum of maintenance and attention and no aeration. So, it is a good animal to expose individually and in large numbers -- you can work with 400 to 500 fish in an experiment if you wish. It is a self-fertilizing hermaphrodite, so that you have sort of a genetic pedigree from the animal to begin with, and you can dose the parents or the adults and then follow the development of individual young from time of egg laying through maturity.

This fish has a lot of potential use in teratogenic studies. We are monitoring this fish for rates of bone and vertebral anomalies, somatic anomalies reflecting teratogenic effects in offspring of adults exposed to dibutylphthalates.

The kinds of effects that we've found most frequently from dibutylphthalates have been vertebral anomalies. We grade the fish by severity of expression, which distinguishes exposed from nonexposed control fish. Interpretation of results is a little more complicated than even in the carcinogenesis problem.

DR. KRAYBILL: The young Rivulus, I think, John, is the one that you can put sections of the whole fish on slides. Can you do that with a mouse or rat?

DR. COUCH: You could do it with mice, I suppose. It would be a much more difficult job.

DR. KRAYBILL: But you can actually see serial sections of the whole fish right there on slides. Dr. Weisburger?

DR. WEISBURGER: I was wondering, does Dr. Harshbarger know what those organics are in Torch Lake? I mean, we've heard so much about the mining dumps being disposed of there, but how about the organics. Any clues?

DR. HARSHBARGER: I learned of the organics recently when Florence Gregoric (Monograph Editor for the Journal of the National Cancer Institute) sent me a clipping from the December 8, 1983 Copper County Sentinel which stated that the Calumet & Hecla Mining Company dumped an estimated 150,000 gallons of waste chemicals into Torch Lake on a daily basis for decades. .pa Little was said about what those chemicals were, but that is something that ought to be checked out.

DR. KRAYBILL: I think that's a good question because those people up in Western Michigan or Michigan Tech are talking about copper and inorganics and I think they'd better be looking at the organics.

DR. STALLARD: I am Dr. Stallard. I used to be medical director for that company, which is Calumet & Hecla.

Torch Lake is a place where stamp sands were dumped for dozens of years and later on a reclamation plant was built at Lake Linden, which is where Torch Lake is, and the organics probably come from the flotation agents, which are pine oils and other organics of complex nature. It was not a problem that I was concerned with at the time but, yes, there would be significant organics in those settings.

DR. KRAYBILL: Dr. Longfellow?

DR. LONGFELLOW: If you use the fish for a model, do you have any feeling for the best route of administration to go with? I mean, Ed was referring earlier to actually incubating the fish.

Alternatively, I know Ron Sonstegaard, in Canada, works with Teleost fish and is injecting picogram amounts directly into the eggs and then watching those develop. And there are other possibilities.

Obviously, a lot of these are probably getting their exposure from feeding. What about, for instance, feeding up a batch of brine shrimp in a small volume with the chemical that you want and then tossing that in as the food source, or various other permutations on this theme. A lot of the pollutants are lipophilic organics, so you might be able to come up with something where you're not exposing the laboratory to a lot of clean-up problems.

DR. COUCH: Dr. Kimura probably could address that better than any of us here. He has been working on dose response in fish in Japan for some time.

With a lot of the fish research, we have been working with harsh, potent, known carcinogens. You saw the list of those presented. These have usually been used in fairly high doses relative to what might be found in any environmental samples. The reason for this, of course, was to see if there was potential for response, period, in the species tested.

At Gulf Breeze, when we started this project, I went out on a limb a little bit. Being an environmental scientist with an environmental agency, I was more concerned with environmentally realistic exposure concentrations to the animals. We already had, even at that time, a pretty good idea that certain fishes would respond positively to carcinogens and develop neoplasms.

But the question that we were interested in more, and I hope NCI is still interested in, is what are the relative environmental doses and what do they mean to the organism in terms of exposure from fish to man.

So, we did exposure with some of the agents in the low parts per billion range and we got some interesting lesions, but we didn't get very many neoplasms or the selected agents active enough. It could have been that our exposures were not long enough. But with the PAHs we exposed oysters for 19 to 24 months in the 10 parts per billion range, for example. You get some interesting lesions that are difficult to interpret, but we got very few bona fide neoplasms, that is, indisputable neoplasms at these low concentrations.

So that is a critical question right now, I think, in terms of the use of fish as assay organisms. How will they prove in terms of threshold and dose response in a broad spectrum? We are going to have to address that, those of us who are working experimentally with fish.

However, I think it is an agent-related thing, too. Aflatoxins in a Rainbow trout will produce tumors at very low concentrations (low parts per billion range), and with a very short exposure period to embryos (a matter of hours).

But not all fish may react like the Shasta strain of the Rainbow trout to carcinogens. So, this is one reason we are looking at a broad spectrum of fishes for inherent sensitivity.

I think, when we are dealing in the dose of 10 to 25 to 50 parts per million range of an overt, harsh carcinogen, with any of these fish we are going to get probable tumors and certainly lesions. But the question still remains: what happens when you go down to the parts per billion, and parts per trillion range, in terms of realistic dose response in the animals?

DR. HAWKINS: I think feeding studies can be a little bit messier than studies in which you dissolve the toxicant in the fishes' habitat water. In our future studies we'll be using our exposure box and we'll be exposing these fish to mixtures of drinking water contaminants, one concentration of which will be the level of the contaminants that are found in municipal waters. That will probably be the lowest concentration. The highest level will be the maximum that they can tolerate over the period of time of the test.

DR. COUCH: The exposure period can be lengthened significantly with fish.

DR. KRAYBILL: We are up to about 50 carcinogens in the drinking water and about 68 mutagens and 18 promoters, so it's not shocking. Chloroform, of course, we've identified years ago. It's in the IARC Monograph as a carcinogen, but by gavage in the rodent in high doses.

But let me ask you all here a question, and I said this to Dr. Overstreet one day: what will happen, or what are we going to do if we get a response to these critters down at the low doses that John is talking about here at the levels in the drinking water?

DR. COUCH: You've got to keep in mind, Herman, that one advantage a fish offers is that you can put them in the water and leave them there as long as you want at these low dose sublethal concentrations and increase your exposure time significantly to environmentally realistic concentrations. That may be an advantage with the fish.

DR. KRAYBILL: But to the lay public who see this, what will they say when we're getting up -- the highest, I guess, is chloroform at 500 micrograms per liter in the city water of Miami -- if you're coming through with a response at 50 to 100 micrograms.

Well, maybe we won't get that. Maybe I'm borrowing trouble. But that's a practical question. Of course, that's what the project's oriented toward.

But anyway, as I said earlier, we didn't put all the eggs in one basket. If they're promoters, that's another story. So, what Bill Hawkins is doing here using MAM plus these contaminants in the drinking water may give us some very interesting findings also.

DR. KRAYBILL: Thank you very much. We will meet again tomorrow morning.

(The Workshop adjourned at 4:42 p.m., Thursday, March 22, 1984.)

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Friday Morning, March 23

SESSION V - EPIDEMIOLOGY
Chairman: Dr. Philip J. Landrigan
National Institute for Occupational Safety and Health

SESSION V - EPIDEMIOLOGY
Chairman: Dr. Philip J. Landrigan, NIOSH

The workshop was reconvened, pursuant to notice, at 8:30 a.m., Dr. Philip J. Landrigan, NIOSH, presiding.

PROCEEDINGS OF THE
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PRESENTATION:

Status of the Kansas Case-Control Study of
Soft-Tissue Sarcoma and Lymphoma

Shelia K. Hoar, Sc.D.
Aaron Blair, Ph.D.

National Cancer Institute

THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND OCCUPATIONAL CANCER STUDIES

March 22-23, 1984

Status of the Kansas Case-Control Study of
Soft-Tissue Sarcoma and Lymphoma

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I am here today to tell you about a case-control study of soft-tissue sarcoma (STS), non-Hodgkin's lymphoma (NHL), and Hodgkin's disease (HD) that is being funded through the NCI/NIOSH Interagency Agreement. Working with me on the study are Dr. Aaron Blair from NCI and Dr. Fred Holmes and Cathy Boysen from University of Kansas. As you are probably well aware, this project was begun in response to 1) reports that certain herbicides and herbicide-contaminants were associated with teratogenesis, mutagenesis, and carcinogenesis in animal and bacterial experiments (Young AL et al., 1978) and 2) the epidemiologic findings from Sweden that persons exposed to herbicides may be at excess risk of cancer. First came the

retrospective cohort study of railroad workers exposed to amitrole and the phenoxyacetic acids, 2,4-D and 2,4,5-T, that showed elevated rates of cancer incidence and mortality (Axelson O and Sundell L, 1974; Axelson O et al., 1980). Then came the well known case-control studies by Hardell and his collaborators revealed associations between exposure to phenoxyacetic acids and chlorophenols and STS, NHL, and HD (Hardell L and Sandstrom A, 1979; Eriksson M et al., 1981; Hardell L et al., 1981). For all three cancers, risks were increased five- to six- fold, regardless of whether exposures were contaminated by polychlorinated dibenzodioxins and dibenzofurans.

In epidemiology, a 5- to 6- fold increase in risk is a remarkable finding, and is cause for great concern. We knew there is widespread potential for exposure to phenoxyacetic acids and chlorophenols. In addition to herbicide formulations these chemicals appear in blue stain retardants used in sawmills, slime control preparations in paper and pulp manufacturing, cutting oils and fluids, wood preservatives, waterproofing agents for leather and textiles, and in medications. If the reported epidemiologic associations were causal and as strong as suggested by the Swedish research, these agents could be responsible for a large proportion of the cases of STS, NHL, and HD in the United States.

At NCI we considered various approaches to further investigate these possible relationships. Some cohort studies of pesticide applicators are underway; Dr. Blair recently reported a duration-related association between lung cancer and employment as an applicator (Blair A et al., 1983). But these cohorts are far too small to

enable us to study any disease as rare as STS. We decided to conduct a case-control study of STS, NHL, and HD giving particular attention to herbicide exposure.

Herbicides are often used in forestry work and in agricultural applications. The effects of forestry exposures were being investigated in the Pacific Northwest, so we concentrated our efforts on agricultural uses. The herbicides of interest are used during the cultivation of several different crops, for example, wheat, corn, sorghum, soybeans, and alfalfa and to control pastureland. But the herbicides are often accompanied by extremely heavy use of insecticides. Study results could possibly be confounded. However, relative to other crops, wheat requires a large amount of herbicides while use of insecticides and fungicides is considerably less.

Kansas, a major wheat-producing state, was chosen as the site for our study. 2,4-D has been the most commonly used herbicide in Kansas although substantial amounts of 2,4,5-T have also been used, as well as a myriad of other chemicals. A 1981 report from the Ohio Agricultural Research and Development Center entitled Pesticide Use on Major Crops in the North Central Region in 1978 stated that 5,168,000 pounds of 2,4-D and 1,256,000 pounds of 2,4,5-T were used in Kansas (Waldron AC and Park EL, 1981). 2,4,5-T use was about 1/4 the 2,4-D use. These amounts were far greater than the amounts used in any of the other North Central states.

Kansas was an attractive state for a case-control study for another

reason as well as its heavy herbicide use: it has a population-based cancer registry. The registry could provide incident cancer cases helping us avoid a death certificate-based study. This was essential because of the extremely low accuracy with which sarcomas and lymphomas appear on death certificates. NCI's Biometry Branch published a paper showing that only around 50% of STS cases who die have STS on their death certificates and only around 50% of persons with STS on their death certificates also have a diagnosis of STS in their hospital records (Percy C et al., 1981).

So, our case group consists of white men, aged 21 and older, diagnosed with STS, NHL, or HD, while residing in Kansas. During 1976-1982, there were 200 men with STS and 173 men with HD, and we selected a sample of 200 men from those diagnosed with NHL during 1979-1981. Late in the study, we learned that one of the NHL cases was ineligible and had to be eliminated. It was too late to replace him, so the final number of the NHL cases is 199.

The controls are white men from the general population of Kansas. Three controls were matched to each case on age ($\pm$ 2 years) and vital status. For cases who are currently alive, controls aged 65 years or older were selected from the Health Care Financing Administration file (the Medicare file); whereas, controls aged 64 years or younger could be selected by telephone using a two-staged random digit dialing technique (Waksberg J, 1978). For cases who have died, the controls were selected from Kansas state mortality files with the additional matching factor of year of death. Persons whose cause of death is STS, NHL, HD, a malignancy of an ill-defined

site (ICDA code 195), homicide, or suicide were excluded. The next-of-kin were interviewed for deceased subjects. One-half of the STS and NHL cases died before the initiation of the study while only 1/3 of the HD cases have died.

As I mentioned, the goal is to have 3 suitably matched controls for each case, however the same controls can be used for comparisons with the three different case series, that is the sarcomas, NHL, and HD cases. Multiple use of the controls meant we needed to select only 1005 controls instead of approximately 1700 that would have been required otherwise. We also selected 70 men diagnosed with colon cancer in 1981 as a "cancer" control series to evaluate possible recall bias among cancer patients, mimicking Hardell and his collaborators.

Pathology specimens have been obtained and are being reviewed for confirmation of the diagnosis of STS, NHL, or HD. This is important for 2 reasons: 1) diagnosing these conditions accurately is difficult and 2) the original pathologists used a wide variety of schemes for categorizing the tumors into subgroups. We need consistent diagnoses and subgroup terminology. The subgroups may be important with respect to etiology. Some subgroups may be associated with herbicide use; others may not be. We are using the WHO's International Classification of Disease for Oncology (1976) codes for STS, HD, and colon cancer cases and the NCI "Working Formulation" for NHL (NHL Pathologic Classification Project, 1982).

So far, we have had about 60% of the specimens reviewed and are

continuing with this effort. I want to point out the large number of STS cases, 20%, that were determined not to be STS by the review panel of pathologists (Table 1). This finding emphasizes the need for pathological confirmation in any study of STS. The pathologists have judged that some of the cases belong, in fact, to one of the other case series in the study. Most often, this involved determining that a HD case was actually a NHL case. A few subjects were found to have malignancies that are not eligible for the study, for example multiple myeloma. Several subjects' tumors were considered benign- a STS case was rediagnosed as nodular fasciitis. If the number of these misclassified subjects turns out to be large enough, we may be able to evaluate observation and recall bias in these people with conditions supposedly unrelated to herbicide use. But hopefully this won't occur or we will have problems with too small sample size. Currently 11% of the cases reviewed have ineligible malignancies or benign conditions.

The cases and controls, or their next-of-kin, are being interviewed by telephone concerning data and place of birth, marital status, education, religion, ethnicity, height, weight, farming practices, non-farming occupations, military service, pesticides used at home or in the yard, consumption of tobacco, coffee, alcohol, and raw milk products, present and past medical conditions, medical treatments, trauma, familial cancer, and the number and health of offspring.

Our questions on farming practices included the calendar years working or living on farmland that grew any of 4 specific crops

(wheat, corn, sorghum, or pasture), the farm names, locations, and sizes, herbicides used, years and acres treated, names and locations of companies where herbicides were purchased, method of application, days/year exposed, use of protective equipment, etc. Analogous questions were asked with respect to insecticide use on crops and animals, farm service occupations such as aerial applicators, and home or garden pesticide use. We have just completed the interviewing phase of the study. Our overall response rate is 93%, a testimony to the excellent effort made by the staff in Kansas (Table 2).

A key component of our study is our plan to obtain corroborative evidence of the self-reported herbicide exposure. Using information from the questionnaires we will contact the farm suppliers and ask about the crops grown and the herbicide and insecticide purchases for about 120 of the study subjects. The person asking the questions of the supplier will not know the chemicals, if any, reported by the subjects. An interesting feature is that we will also attempt to verify that farmers who reported insecticide use but not herbicide use did not, in fact, purchase any herbicides from their insecticide supplier. There are limitations to this system of which we are well aware, for example, the farmer could easily have used more suppliers than reported in the interview. But in the extremely rural areas the problem will be minimal. And we can certainly learn something about the accuracy of self-reported data if the supplier does report herbicide purchases. This important verification step will allow us to crudely evaluate potential observation bias, a criticism of the Swedish studies (Cole P, 1980).

We developed a questionnaire for the suppliers, gained the cooperation of the head of the Extension Service in Kansas, conducted a small pilot study of our procedures, and conducted the main survey. We had excellent cooperation from the suppliers. Dr. Robert Robel from Kansas State University conducted interviews with 107 of 128 suppliers. The remaining suppliers either could not be located, had gone out of business, or, in one instance, was under indictment for fraud.

At this time, only the data from the first 20 corroborative surveys in the pilot study are available. Comparisons between the data from the 20 suppliers and the 20 corresponding subject interviews are, quite frankly, not very encouraging. The concordance in crops grown and use of pesticides overall is poor, not much better than 50-60% agreement. However, that number will improve when we restrict the data to the study subjects' experiences as an adult. The subject interview records information on childhood and adult life, while the supplier may only be familiar with the adult experience. When the data are restricted to a common time period, the concordance should increase. Also, there will be improvement when we consider whether the pesticides were applied by custom/commercial applicators. The suppliers will not be able to corroborate such exposures. The degree of concordance may give us an indication of how vigorously we should interpret the results of the study.

As a final summary of our status, all interviewing and verification activities are complete. We are finishing entering and editing the

the data on the computer. We are developing analytic techniques, for example, creating indices of herbicide exposure, and hope to rapidly analyze the data and report some results by the fall.

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P A T H O L O G Y

ST S : 104/200 R E V I E W E D
21/104 (20%) D I A G N O S E D N O T S T S

N H L : 122/199 R E V I E W E D
7/122 (6%) D I A G N O S E D N O T N H L

H D : 123/173 R E V I E W E D
13/123 (11%) D I A G N O S E D N O T H D

TABLE 1

	<u>I D E N T I F I E D</u>	<u>I N T E R V I E W E D</u>	<u>R E S P O N S E R A T E</u>
S T S	200	188	94%
N H L	199	190	95%
H D	173	158	91%
C O N T R O L S	1005	925	92%
C O L O N C A.	70	64	91%
T O T A L	1647	1525	93%

TABLE 2

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Epidemiological Study of Populations
Previously Exposed to Hexachlorobenzene (HCB)

Carl R. Morris
Environmental Protection Agency

Ayhan Gocmen
Hacettepe University
Ankara, Turkey

Henry A. Peters
Derek J. Cripps
George T. Bryan

University of Wisconsin
Madison, WI

EPIDEMIOLOGICAL STUDY OF POPULATIONS
PREVIOUSLY EXPOSED TO HEXACHLOROBENZENE (HCB)

Carl R. Morris^a, Ayhan Gocmen^b, Henry A. Peters^c, Derek J. Cripps^c, and George T. Bryan^c. ^aU.S. Environmental Protection Agency, Washington, D.C., ^bHacettepe University, Ankara, Turkey, and ^cUniversity of Wisconsin, Madison, Wisconsin.

A severe outbreak of porphyria occurred in Turkey between 1956 and 1958 during which time the probable relationship to the fungicide, hexachlorobenzene (HCB), became known. The fungicides from Western Europe, Chlorable and Surmesan A, containing 10% hexachlorobenzene, had been imported to control a wheat fungus and was added to some stores of seed wheat. This fungicide coated wheat was intended for planting and was distributed primarily in southeastern Turkey. During this time period, some of the seed wheat was used by some individuals in this population for bread or bulgar. It was estimated that more than 4,000 Turks who consumed this bread developed porphyria of varying severity.

Over the past 5 years, we have been conducting a study of some members of this affected Turkish population which was inadvertently exposed to the hexachlorobenzene (HCB) contaminated wheat over 25 years ago. Since this project was initiated in 1979, over 200 of the affected Turks have been identified and clinically examined with urine, stool, and blood assessment of porphyrins, HCB, and routine laboratory tests.

Clinical examinations and surveillance of these individuals is continuing. Many of the affected Turks continue to exhibit various manifestations of their initial chemically-induced disease. Clinical symptoms of their disease are still severe. A summary of the clinical symptoms of these affected individuals is presented in Figure 1. Dermatologic symptoms mostly attributable to the previous photosensitizing effect of porphyrins include hyperpigmentation (71%), severe scarring (86%), hirsutism (48%), pinched facies (42%), and fragile skin (38%). Short stature (45%) and small hands (68%) were observed as well as a painless arthritis (67%). Porphyric and neurologic symptoms include general weakness (66%), parasthesias (55%), cogwheeling (29%), sensory shading (64%), myotonia (50%), and enlarged liver (4%).

In addition to the dermatologic and orthopedic symptoms, a sizable number of these affected individuals demonstrated enlarged thyroids. Of the 132 males examined, 33 had enlarged thyroids (25%) as compared to the 72 females of which 60% (43 individuals) demonstrated enlarged thyroids.

The next few slides exemplify some of these clinical observations.

SLIDE 2 PLEASE.

This slide demonstrates the facial scarring observed on several of these affected individuals as well as the hirsutism. SLIDE 3 PLEASE.

This demonstrates the short fingers and the small hands. SLIDE 4 PLEASE.

In addition to the dermatologic and orthopedic symptoms, a high percentage of the females and males presented an enlarged thyroid. This example is one of our more extreme cases.

We are presently investigating the potential causative factors of these thyroid enlargements and hope to be able to present those findings at a future meeting of the Workshop.

In addition to the clinical observations on the affected individuals, we are also quantifying hexachlorobenzene levels in maternal milk, fat, and plasma in present survivors and children born since 1955. A total of 186 urine specimens and 170 stool specimens from affected Turks have been evaluated for porphyrins as of May 1983. Twenty-three subjects gave a vague history of having excreted red or brown urine in the previous two years, but in only three of these patients were porphyrins elevated.

The mean values are summarized in the next slide (SLIDE 5 PLEASE) with the range of values listed in parentheses, and compared with 37 control Turks and 40 U.S. controls.

Human milk samples have been taken from several of the affected individuals and found to contain increased levels of HCB. We have been able to obtain multiple specimens from two individuals. HCB was highest in mothers nursing for the first time, whereas after three or four babies had been nursed, HCB concentrations fell below the average suggesting that the toxic burden was decreased by means of lactation. WHO has established the maximum allowable limit of HCB in cows' milk at 0.02 ppm. Only 20 of 140 samples of human milk collected in Turkey have been 0.02 ppm or less. The "controls" which we collected were from the HCB contaminated area of Turkey and some are siblings of porphyric patients. Controls from other areas of Turkey are being collected and analyzed.

Thyroid function tests have also been conducted. Approximately 50% of the female patients with enlarged thyroids were found to have one or more abnormally low values of T3 or T4.

Time does not permit a complete presentation of all the various aspects of these studies, however, I would like to indicate that, in addition to the above clinical studies, experimental animal models are being utilized to correlate the clinical findings.

The children that were initially exposed to the chemical died under the age of one year from a condition known as Pembe Yara or pink sore. These children died with convulsions, weakness, and an unusual toxic erythema. Utilizing animal models, Pembe Yara-like symptoms have been observed in experimental studies in which HCB is fed mid-gestation (3 weeks) to pregnant rats. High concentrations of HCB in the maternal fat and liver as well as fetal tissues are also detectable. Our present studies suggest that these conditions are probably related to transplacental absorption of HCB and consumption of maternal milk.

A complete discription and discussion of these studies will be presented in the final report of the current research program which is due in May, 1984.

In addition to the studies cited above, the project has also included the conduct of a subchronic and chronic exposure study of HCB in weanling male and female Sprague-Dawley rats. Preliminary results of these studies are presented in the next few slides.

HCB was fed ad libitum to male and female weanling Sprague Dawley rats (N=94 per group) for up to 2 years at level of 0, 75, or 150 ppm. At weeks 0, 1, 2, 3, 4, 8, 16, 32, 48, and 64, four animals from each group were killed for histological examination by light microscopy. At 4 weeks hyperemia, congestion, swelling, and parenchymal degeneration was observed in the kidneys of exposed animals. At 32 weeks, severe parenchymal degeneration and epithelial necrosis accompanied by proximal convoluted tubular regeneration and preneoplastic foci were evident. At 64 weeks and beyond, renal adenoma and/or renal cell carcinoma were evident. Slide 6 presents a summary of those findings. (SLIDE 6 PLEASE).

In addition to these renal cell adenomas and renal cell carcinomas, liver hepatomas, hemangiomas, hepatocarcinomas, bile duct adenomas and bile duct carcinomas were found. A generalized leukemia involving thymus, spleen, liver and kidney were also observed in the HCB exposed animals. Slide 7 presents a summary of the hepatocellular effects. (SLIDE 7 PLEASE).

In addition to the histopathologic evaluations, fat samples were taken for HCB analysis. The results of these analyses are presented in the next two slides.
(SLIDE 8 PLEASE).

This slide depicts the levels of HCB in fat from exposed male rats. I believe these studies demonstrate our concern for the potential increase in body burden to this chlorinated hydrocarbon. (SLIDE 9 PLEASE).

This slide demonstrates the distribution of HCB to body fat in the non-pregnant female rat. Of particular interest is the 80-100 day lag period before there is a change in slope of the distribution curve. A great deal more attention needs to be focused on this phenomenon which may be a general observation that is applicable to any chemical which tends to accumulate in body fat.

In conclusion, these studies as well as others are of particular interest to EPA as we continue to assess impacts of HCB and other chlorinated hydrocarbons on human health and the environment. We believe that the clinical studies presented here warrant additional study with particular attention directed toward the families of the affected Turks who can serve as a control for this population study. Further followup study is necessary to delineate the causative factors of the thyroid enlargements of many of the affected Turks and to determine what the long range impact will be on this or other populations with respect to their potential to develop other adverse effects with time. We also

have a considerable interest in exploring methodology by which these body burdens may be reduced. We are currently interested in the potential correlations between these animal models and the human experience.

I would like to thank the Workshop Organizing Committee for inviting us to participate in this week's activities and would welcome any questions.

SLIDE 1

SUMMARY OF SYMPTOMS

Dermatologic

Hyperpigmentation	140/186	= 75.3%
Severe scarring	159/186	= 85.5%
Hirsutism	90/186	= 48.4%
Pinched facies	77/186	= 41.4%
Fragile skin	74/186	= 39.8%

Orthopedic

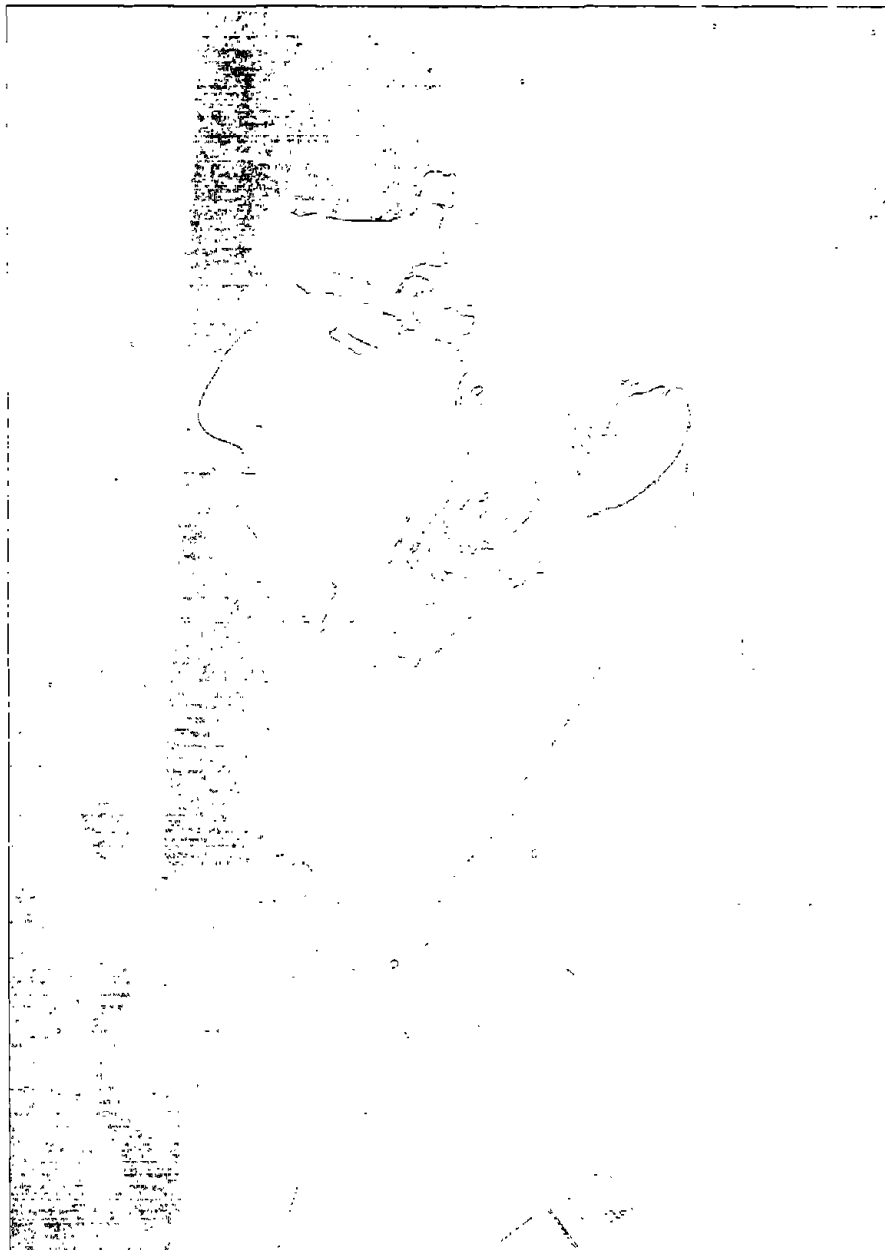
Short Stature	86/186	= 46.2%
Arthritis	125/186	= 67.2%
Small hands	123/186	= 66.1%

Porphyric and Neurologic

Weakness	132/186	= 70.9%
Paresthesias	104/186	= 55.9%
Cogwheeling	42/150	= 28.0%
Sensory shading	94/150	= 62.6%
Myotonia	48/101	= 47.5%
Recent Porphyrinuria	23/186	= 12.4%
Enlarged Liver	8/186	= 4.3%

Thyroid Enlargement

Enlarged Thyroid	71/186	= 38.2%
<u>Male</u>	28/116	= 24.1%
<u>Female</u>	43/70	= 61.4%







SLIDE 6

RENAL TUMORS IN RATS (R) CHRONICALLY EXPOSED TO
 HEXACHLOROBENZENE (HCB). R.W. Lambrecht, E. Erturk,
 E.E. Grunden, H.A. Peters, C.R. Morris, and G.T. Bryan.
 University of Wisconsin Center for Health Sciences, Madison, WI
 53792, and EPA, Washington, D.C. 20460.

Tumors	HCB Concentration in Diet					
	0 ppm		75 ppm		150 ppm	
	M	F	M	F	M	F
Renal Cell Adenoma	7/54	1/52	41/52	7/56	42/56	15/54
Renal Cell Carcinoma	0/54	1/52	0/52	2/56	0/56	2/54
Other	0/54	1/52	0/52	0/56	1/56	1/54

(Supported in part by NCI Grant CA 14520 and NCI/EPA Contract
 68-01-5002.)

Proc. Am. Assoc. Cancer Res.
 Vol. 24
 March 1983
 p. 59
 Abstract No. 233

SLIDE 7

HEPATOCARCINOGENICITY OF CHRONICALLY ADMINISTERED
 HEXACHLOROBENZENE IN RATS. R.W. Lambrecht*, E. Erturk,
E.E. Grunden*, H.A. Peters*, C.R. Morris*, and G.T. Bryan. Univer-
 sity of Wisconsin Center for Health Sciences, Madison, WI 53792.

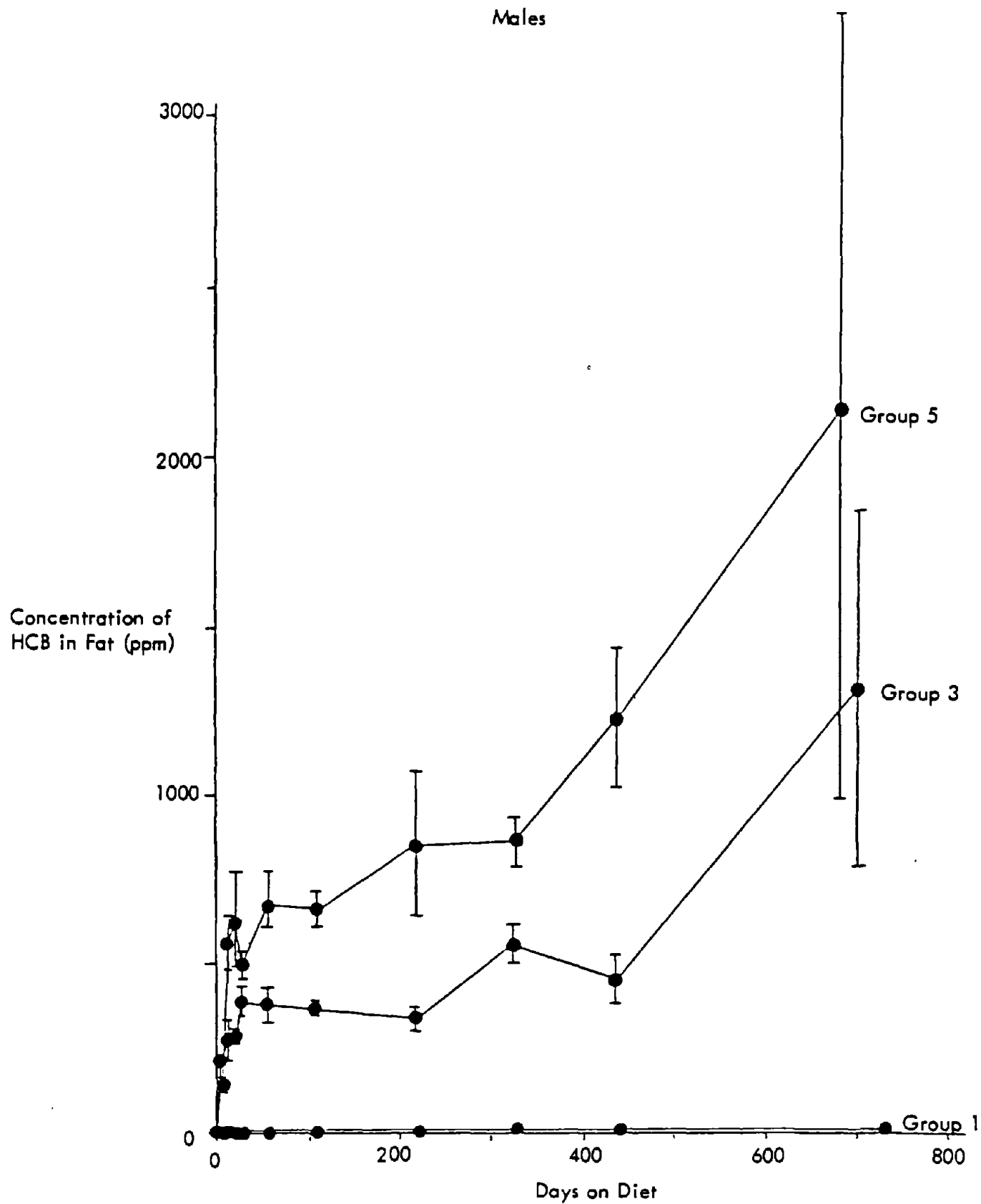
Lesion	0 ppm HCB		75 ppm HCB		150 ppm HCB	
	M	F	M	F	M	F
Hepatoma/hemangioma	0/54	0/52	10/52	23/56	11/56	35/55
Hepatocarcinoma	0/54	0/52	3/52	36/56	4/56	48/55
Bile duct adenoma or carcinoma	0/54	1/52	2/52	19/56	2/56	29/55

(Supported in part by NCI Grant CA 14520 and NCI/EPA Contract
 68-01-5002.)

Federation Proceedings
 Vol. 42, No. 4
 March 5, 1983
 p. 786
 Abstract No. 2889

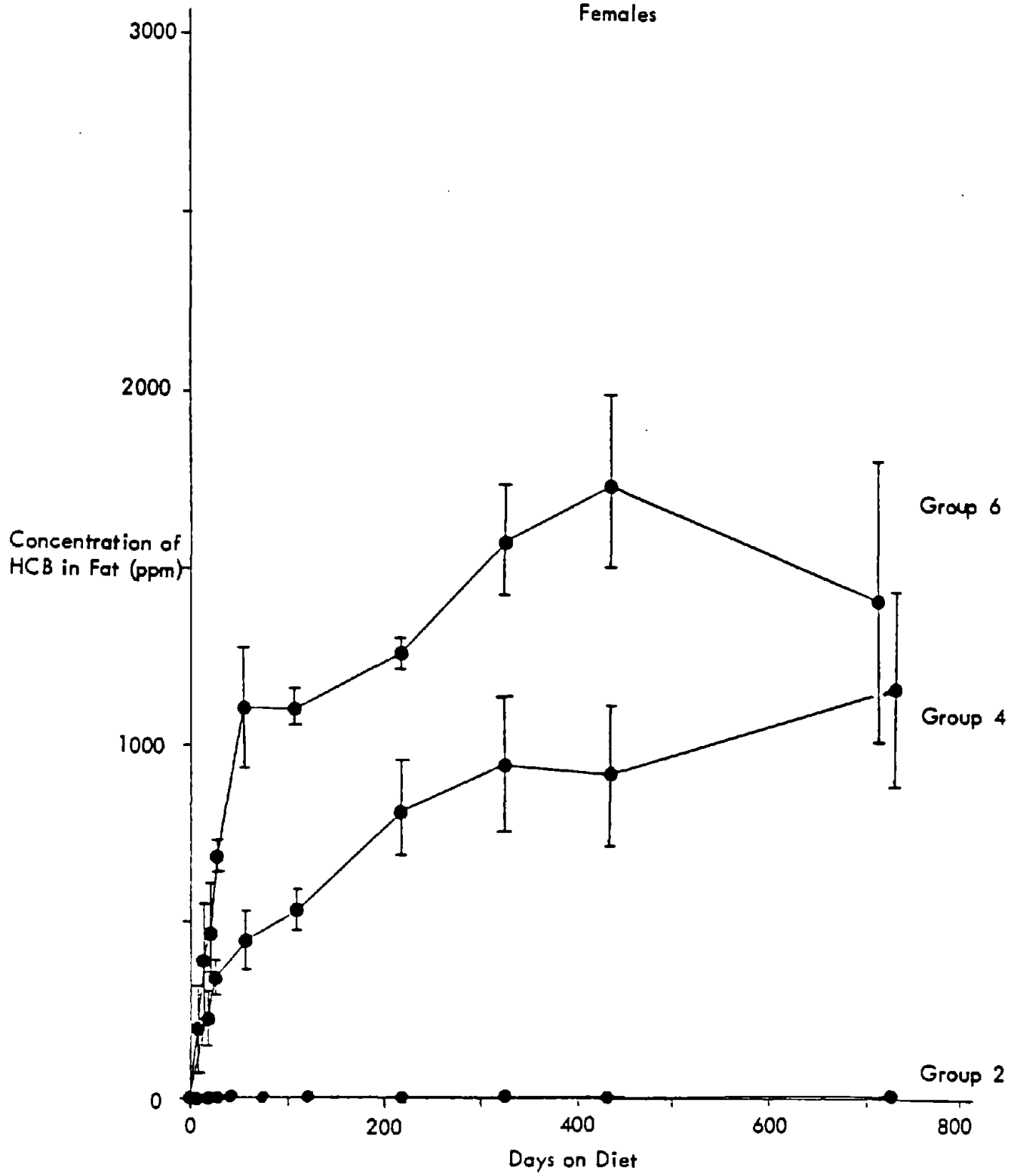
SLIDE 8

Males



SLIDE 9

Females



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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Possible Teratogenic and Carcinogenic Effects
of Pesticides on Human Health

Dr. Casey Jason
Environmental Protection Agency

Possible Teratogenic and Carcinogenic Effects of Pesticides on Human Health

Dr. Casey Jason, EPA

First of all, as I was just telling somebody next to me, it is a little ironic that I should be coming to NCI to speak about a study that is largely a reproductive outcome study; however, I think some of the principles are generally applicable. What we learn from a study of this type is also important to understanding a little bit more perhaps about cancer principles and cancer study formats.

Initially, it was proposed to look at both cancer and adverse reproductive outcomes in the floriculture industry in Bogota, Colombia.

At first glance you might think that given the small numbers of people involved that this might not really be feasible. As it turns out, there are approximately 13,000 floriculture workers exposed to pesticides in Bogota, Colombia, and of these more than half are actually women, most of whom are of reproductive age.

In terms of cancer outcomes, I'm sure most of you have already thought the numbers are going to be diminishingly small, and we did indeed find that to be the case.

However, in looking at the number of major malformations in this group of women, taken as a prevalency study, we found almost 600 infants with major malformations. This is taken over greater than a 10-year period.

A questionnaire format was utilized that went out to all of the parents involved in the pesticides industry.

Thus, it was decided to pursue adverse reproductive outcomes. I would like to thank just a few people who helped us refine this study and, I think, make it a much more reasonable one to interpret.

First of all, Tom Mason, who is right out here now, has been a tremendous help. In addition, Bob Miller at the NCI, and Lou Holmes over in Boston, and Leon Gordis over at Johns Hopkins. We all worked together with Nubio Munyoz and the people at the University of Miami to reformat this study. We all desired to put it into something that could potentially be used as a model for looking at adverse reproductive outcomes in a study population of this type.

There were 560 children with malformations, according to the questionnaire-elicited information. We had a geneticist in Bogota, Colombia -- our NIH equivalent there -- go as part of a small group to examine each of these children, document exactly what the type of malformation was, and in the final questionable cases had Lou Holmes, from Boston, fly in just three months ago to verify just exactly what the malformation type was and whether it formed an unusual syndrome.

A careful questionnaire of each family was administered by trained individuals in questionnaire administration, and in this case a careful reproductive history, a pesticides exposure history, and other risk-factors history were done.

In many ways, this was modeled after the NIH diabetes-in-pregnancy study format.

The comparisons that we thought might be most fruitful were three types. The first takes those individuals with major malformations whose mothers were exposed during the first trimester of pregnancy. And a comparison group would in this case be those mothers that were either exposed at another portion of pregnancy or not exposed at all during their pregnancy but who also had malformed infants. We thus attempted to control for some of the biases that might be introduced with having a control group of normal infants' mothers.

A second comparison, using the mentally retarded infants as well as other neurologic deficits, was done. In this case the group being investigated were all of those infants having neurologic abnormalities whose mothers were exposed at any time during pregnancy. The comparison group in this case were mothers having malformed infants but not having exposure during the critical time during gestation. The third comparison was one suggested by the NIH in Bogota and which we decided was well worth investigating: a two-to-one control to exposed match, where the control group consisted of normal infants. The group being investigated, of course, were malformed infants whose mothers were exposed during the first trimester.

So you will see a similarity between the first and the third group; the comparison group being the one that changed.

Finally, as I'm sure everyone here is quick to recognize, the floriculture industry in Bogota, Colombia, involves something like 100 different pesticides, fungicides, nematocides, and various and sundry other things, many of which could impact reproductive outcome.

So it was decided to investigate one particular chemical of interest to the agency, a priority chemical, captan. It is the primary fungicide used in Bogota, Colombia; secondly, one of its metabolites has a very interesting resemblance to thalidomide.

We did not feel, necessarily, that we would have infants with phocomelia -- shortened limbs -- or that we would be able to necessarily correlate agent with outcome. We felt that it was an important principle to find out whether we could use a certain chemical as a surrogate and get usable information, and then, using certain industry records for spraying, to try to use some type of application of that principle for estimating the type of exposure that might have occurred in a specific occupation in the floriculture industry.

The first slide shows the many groups involved in this study.

(Showing of viewgraphs)

This just gives you the idea of the expanse of this study. I think if it weren't for people like Bob Miller, Tom Mason, and others, it could easily have been impossible.

You notice EPA isn't there. However, we were actually involved.

Colombia was the NIH equivalent that did the investigation of the infants, administered the questionnaires, and, in conjunction with their geneticist and Lou Holmes, came up with the final tally of major malformations.

NCI here, through Tom Mason, administered funding. PAHO in Washington, and IARC in Lyon that developed the questionnaire format, incorporated certain segments from the NIH diabetes reproductive study, and worked with the Bogota group in administering the questionnaire.

The University of Miami in this case worked on the captan metabolite measurements. I will quickly describe their work, because we are in the process of evaluating the various comparisons that I have previously mentioned. Those will be ready within the next four months. By the next meeting we should have some interesting comparisons for you.

(Change of viewgraphs)

Just to give you an idea of why captan might be an interesting compound to look at, please look at the model compound thalidomide. There are few animal species - rabbits, for example, is one - that show an adverse reproductive outcome similar to that in humans. As you can see, the tetrahydrothalimide up at the upper right there shows a rather striking resemblance to this compound. I must admit, however, animal studies to this date have not shown a similar impact on reproductive outcome to that seen in thalidomide. However, as we all know, thalidomide didn't show a result except in two animal species of all those tested.

(Change of viewgraphs)

This will just give an idea of the amount of pesticides being used. These are kilograms per year, and as you can see we have something like almost 24,000 kilograms per year of captan alone being used. The only way they qualified for this list was if they were using more than 1000 kilograms per year. All told, a given industry used anywhere from 90 to over 100 different chemicals. I could also show you pictures that are rather dramatic, where workers are completely white with pesticides and fungicides that are being sprayed even while they are picking and cultivating flowers. This occurred on a daily basis, with cyclic exposure to different agents.

Over the past 10 years, the conditions have become better. The use of respirators is encouraged, but when Lou Holmes went to look at some of the mothers of infants at the industry site, he found that respirators were hanging from their chins rather than being used at all, and their faces were white and their hair white from the use of various pesticides.

(Change of viewgraphs)

DR. JASON: This gives you an idea of the pesticide volume.

We thought captan in the form of tetrahydrothalimide would be very interesting to measure in urine. Early experimental work, which had not been fully developed, indicated that it could be, albeit in fairly high amounts.

A pilot study was done in Florida by the University of Miami, where nothing was found in the urine of people who had recently sprayed captan; so we decided to do an animal study in rats.

(Change of viewgraphs)

What was done here -- this is the dosing for the rats. In this case, this was done at 0.001 LD50 and what we found was something very interesting and which accounted -- using the rat as the model for the human -- for why the University of Miami group was not able to find any measurable metabolite in the urine of the humans. It is because it was all excreted within 8 hours, the peak coming at 6. These people were being measured several days later.

This shows you very dramatically how the excretion occurred.

(Change of viewgraphs)

So when we were sampling at 24 hours -- this is parts-per-million on the Y-axis -- we had very little chance of detecting it in the urine. This indicated to us immediately that if we were to do a pilot in the floriculture industry, we would have to sample over the period of hours that the person was actually working rather than the following morning, when we would expect it would probably be cleared.

So realizing that captan is only one out of a hundred, and that it couldn't necessarily always be used as a surrogate, we still felt that it would be important, relative to given occupations, to show whether or not individuals in that industry had measurable levels in the urine which we could correlate with personal-air samples on their body to give us some crude idea as to the possible body burden, given a personal air sample showing a certain level of captan.

(Change of viewgraphs)

This viewgraph demonstrates that less than 5 percent or around 5 percent of the administered dose of captan is cleared in the urine. So it is a diminishingly small amount, but none was found in the adipose of the animal per se, and very low levels were also found in the serums. So the urine offered a way to get measurable levels if adequate amounts were administered to the animal. Now, if we could just very quickly go to the human.

(Change of viewgraphs)

These are the groups. We took two of the largest industries -- actually, the University of Miami, in conjunction with IARC, selected two of the more than 40 industries that were larger and agreed to have them come, and in this case, selected from the cultivators, sorters, propagators and office workers, to measure the excretion of captan during a given day of work at these two floriculture industries.

(Change of viewgraphs)

These were selected at random from the industry files. What we found was essentially that two groups, cultivators and sorters, were the only ones with detectable levels, and these were measured through time, essentially over two-hour blocks of time.

Those that we expected not to have any, didn't have measurable levels, but we were able to find detectable levels in approximately five individuals.

The detectable levels were approximately 30 ppm, and these were areas where essentially it was discovered in the urine but it was difficult to quantitate, so it was at the threshold of detection.

The last viewgraph, which is not shown, demonstrates the correlation with the air sampler. There was one there where it was measured in micrograms-per-cubic meter.

What it showed, essentially, was that for the cultivators and the sorters, very high air levels were present. These correlated well with the two groups that also showed detectable levels in the urine.

The main purpose for doing this, of course, was to investigate the possibility of using a surrogate chemical. I'm sure we could have thought of other blood measurements, but non-invasive techniques were the ones strongly recommended by industry. And because captan was a priority chemical, we decided that it might be an ideal one to use.

However, of course, in a study of this type, merely to even do this type of analysis without having a correlate with an adverse reproductive outcome is rather lame.

At this point, we are not proceeding any further with the pilot measurement of captan analysis for other occupational groups but, rather, are very carefully going over our data on the adverse reproductive outcomes.

If we should find an increase in a specific adverse reproductive outcome, or a suggestion that one is occurring, a much more focused study will occur, and a correlation will be done with industry spraying records, which are available for the majority of the industries.

Thanks very much. I will be very happy to answer any questions.

DR. LANDRIGAN: I can testify to the severity of that pesticide spraying from my own clinical experience. We were asked a few years ago to evaluate cases of frank organo-phosphate poisoning in USDA inspectors at the Miami Airport, and their only function was to open the boxes and check the plants, not to dust them.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Proportionate Mortality Study of Workers
in the Garment Industry Exposed to Formaldehyde

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ABSTRACT

In order to evaluate the human carcinogenicity of formaldehyde, we conducted a proportionate mortality study of garment workers engaged in the production of shirts from formaldehyde treated cloth. This study included 3 plants, and was based upon 256 deaths identified from a death benefit insurance fund. No deaths due to nasal cancer were observed, and the mortality from respiratory cancer (11 cases, PMR = 95) was slightly less than expected. Statistically significant ($p < .05$) elevations in proportionate mortality were observed for malignant neoplasms of the "buccal cavity" (3 cases, PMR=750), for "biliary passages and liver" (4 cases, PMR=313) and for "other lymphatic and hematopoietic sites" (4 cases, PMR=400). A proportionate cancer mortality (PCMR) analysis also was conducted, and cancer of the "buccal cavity" (3 cases, PCMR=682), and other "lymphatic and hematopoietic sites" (4 cases, PCMR=342) were still significantly elevated. The observed excesses in cancer mortality were primarily experienced by white females who made up the major portion of the workforce, and workers with more than 10 years of latency and duration of exposure a criterion for inclusion for most workers in the study group. The neoplasms observed were not equally distributed among the three facilities included in the study. Because of the small number of deaths involved and the lack of consistency with other studies, we believe that these findings should be viewed cautiously, pending the outcome of more definitive studies.

INTRODUCTION

Formaldehyde, commonly used as a disinfectant and tissue preservative, and as a feedstock in the synthetic chemical industry, has been shown to be carcinogenic in rodents. In a study conducted by the Chemical Industry Institute of Toxicology rats exposed by inhalation to 6 and 15 parts per million (ppm) of formaldehyde, and mice exposed to 15 ppm, developed squamous-cell carcinomas of the nasal passages (Chemical Industry Institute of Toxicology, 1982). In experiments at New York University inhalation of formaldehyde alone or in combination with hydrogen chloride induced squamous-cell nasal carcinomas in rats (Albert et al, 1982). In addition, formaldehyde has been shown to be mutagenic in a number of cellular test systems (Auerbach et al, 1977), and is highly reactive with nucleic acids (DNA) and proteins (Feldman, 1975; Grafstrom, 1983).

A number of mortality studies of occupational groups exposed to formaldehyde have been initiated, and several have recently been completed (Wong, 1980; Walrath and Fraumeni, 1983; Levine et al, 1983; Fayerweather et al, 1982; Marsh, 1982; Liebling, 1982). None of these studies have detected an association between formaldehyde exposure and either nasal or lung cancer. These studies were all extremely limited in their ability to detect an excess of nasal cancer, even if such an association existed, because of small sample size, intermittent exposures, and insufficient documentation of exposures. A few of these studies have detected statistically significant ($p < .05$) excesses of other cancers. In particular, excesses of prostatic cancer among formaldehyde production workers (Wong, 1983), cancers of the

skin, kidney and brain among embalmers (Walrath and Fraumeni, 1983), and cancers of the buccal cavity-pharynx and colon among workers involved in the production of formaldehyde resins (Liebling, 1982), have been observed. Because of the inconsistency between the observations from these investigations and other studies of formaldehyde exposed workers, their findings must be viewed as inconclusive.

BACKGROUND

In 1979, we initiated a survey of workers exposed to formaldehyde in various industries in order to identify a suitable population for an epidemiologic study. The results of the survey suggested that garment workers, who are exposed to the offgassing of formaldehyde from cloth treated with formaldehyde resins, would be a suitable group for study. This group was relatively large, had reasonably continuous exposures, and was not known to be exposed to other potentially carcinogenic agents at the work site (Elliott, 1980; Elliott, 1983). The study was planned to have two phases: an initial phase consisting of a proportionate mortality study (PMR), followed by a retrospective cohort mortality study. Following are the findings from the initial phase (PMR) of this investigation.

MATERIALS AND METHODS

This study is based upon deaths reported to a death benefit insurance fund for three shirt manufacturing facilities, which will be henceforth referred to as facilities A, B and C. This insurance fund is jointly operated by the union and companies involved. Plants B and C are located in Pennsylvania, and plant A is located in Georgia. These facilities produce shirts from cloth, which is received pre-treated with formaldehyde resins to impart crease resistance, color fastness and shrink resistance. Workers in these facilities are currently exposed to small amounts of formaldehyde which offgasses from the cloth. All three sites have used formaldehyde treated fabrics since 1958. Formaldehyde measurements recently taken at plants A and B indicate that virtually all of the employees working in the production of these garments are exposed to air levels that range from 0.1 to 1.0 ppm. Past exposures to formaldehyde, although undocumented, are believed to have been higher, since improvements in the resin systems used have greatly reduced the amount of free formaldehyde contained in the cloth. No other potentially confounding chemical exposures were observed in these facilities (Elliott, 1980; Elliott, 1983).

Workers whose survivors are eligible for death benefits were included in this study if they met one of the following criteria: (1) worked at least six months and died within 6 months of the last date worked; (2) worked at least 6 months and were disabled on the job; or (3) retired between age 62-65 and had worked at least 20 years if retirement was prior to 1975, or for 10 years if retirement was after 1976.

Deaths for inclusion in this study were identified at the insurance fund by the number of the local union that represented these facilities. A total of 304 death benefit files for individuals from these locals dying between January 1, 1959 and March 23, 1982 (date of record copying) were copied. However, 48 of these deaths were subsequently eliminated, 23 because their last date of union membership was prior to 1958, the year formaldehyde treated cloth was first introduced in these plants, and another 24 were eliminated from the study because they had never worked at the study facilities. Although these individuals did belong to a union local which represented one of the study sites, this local also represented other garment facilities that were not included in the study. Finally, one subject was eliminated because we were unable to obtain this person's death certificate resulting in a final study size of 256 deaths. The underlying cause of death was coded by a nosologist using the revision of the International Classification of Diseases in effect at the time of death.

A proportionate mortality (PMR) analysis was performed using a computer program developed at NIOSH (Waxweiler et al, 1983). This program computes a proportionate mortality ratio by dividing the number of deaths observed in cause specific categories, by the number of deaths expected based upon the proportion of deaths in this category in the U.S. general population. The PMR is standardized for the potentially confounding effects on mortality of sex, race, age and calendar time. The PMR analysis was stratified by duration of exposure (years exposed), latency (years since first exposure until death), and plant site. For duration and latency, exposure was

assumed to begin either on the date of initiation into the union, or January 1, 1958, whichever occurred later. Exposure was assumed to have ended on the last date of membership in the union.

A proportionate cancer mortality (PCMR) analysis also was performed. This analysis is essentially the same as the PMR analysis, except that the analysis is restricted to cancer deaths alone. This analysis is useful because it has been demonstrated that cancer mortality rates of working population are usually less affected by the "healthy worker effect", than are mortality rates for the nonmalignant causes of death (McMichael, 1976). A potential bias in the proportionate mortality ratio is often introduced by a deficit in cardiovascular diseases and other causes of death which are subject to the "healthy worker effect", thereby inflating the PMR for other causes of death, and particularly for malignant neoplasms.

The statistical significance of the PMRs and PCMR's was tested using a Fisher's exact test based upon the Poisson distribution. A one tailed p-value of 0.05 was used as the minimum criteria for statistical significance. Ninety percent Fisher exact confidence limits were also computed. Statistical tests, confidence limits, and PMRs were not calculated for death categories containing only one observed death.

RESULTS

The frequency distributions of deaths according to sex, race, plant site, age at death, and duration of employment are presented in Table I. The study population was predominantly white (95 percent), female (75 percent), from plant A (59 percent), and over 55 (67 percent) at the time of death. Given the eligibility requirements for death benefits discussed earlier, it was not surprising that duration of employment was generally long with a median of 20 years.

Table II presents the overall mortality experience for the study population. No deaths due to nasal cancer were observed in this investigation. Statistically significant excesses in mortality were observed for cancers of the buccal cavity (3 cases, PMR=750), biliary passages and liver (4 cases, PMR=313), and other lymphatic and hematopoietic sites (4 cases, PMR=400). All three of the buccal cavity malignancies were cancers of the parotid gland. One of these deaths was an epidermoid cell carcinoma, another was an adenocarcinoma and the third did not have any histology specified on the death certificate. Hospital pathology reports were requested for all three deaths. However, only one report was obtainable, which confirmed the case of epidermoid cancer. Three of the tumors in the category on biliary passages and liver were cancers of the gall-bladder, and the fourth was cancer of the bile duct. In the category of "other lymphatic and hematopoietic sites", three of the deaths were multiple myelomas and the fourth was a diffuse poorly differentiated

lymphoma. Statistically non-significant excesses in mortality were evident in many of the malignant categories. There were fewer deaths observed than expected in a few cancer sites; respiratory system (11 cases, PMR=90), stomach (2 cases, PMR=88) and urinary organs (2 cases, PMR=92). There was a slight excess in mortality due to diseases of the circulatory system (93 cases, PMR=104), and respiratory system (14 cases, PMR=118). Marked deficits in mortality were observed for diseases of the digestive system (1 observed vs. 9.9 expected), and other causes of death (10 observed vs. 15.46 expected).

The results from the proportionate cancer mortality analyses are presented in Table III. Generally the magnitude of the excesses for malignant neoplasms observed in Table II were reduced by using this approach. Statistically significant excesses remained, however, for cancers of the buccal cavity (3 cases, PCMR=682) and for the category of other lymphatic and hematopoietic sites (4 cases, PCMR=342). Cancer of the biliary passages and liver was still in excess (4 cases, PCMR=274), but not statistically significant.

The proportionate mortality analysis for selected malignancies was stratified by sex and race (Table IV). The pattern in cancer mortality observed for the entire study group (Table II) was mirrored by the white female category. White women had a statistically significant excess in mortality due to cancers of the buccal cavity (2 cases, PMR=738) and the other lymphatic and hematopoietic category (3 cases, PMR=387). White women also had a non-significant excess of cancers of the biliary passages and

liver (3 cases, PMR=287). A statistically significant excess in mortality due to all malignant neoplasms combined was evident for both white (74 cases, PMR=126), and black (4 cases, PMR=338) women.

In Table V the proportionate mortality experience of the study population is subdivided by plant site. Plant A had statistically significant excesses in cancer mortality due to all lymphatic and hematopoietic sites (8 cases, PMR=234), and for the other lymphatic and hematopoietic sites (3 cases, PMR=507) in both instances reflecting the unusual finding of multiple myeloma at this site. Plant B had a statistically significant excess in mortality due to cancer of the buccal cavity (2 cases, PMR=2484) since two of the parotid tumors had occurred among employees at this location. Plant C had a statistically significant excess of cancer of the biliary passages and liver (2 cases, PMR=823) resulting from two cases of gall bladder cancer.

In Table VI the mortality experience of the study population is examined with regard to latency and duration of exposure. Statistically significant excesses were present in the category of greater than 10 years of latency and duration of exposure for all malignancies combined (51 cases, PMR=137), buccal cavity cancer (2 cases, PMR=952), malignancies of the biliary passages and liver (3 cases, PMR=467), malignancies of all lymphatic and hematopoietic sites (8 cases, PMR=283), and for malignancies of other lymphatic and hematopoietic sites (10 cases, PMR=761). There was also a statistically significant excess in mortality due to malignancies of the digestive organs and peritoneum (PMR=194) in the less than 10 years of latency and exposure category.

DISCUSSION

Based upon the findings of the rat inhalation studies (Chemical Industry Institute of Toxicology, 1982; Albert et al, 1982), nasal cancer was considered a priori to be the most likely cancer site to be affected by formaldehyde. No cases of nasal cancer were observed in this investigation. These findings are consistent with the results from other mortality studies of formaldehyde exposed workers (Wong, 1980; Walrath and Fraumeni, 1983; Levine et al, 1983; Fayerweather et al, 1982; Marsh, 1982). However, due to sample size, this study only had a 20 percent chance (power) of detecting a 2 fold excess for this rare tumor site ($\alpha=.05$). In addition to statistical power, this study may not have observed any cases of nasal cancer because the latency period was too short, or because the intensity of formaldehyde exposures was too low. This study was also negative for respiratory cancer, for which there were fewer cases observed than expected (11 vs. 12.2).

In this study, statistically significant elevations in proportionate mortality were observed for malignant neoplasms of the buccal cavity (3 cases, PMR=750) due to an excess of cancers of the parotid gland, for biliary passages and liver (4 cases, PMR=313) due to an excess of cancers of the gallbladder, and for other lymphatic and hematopoietic sites (4 cases, PMR=400) due to an excess of multiple myelomas. These excesses are based upon relatively small numbers of deaths (3 or 4), making the estimates of relative risk (PMRs) imprecise, as reflected by the wide confidence intervals for these sites in Table II.

The increased mortality in certain cancer sites in the PMR analyses was also evident in the proportionate cancer mortality (PCMR) analysis. However, the magnitude of these excesses was reduced in this analysis and the findings were only statistically significant for buccal cavity (PCMR=682), and other lymphatic and hematopoietic sites (PCMR=342). The fact that these sites were significantly elevated in this analysis, indicates that the excesses of these sites observed in the PMR analysis can not be fully attributed to deficits in the nonmalignant causes of death inflating the PMRs for malignant sites.

The observed excess in cancer mortality was most pronounced among white females who made up the major portion of the study population (73 percent). The fact that the observed excesses were primarily among workers with greater than 10 years of latency and exposure, supports the hypothesis that these findings could be related to occupational exposure to formaldehyde, since the induction of cancer in humans is usually related to duration of exposure, and requires a long period of time from first exposure to the development of the disease (latency). Alternatively, these observations may be explained by the fact that the majority of the study subjects had more than 10 years of latency and duration of exposure because of the eligibility requirements for receiving death benefits. It is worth noting that the maximum latency achievable in this study was 24 years, and that this study would be unable to detect an excess of cancer if there were a longer latency period than the 24 years covered by this study.

The excesses in cancer mortality were unevenly distributed among the plant facilities. The parotid gland tumor excess was from plant B, the gall bladder and biliary passages cancer excess was from plant C, and the multiple myeloma excess was from plant A. Based upon our industrial hygiene survey results, we believe that the processes and formaldehyde exposures were very similar at these facilities (Elliott, 1980; Elliott, 1983). This differential in cancer site excesses among the plant locations, despite relatively uniform exposure to formaldehyde, argues against the hypothesis that these excesses were related to formaldehyde exposures. On the other hand, because of the small study size of each of the plant locations the statistical power of each location for these cancer sites was exceedingly low.

Significant elevations in mortality due to the cancer sites observed have not been consistently observed in other studies of formaldehyde exposed workers, although excesses of oral pharyngeal and lymphatic cancer have been observed in a few of these studies. A statistically significant ($p = .01$) increase in risk due to lymphatic and hematopoietic neoplasms (SMR=242) was observed in a mortality study of British pathologists (Harrington, 1975). In a PMR study of New York State embalmers and funeral directors, Walrath and Fraumeni (1983) observed a two fold (non-significant) increase in mortality due to cancers of the buccal cavity and pharynx (PMR=201) among persons licensed only as embalmers. Six of the seven deaths in this category were malignancies of the buccal cavity, and one was a salivary gland tumor (personal communication J. Walrath). According to U.S. Vital Statistics among white males in 1975, only 30 percent of the deaths in the

category of buccal-pharyngeal cancer were due to cancers of the buccal cavity. Thus, if the analysis had been restricted in the Walrath study to buccal cavity cancer, a much larger increase in mortality would probably have been observed. In our study, however, the tumor type was parotid gland which is not entirely consistent with the types observed in Walrath's study. Finally, Liebling (1982) in a PMR study of chemical workers observed a statistically significant ($p = .05$) excess of cancer of the buccal cavity and pharynx (PMR=870) among a high formaldehyde exposure group. However, this excess was based upon only two cases (nasopharyngeal and floor of the mouth), again different from the parotid tumors observed in this investigation.

The observation that all 3 of the buccal cavity cancers were parotid gland neoplasms is highly unusual. According to U.S. Vital Statistics for 1970, among white females only 16 percent of buccal cavity cancers were parotid gland tumors. Despite this fact, the biologic plausibility of this finding being causally-linked to formaldehyde is limited. Cancers experimentally induced in rats by formaldehyde inhalation have involved the tissues receiving direct contact with formaldehyde (nasal cavity). In laboratory rats, which are obligate nose-breathers, the principal site of formaldehyde absorption is the nasal epithelium (Heck, 1982). In humans, who can be either nasal- or mouth-breathers, the tissue primarily receiving exposure can be either the nasal or oral epithelium. However the parotid gland is internal to the oral cavity and communicates with it by a pair of salivary ducts. Given the rapid metabolism of formaldehyde to formic acid at the site of absorption (Heck, 1982) and the location of the parotid glands, it

is speculative as to whether or not any formaldehyde reaches the parotid gland. It is even harder to comprehend how internal sites such as the gallbladder, and the lymph system could receive significant doses of formaldehyde.

There is, as always, the possibility that the excesses observed in this investigation could either be the result of confounding variables that were not accounted for in the analysis, or be due to chance. Since a large number of causes of death were examined in these analyses a few causes would be expected to be significantly elevated on the basis of chance alone. Although tobacco usage and alcohol consumption have been associated with cancers of several buccal cavity sites, these relationships do not apply to cancer of the parotid gland for which there are no known etiologic risk factors (Frie and Holland, 1982). In addition, the fact that lung cancer was not elevated in this study indicates that smoking is unlikely to have contributed to the observed excess of buccal cavity cancer. Multiple myeloma has been associated with exposure to radiation, asbestos and heavy metals, and with employment in the rubber, petrochemical, furniture, leather, and food industries (Schottenfeld and Fraumeni, 1982). Gallbladder cancers have been associated with a history of gallstones (Frie and Holland, 1982). We are unaware of any environmental exposures in these plants, or other factors which could explain the observed excesses, although unrecognized factors such as diet or ethnicity could conceivably be responsible.

In summary, no nasal cancer deaths were observed, but statistically significant elevations in proportionate mortality were observed for cancers

of the buccal cavity (parotid gland), biliary passages and liver (gallbladder), and other lymphatic and hematopoietic sites (multiple myeloma). The fact that these excesses occurred among workers with greater than 10 years of latency and exposure, and the fact that there are no other known environmental factors in these plants which could explain these observations, supports the hypothesis that these excesses are associated with formaldehyde exposure. Alternatively, the fact that elevations in mortality for these tumors have not been consistently observed in other studies of formaldehyde exposed workers, the fact that these excesses were not consistently present in each of this investigation's plant locations, and the lack of biologic plausibility for these effects argues against this hypothesis. Because of these inconsistencies, and the small number of deaths involved, we believe that these positive findings should be viewed as tentative, and that conclusions on the human carcinogenicity of formaldehyde must await the results of more definitive investigations that are now in progress.

Acknowledgement

The authors would like to express their appreciation to Mr. Eric Frumin, Director of Health and Safety of the Amalgamated Clothing and Textile Workers Union, for his cooperation and assistance in the conduct of this investigation. We would also like to recognize the excellent work of Mrs. Judy Edelbrock and members of her staff in the Industry Wide Studies Branch of NIOSH, who obtained the death certificates and coded the data for this study.

TABLE I

DISTRIBUTION OF DEATHS ACCORDING TO
SEX, RACE, PLANT SITE, AGE AT DEATH,
AND DURATION OF EMPLOYMENT

<u>VARIABLE</u>		<u>FREQUENCY</u>	<u>PERCENT</u>
Sex	Male	64	25.0
	Female	192	75.0
Race	White	244	95.3
	Black	11	4.3
	Unknown	1	0.4
Plant Site	A	150	58.6
	B	55	21.5
	C	51	19.9
Age at Death	18-35	10	3.9
	36-55	73	28.5
	56-75	144	56.3
	75+	29	11.3
Duration of Employment			
	0-5	23	9.0
	6-10	24	9.4
	11-15	36	14.1
	16-20	46	18.0
	21+	127	49.6

TABLE II
PROPORTIONATE MORTALITY RATIOS (PMR)
FOR ALL CAUSES OF DEATH^{a, b}

CAUSE OF DEATH	90 percent			
	OBSERVED	EXPECTED	PMR	CONFIDENCE
	O	E	O/E x 100	LIMITS
All Malignant Neoplasms	87	73.26	118	99, 142
Buccal Cavity & Pharynx	3	1.31	229	62, 592
Buccal Cavity	3	.40	750**	204, 1939
Digestive Organs & Peritoneum	22	17.49	126	85, 180
Esophagus	1	.90	-	--
Stomach	2	2.27	88	16, 277
Intestine except Rectum	8	7.32	109	54, 197
Biliary Passages & Liver	4	1.28	313*	107, 715
Liver not specified	2	.47	426	76, 1340
Pancreas	4	3.27	122	42, 280
Peritoneum & Digestive Organs	1	.27	-	--
Respiratory System	11	12.20	90	53, 157
Trachea, Bronchus & Lung	11	11.62	95	53, 157
Breast	17	14.58	117	74, 175
Female Genital Organs	12	9.96	120	69, 195
Urinary Organs	2	2.17	92	16, 290
Other & Unspecified Sites	10	9.41	106	58, 180
Skin	2	1.12	179	32, 562
of Brain & Other Parts Nervous System	1	2.14	-	--
Connective Tissue	1	.33	-	--
Other Unspecified Sites	6	5.83	103	45, 203
All Lymphatic & Hematopoetic	10	6.13	163	88, 277
Lymphosarcoma & Reticulosarcoma	1	2.05	-	--
Hodgkin's Disease	1	.70	-	--
Leukemia & Aleukemia	4	2.38	168	57, 385
Other Lymphatic & Hematopoetic	4	1.00	400*	137, 915
Benign Neoplasms of the Brain	1	.77	-	--
Diabetes Mellitus	5	5.69	88	35, 185
Diseases of Blood & Blood Forming Organs	1	1.03	-	--
Diseases of Nervous System & Sense Organs	21	21.03	100	67, 144
Diseases of the Circulatory System	93	89.84	104	87, 123
Respiratory Diseases	14	11.87	118	71, 184
Diseases of the Digestive System	1	9.89	-	--
Diseases of the Genito-Urinary System	2	2.91	69	12, 216
Diseases of the bones & organs of movement	1	.46	-	--
External causes of death	19	20.69	92	60, 135
Unknown causes	1	3.06	-	--
Other causes	10	15.46	65	35, 110

a. PMRs were not calculated for death categories containing only one observed death.

b. Standardized for age, sex, race, and calendar time.

* p .05 (one tail)

** p .01 (one tail)

TABLE III
PROPORTIONATE CANCER MORTALITY RATIOS (PCMR) FOR ALL MALIGNANT NEOPLASMS^{a,b}

CAUSE OF DEATH	OBSERVED O	EXPECTED E	PMR O/E x 100	90 percent CONFIDENCE LIMITS
Buccal cavity & Pharynx	3	1.44	208	57, 538
Buccal Cavity	3	.44	682**	185, 1758
Digestive Organs & Peritoneum	22	20.19	109	74, 156
Esophagus	1	.99	-	--
Stomach	2	2.57	78	14, 245
Intestine	8	8.61	93	46, 168
Biliary Passages & Liver	4	1.46	274	94, 627
Liver not specified	2	.54	370	66, 1166
Pancreas	4	3.74	107	37, 245
Peritoneum & Digestive Organs	1	.33	-	--
Respiratory System	11	13.14	84	47, 139
Trachea Bronchus & Lung	11	12.53	88	49, 145
Breast	17	19.27	88	56, 132
Female Genital Organs	12	13.20	91	52, 147
Urinary Organs	2	2.34	85	15, 269
Skin	2	1.33	150	27, 473
Brain & Other Parts Nervous System	1	2.52	-	--
Connective Tissues	1	.38	-	--
Other & Unspecified Sites	6	6.23	96	42, 190
All Lymphatic & Hematopoietic	10	6.94	144	78, 244
Lymphosarcoma & Reticulosarcoma	1	2.37	-	--
Hodgkin's Disease	1	.76	-	--
Leukemia & Aleukemia	4	2.64	152	52, 347
Other Lymphatic & Hematopoietic	4	1.17	342*	117, 782

a. PCMRs were not calculated for categories containing only one observed death.

b. Standardized for age, sex, race and calendar time.

* p = .05 (one tail)

** p = .01 (one tail)

TABLE IVa,b
PROPORTIONATE MORTALITY RATIOS (PMR)
FOR SELECTED CANCER SITES
BY SEX/RACE CATEGORIES

CAUSE OF DEATH	White Female		White Male		Black Female		Black Male	
	OBS	PMR	OBS	PMR	OBS	PMR	OBS	PMR
All Malignancies Combined	74	126*	8	68	4	338*	1	--
Buccal Cavity & Pharynx	2	234	1	--	0	--	0	--
Buccal Cavity	2	738*	1	--	0	--	0	--
Digestive Organs & Peritoneum	19	139	2	62	1	--	0	--
Biliary Passages & Liver	3	287	1	--	0	--	0	--
Respiratory System	8	109	2	46	1	--	0	--
Trachea Bronchus & Lung	8	114	2	49	1	--	0	--
Breast	16	111	0	--	1	--	0	--
Female Genital Organs	12	123	--	--	0	--	0	--
Urinary Organs	1	--	0	--	0	--	1	--
Skin	1	--	1	--	0	--	0	--
Brain & Other Parts nervous system	1	--	0	--	0	--	0	--
Connective Tissues	1	--	0	--	0	--	0	--
Other & Unspecified Sites	6	175	0	--	0	--	0	--
All Lymphatic & Hematopoietic	7	144	2	178	1	--	0	--
Leukemia & Aleukemia	3	160	1	--	0	--	0	--
Other Lymphatic & Hematopoietic	3	387*	0	--	1	--	0	--

a. PMRs were not calculated for categories containing only one observed death.

b. Standardized for age, and calendar time.

* p .05 (one tail)

TABLE V
PMR ANALYSES OF SELECTED CANCER SITES
BY PLANT LOCATION^{a, b}

CAUSE OF DEATH	PLANT A		PLANT B		PLANT C	
	OBS	PMR	OBS	PMR	OBS	PMR
All Malignancies Combined	48	115	19	119	20	128
Buccal Cavity & Pharynx	0	-	2	768*	1	-
Buccal Cavity	0	-	2	2484**	1	-
Digestive Organs & Peritoneum	9	87	7	183	6	175
Biliary Passages & Liver	2	269	0	-	2	823*
Respiratory System	8	112	3	133	0	-
Trachea Bronchus & Lung	8	118	3	139	0	-
Breast	9	111	3	90	5	155
Female Genital Organs	5	90	2	83	5	241
Urinary Organs	2	155	0	-	0	-
Skin	1	-	0	-	1	-
Brain & Other Parts Nervous System	1	-	0	-	0	-
Connective Tissues	1	-	0	-	0	-
Other & Unspecified Sites	4	159	1	-	1	-
All Lymphatic & Hematopoietic	8	234*	1	-	1	-
Leukemia & Aleukemia	3	228	0	-	1	-
Lymphatic & Hematopoietic	3	507*	1	-	0	-

a. PMRs were not calculated for categories containing only one observed death.

b. Standardized for age, sex, race, and calendar time.

* p .05 (one tail)

** p .01 (one tail)

TABLE VI

PMR ANALYSES OF SELECTED CANCER SITES
BY DURATION OF EXPOSURE AND LATENCY a,b,c,d,e

CAUSE OF DEATH	Latency 10 yrs. & Duration 10 yrs.		Latency 10 yrs. & Duration 10 yrs.		Latency 10 yrs. & Duration 10 yrs.	
	OBS	PMR	OBS	PMR	OBS	PMR
All Malignancies (MN) Combined	28	103	8	68	51	137*
Buccal Cavity and Pharynx	1	-	0	-	2	286
Buccal Cavity	1	-	0	-	2	952**
Digestive Organs & Peritoneum	10	194*	1	29	11	124
Biliary Passages & Liver	1	-	0	-	3	467*
Respiratory System	5	154	3	160	3	42
Trachea, Bronchus & Lung	5	163	2	112	3	44
Breast	3	56	2	109	12	163
Female Genital Organs	5	127	0	-	7	148
Urinary Organs	0	-	1	-	1	-
Skin	1	-	0	-	1	-
Brain & Other Parts nervous system	1	0	-	0	-	-
Connective Tissue	0	-	0	-	1	-
Other & Unspecified Sites	0	-	1	-	5	200
All Lymphatic & Hematopoietic	2	88	0	-	8	283**
Leukemia & Aleukemia	2	217	0	-	2	192
Other Lymphatic & Hematopoietic	0	-	0	-	4	761**

- a) For both latency and duration of exposure, exposure was assumed to start either on the date of initiation into the union or 1/1/58, whichever occurred later.
- b) Latency is the time from first exposure until death: Duration of exposure is the time between the first and last date of exposure.
- c) PMRs were not calculated for categories containing only one observed death.
- d) No cancer deaths were observed with latency 10 years & duration 10 years.
- e) Standardized for the effects of age, sex, race, and calendar time.

* p .05 (one tail)

** p .01 (one tail)

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION V - EPIDEMIOLOGY
Chairman: Dr. Philip J. Landrigan
National Institute for Occupational Safety and Health

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. LANDRIGAN: Could I ask the four speakers to come on up and sit at the table? I think it will be easier for people to ask you questions.

DR. LANDRIGAN: Are there any questions?

DR. RICE: Rice. NCI. I would like to ask Dr. Morris some questions about this experimental work with hexachlorobenzene.

You mentioned that among the problems people have experienced are thyroid enlargements, presumably goitrous. Did you see any thyroid pathology in your experimental work?

DR. MORRIS: So far we have only had one individual that has been transported to Ankara, Turkey, to Hacettepe Medical Center, for evaluation and removal of the thyroid tumor. We do not have the pathology report as yet.

We have three or four patients who we hope will consent to having the procedure done next year; however, they are very reluctant. The patient who had her tumor removed has been very concerned because there is now a recurrence of her tumor. We are very concerned that the recurrence may be a problem.

DR. RICE: Well, then, is it not correct to say that the thyroid enlargement is part of the characteristic hexachlorobenzene syndrome in human beings, that shouldn't be considered a major part of the overall picture?

DR. MORRIS: Of hexachlorobenzene?

DR. RICE: Yes.

DR. MORRIS: I think the enlarged thyroid is unusual and unique to hexachlorobenzene.

DR. RICE: Okay. So you do think that is characteristic of human exposure?

DR. MORRIS: Yes.

DR. RICE: What about your rats?

DR. MORRIS: There were thyroid lesions in the rats. However, they were not statistically different from untreated controls, though there was a trend. Thyroid tumors have been observed in the hamster and parathyroid tumors in the mouse. So it is site-specific in the experimental animal, and we are seeing it in the human population as well.

DR. LANDRIGAN: Is Eastern Turkey one of the goitrous belts of the world where there is a high prevalence of background enlargement of thyroids?

DR. MORRIS: There are some areas there, yes. The background tumor rate for the thyroid, we have been told, is around 7-8 percent in that part of Turkey. In our study, males are running 25-30 percent and females are about 60 percent.

DR. LANDRIGAN: Enlargements, but how about actual tumors?

DR. MORRIS: We don't know the character of those enlargements yet.

DR. STARLING: Dr. Starling. Ohio. The short phalanges and the apparent age of the patients indicate that these were small children when the exposures took place. It does resemble, I suppose, the acro-osteolysis that you see in adults in vinyl chloride exposure.

DR. BRESNICK: Bresnick. Eppley Institute. I have a question for Dr. Hoar and also a question for Dr. Morris.

Has there been any attempt to verify the questionnaire, if you will, by analysis for the phenoxyacetic acids of the aquifers or of the well waters in a select area?

DR. HOAR: No.

DR. BRESNICK: Do you think that might be worthwhile? I would tend to think that these would persist for a fair length of time in the well waters and any type of waters.

DR. HOAR: Yes. It might be something EPA with their expertise could investigate. In other epidemiologic studies we collected residential histories and sources of drinking water. We linked the information to EPA water treatment data and looked for associations with cancer. Ken Cantor did this work for the National Bladder Cancer Study. Something similar could be done for pesticides.

DR. STARLING: In regard to those adipose tissue curves, Dr. Morris, it is strongly resemblant of what you might find with an early, small induction of some sort of metabolizing system which tends to saturate and then fade out as the liver becomes either preneoplastic and neoplastic, and both those tended to fit in with your current studies of preneoplasia, preneoplastic foci, and carcinoma. I guess what I would like to ask is, have you looked at blood levels of the hexachlorobenzene during the course of these strange changes in adipose?

DR. MORRIS: We did not look at HCB blood levels in the chronic rat study. In the subchronic study, we did measure for HCB and didn't find any detectable levels of HCB in that shorter time frame.

DR. JASON: Were those denominators lipid that you used for hexachlorobenzene? Some of those measured were adipose, weren't they?

DR. MORRIS: Yes.

DR. JASON: So I assume that the denominator was triglycerine? I have been involved in the dioxin program -- one of the things that occurs to me is you have to keep in mind the general toxicity to the animal, and perhaps actual weight loss that would be first reflected in lipid. So the choice of the denominator becomes critical.

A lipid denominator, or the total lipid pool makes the most reasonable choice for a lipid-soluble agent.

If an animal were to lose weight, the first to be depleted would be glycogen stores and then lipid. Any animal that is chronically malnourished is then going to deplete the lipid stores very rapidly. Depending on how it's cleared, if the hexachlorobenzene is cleared similarly to, let's say, triglyceride or cholesterol, then you might see it keep up with the lipid loss. However, if it isn't cleared as rapidly, you may see an apparent rise that isn't a real rise when you figure the total body burden. Thus these are important things to keep in mind.

DR. COLE: Dr. Cole. Shell. For Dr. Morris, first: Have these individuals with the shortened distal phalanges been X-rayed, and if so, what is the picture on X-ray?

DR. MORRIS: We have not done X-rays on these patients. In fact, in the first few visits to eastern Turkey, we had problems even spinning down the blood. During the second visit, we took an old hand-held chemistry spinner used in chemistry to prepare the blood sample. So X-ray is even more exotic in that part of the world. They would actually have to be transported into medical centers, such as Dyarbakir or one of the larger centers, for that kind of evaluation.

DR. COLE: So the one that went in for the thyroid hasn't had the hands X-rayed then, at the same time?

DR. MORRIS: Not to my knowledge.

DR. COLE: All right. One other question for you: Regarding the females that are now in the childbearing years, are their children showing any of this syndrome of the Pembre Yara, or whatever it is called?

DR. MORRIS: Not that we have been able to observe. It has been interesting, if you look at the history that some of the women gave when they brought their child to the clinic. For example, this might be their third or fourth pregnancy. We have done the maternal milk samples on some of those and have found high levels of HCB in the maternal milk. My guess is that the result is much like we have seen in other situations, where perhaps the first two or three survivors got a large dose of mother's HCB before she finally got her body burden down so the offspring would survive.

DR. COLE: Most of these have had children that died early?

DR. MORRIS: Well, yes. In their history that is the case. One must weigh that carefully, however, because in this part of the world, survival of the offspring is questionable anyway; they have a lot of intestinal diseases that may cause early death of their children. I have seen situations where a

mother kept giving the child all sorts of medicines that she was able to acquire rather than go out and buy some milk. Nutrition is clearly a problem, too. So I can't fully answer that question.

DR. COLE: Thank you. I have one question for Dr. Jason. Number one, what type of flowers are they cultivating? And what initiated the study in the first place?

DR. JASON: Okay. The answer to the second question is a bit longer than the first, and Tom probably can help me out a little bit.

I inherited this project. But the reformatting of the study was largely because it was a very expansive study and had a number of elements that we couldn't interpret.

The reason we chose reproductive outcome was because we did have relatively well-recorded information on major malformations; we felt we could explore that more intelligently. My own bias, of course, would be to also investigate fertility and the spontaneous abortion rate, using a prospective method that would be very sensitive to early prenatal loss. But I think that not given that ability in this country, this represented the next most reasonable alternative.

Now, to your first question on what kind of flowers: chrysanthemums, roses, the whole variety. They are the major supplier for the U.S. flower industry. Bogota, Colombia, I think contributes over 50 percent -- somebody has suggested to me 70 percent -- of the flowers that come to the U.S.

I'm sure Phil probably knows more about this than I. He has been a consultant in these areas. The thing that impressed me the most was that in all of the information I had and in all of the discussions with the people there, these people many times were covered with practically no protection.

DR. COLE: Is it year-round?

DR. JASON: Exactly. They supply us throughout the whole year.

Your next question might be: Well, in this case, if male workers are exposed, how in the world do we expect to find malformations? Because, if anything, problems in fertility would be expected. Some chromosomally abnormal births might also occur.

We are actually looking for an increase in chromosomal abnormalities and for the more classical major malformations -- not necessarily, but also, those that might be genetically transmitted.

DR. COLE: But is there a suggestion of a problem?

DR. JASON: We don't know.

DR. LANDRIGAN: You are not doing semen analysis -- that would probably not be culturally acceptable?

DR. JASON: The industries are reluctant, at best, as you can imagine, Phil. I think the semen analysis would certainly be an ideal thing to do for the male workers. I think maybe as the next step, if we were to find something interesting, that could be used.

DR. LANDRIGAN: I am just very pessimistic, I guess, about your ability to find either increased rates of stillbirths or spontaneous abortion . . .

DR. JASON: Oh, we won't, using questionnaire methods. We are not looking for that.

DR. LANDRIGAN: . . . or malformation in a population with probably a 20-25 percent infant mortality rate.

DR. JASON: There are several groups right now looking at the spontaneous abortion rate, for example, a colleague of mine, Alan Wilcox over at NIEHS. We at EPA are also looking at it, using a very sensitive method in urine, carboxy terminal determination of HCG, which can detect a positive pregnancy as early as 9 days developmental age. I think that kind of analysis prospectively could give those types of answers, but certainly the retrospective analysis of spontaneous abortion rates, even in the best of settings, has serious problems.

Semen analysis offers another way to go after that using the male component of the problem. We are evolving, and we are trying to address the very question that Phil is raising. Perinatal mortality is another tough one, but we feel we are making some progress, and within the next year or two we might suggest that type of abortion study format in the floriculture industry also.

DR. MASON: Tom Mason, NCI. I have a question for Dr. Stayner: How much of your gall bladder excess may simply be explicable in terms of the regional differences and regional distribution patterns of mortality from gall bladder cancer? I would just like some comments, since you know where I am coming from.

DR. STAYNER: I personally, of all the observations, find this the hardest to swallow in terms of plausibility.

DR. MASON: I think it is explicable in terms of regional patterns of excesses of gall bladder mortality, definitely among the female work force. And I think you would be well advised in this particular instance to use some regional comparison rate.

DR. STAYNER: I will have to look at it. But two of the plants were in Pennsylvania, and one is in Georgia. I don't know if that gives you any clue off the top of your head, but I will have to look at your book.

DR. LANDRIGAN: In regard to the parotid tumors you mentioned, Les, do you have any further detail on the pathology -- that is to say, did they arrive in the gland or in the duct, by the ampulla?

DR. STAYNER: I forget exactly how many of them we were successful in getting the pathology for, but one of them was a squamous carcinoma, which I guess is rare for a salivary gland tumor, and another one was adenocarcinoma. I think the third one, we were not able to get any information on.

DR. LANDRIGAN: Perhaps these last two questions from the people who are now on their feet, and then no more, please.

DR. STEENLAND: Kyle Steenland, NIOSH. Dr. Jason, about the flowers in Colombia. I was in Colombia last summer, and we did a small kind of study with some students, looking at inhibition of cholinesterase in these flower workers who were also exposed to organophosphates. I think you had malathion on the list.

DR. JASON: Exactly.

DR. STEENLAND: We found that the flowers are in open sheds. They are not enclosed.

DR. JASON: That's right.

DR. STEENLAND: And there are many different ones. The sprayers went around on a weekly or a biweekly basis from shed to shed, so that the women were not usually in those sheds when they were sprayed, where we were -- and we had a particularly good one.

DR. JASON: Yes.

DR. STEENLAND: In other words, there was an intermittent exposure, and the inhibition of cholinesterase we didn't find it. It's also an effect that may go away after a period of time. With captan, I think you mentioned a few hours, and it may be a much more difficult thing to measure.

I would question why you would focus -- I don't know the expense of measuring captan in the urine, but why you would focus on that. All we did was little pinpricks, and it may have been more.

DR. JASON: Well, captan was chosen partly because there is very little in the literature right now about captan, and it's one of our highest priority chemicals. As I'm sure you can understand, I'm from the EPA, so we do have priority chemicals and certain issues that people are interested in.

Also, one of captan's metabolites, tetrahydrothalidimide, essentially looks very much like thalidomide. The literature is not all in on animal work in this area; so for two reasons it was felt that would be an interesting one to do, independent of the floriculture industry, per se.

DR. STEENLAND: Actually, my question has nothing to do with captan. I was curious about two things: one is, I didn't understand the definition of the control groups, and I don't understand what you hope to find or how you are going to find it.

Secondly, if you do find something, I'm not sure how you'll know among this whole gamut of chemicals what in the world you've got.

DR. JASON: That's exactly right. I think your third comment is more valid than your first. The gamut of chemicals, you're right, is a gamut. It's just that. And I think the problem of associating it with any particular chemical is probably 95 times greater than your usual problem, because we have 95 different chemicals.

Next, it's probably going to be an embryotoxic influence rather than a specific malformation. I agree with you.

But in terms of the comparison groups, number one, I think that it was an amalgam of ideas as to how we should organize. The review panel felt that biases introduced by a mother's response when she has a malformed infant vs. that of a mother without a malformed infant could be alleviated by comparing specific malformation entities against a control group containing other types of malformations.

So the first control group that made the most sense to us was to take two groups of malformed children and talk to their mothers. In the first, the exposure during the first trimester for a mother with a child having a specific malformation is compared to mothers having malformed infants but with no exposure during the first trimester.

Now, the second comparison group was neurologic disease. The literature does not say that neurologic disease is only the result of exposure during the first trimester. It would appear that it could be exposure during the first two years, or for that matter, for life. So in this case we decided to use the total exposure during the pregnancy as an index, and then in that case the comparison group would be other malformed infants whose mothers were essentially not exposed.

The third comparison group was the one that NIH in Bogota suggested. They wanted a comparison group of normal infants. Realizing the biases inherent in that, we still felt that a two-to-one match, if they wanted to look at that, would be worthwhile.

DR. LANDRIGAN: One more quick question, please.

DR. O'BERG. I am Maureen O'Berg from DuPont. I have a question for Leslie Stayner. You mentioned the Monson Program, and of course that is a program that has been used rather extensively in these kinds of studies and has been referenced in literature, and now you have a new program. I wonder if you could briefly explain what you see the differences to be and also what does that mean for those of us who go back and look at results from the Monson Program in past literature?

DR. STAYNER: Well, it's very similar in certain respects, but I think it is very similar to our Life Table Program, if you are familiar with that at all. One of the nice features of it is that there are a lot of nice reports in it that show you errors in data fields.

I had run this data on the Monson Program, and I also found a few bugs in working with females; particularly, some of the female cancers were ending up in the wrong categories.

The NIOSH program is also nice in that it gives you duration of exposure and latency analysis, which you don't get from the Monson Program.

The categories of death are more detailed than what you would get from the Monson Program. For example, the buccal cavity cancer, which I was able to look at here, you can't look at it in the Monson Program. It is grouped together with buccal cavity and pharynx. So if you want to look at just buccal cavity alone, you can't do it with Monson's.

DR. O'BERG: But there is nothing with the way the person-years are calculated that would make the numbers come out differently, for instance?

DR. STAYNER: Well, it's not person-years.

DR. O'BERG: Well, whatever you are using there, like the PMRs.

DR. STAYNER: No, that's analagous, I think.

DR. O'BERG: All right. Thank you.

DR. LANDRIGAN: The PMR program that we have developed will essentially represent a companion to the NIOSH Life Table, which was developed and written up last year. Jay Beaumont, who is standing over on the far wall, is the team leader in that effort. Any quick comments? And then Jay will be available for anyone who wants to go into detail on it afterwards.

DR. BEAUMONT: I just want to say that the basic functions of the calculations of the person-years and also the standard death rates are very similar, almost identical between the two programs. So in terms of the basic analysis, it's the same.

The NIOSH program has 89 causes of death, vs. about 50 in Monson's. We take into account gaps in work histories and can also do a cumulative dosage in several different ways. We can handle ICD codes, either in the revision in effect at the time of death, or converted to one revision like Monson does. It basically has more bells and whistles.

DR. LANDRIGAN: It will be written up in the literature within the next year just as the Life Table Program was. If anyone wants one sooner or wants to get in touch, just write to Jay at NIOSH and send a completed 1040 form. Thank you.

DR. MORRIS: A commercial? We are going to be doing, through this NCI-EPA collaborative program, an international symposium on hexachlorobenzene in June of 1985. Dr. Kraybill and myself are going to be co-chairing, at least from this side's point of view, we would welcome any suggestions anyone might have with respect to papers or speakers. Thank you.

* * * *

(Whereupon, the workshop was recessed, at 10:05 a.m.)

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Friday Morning, March 23

SESSION VI - EXPERIMENTAL STUDIES
Chairman: Dr. Edward Bresnick
Eppley Institute for Research
in Cancer and Allied Diseases

SESSION VI - EXPERIMENTAL STUDIES
Chairman: Dr. Edward Bresnick, Eppler Institute

DR. BRESNICK: My name is Bresnick. I am to chair this sixth session of this conference. The first paper of this session is Carcinogenic Potential of Foundry Pyrolysis Effluents. It's by Dr. Moorman of NIOSH.

PROCEEDINGS OF THE
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PRESENTATION:

Carcinogenic Potential of Foundry Pyrolysis Effluents

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CARCINOGENIC POTENTIAL OF FOUNDRY PYROLYSIS EFFLUENTS

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ABSTRACT

Recent epidemiologic studies in the United States and abroad indicate that certain foundry workers, particularly molders and metal pourers, have a higher than normal risk of lung cancer. The current study was conducted as part of an effort to determine whether the increased risk is due to the presence of carcinogens in mold emissions. Emissions from molds composed of four types of binders, including furan (urea-formaldehyde-furfuryl alcohol), green sand with sea coal, urethane (phenolic-isocyanate), and phenol-formaldehyde resins in shell molds were collected, analyzed, and tested for their ability to induce cancer when administered repeatedly to the respiratory tracts of hamsters by intratracheal instillation.

The maximum tolerated dose was instilled weekly for 18 weeks. The dose was 10 mg per instillation for green sand, shell, and furan. The urethane mold effluent, being more acutely toxic, required a 5 mg/installation. A positive control received 3 mg BaP + 3 mg ferric oxide. A second group received half the maximum tolerated dose.

The mold effluent-treated groups all demonstrated very low incidences of tumors even though the life-holding period was held to 98% mortality.

Acknowledgment:

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INTRODUCTION

Numerous investigations of the mortality rates of foundrymen have indicated that workers in certain areas of the foundries experience an elevated risk of lung cancer. Several of these studies indicated that molders and casters are the foundry workers at highest risk of dying from lung cancer. In most cases these data reflect conditions which were present in the foundries at least 30 years ago. At that time, molders were pouring metal into the molds that they produced. Today, it is unusual for molders to pour metal into their own molds; however, a new group of worker (referred to as casters or pour-off men) have assumed this responsibility. Other changes which have occurred in the foundries are the use of new molding systems composed of synthetic chemical binders. Most epidemiological studies do not reflect the exposure of workers to fumes from molds composed of these new synthetic chemical binders; it is only recently that the chemical and physical properties of emissions from these and from the more traditional green sand molds have been studied.

The major health hazards in the foundry industry are respiratory diseases due to high concentrations of dusts and gases. Table I lists the major reports on this subject. The first three investigations were based on census figures and death records. In these studies, it was found that foundry workers and, in particular, molders and casters have higher lung cancer rates than workers in other occupations. In 1938 Turner and Grace reported that foundry workers in Sheffield, England, had the highest lung cancer rates among the 12 occupations

examined(1). In the following year, the Registrar General of Great Britain reported that molders and metal pourers ranked fourth among occupations with high lung cancer rates in London and Wales(2). Milham later reported similar findings for foundry workers in the State of Washington(3). In a recent NIOSH study, based on the death records from the International Molders and Allied Workers Union's death benefit program, Egan and coworkers reported that there is a 1-1/2 fold excess of lung cancer cases among foundrymen(4).

The two epidemiological studies which are perhaps the best known were performed in Finland by Koskela et al.(5) and in Canada by Gison and coworkers(6). Both studies found that molders and cleaning room operators were at a two- to threefold higher than normal risk of developing lung cancer. The Canadian group also found that crane operators are at an elevated risk of lung cancer, but this was not found to be the case in another study of crane operators in steel foundries performed by Lerer et al.(7). Finally, in a recent report of the lung cancer rates in workers in a U.S. foundry, Decoufle and Wood reported that there was a twofold excess of lung cancer in workers with more than five years' exposure in the foundries(8).

Benzo(a)pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAH) have been identified in condensates on particles emitted from molds using both traditional binders and the modern synthetic chemical binders. The concentrations of BaP liberated from dry sand and green sand molds were substantially higher than those liberated from molds composed of the newer synthetic chemical binders(9). Several reports indicate that the addition of sea coal to green sand molding aggregates does not contribute to the formation

of PAH but that coal tar pitch may be a major source of the PAH liberated from green sand molds(10,11,12). In vitro bioassays, performed with organic extracts of dusts collected from a green sand foundry, indicate that the mutagenic activity of samples is greatest when the foundry is operating at full capacity. At such times, compounds other than PAH make the greatest contribution to the mutagenic activity of the extracts of the dust samples(13).

We hypothesize that an elevated risk of lung cancer exists in certain areas of foundries as a result of exposure to thermal decomposition products evolving from pyrolyzed mold sand binders. Furthermore, the complex emissions vary with different binder composition, resulting in different carcinogenic potential.

The purpose of this project was to collect emissions from green sand molds and from molds composed of some widely used synthetic chemical binders in preparation for bioassays of their carcinogenicity in hamsters. Emissions were collected from molds composed of four types of binders, including furan (urea-formaldehyde-furfuryl alcohol), green sand with sea coal, urethane (phenolic-isocyanate), and phenol-formaldehyde resins in shell molds. The emissions were collected, analyzed, and prepared for tests of their ability to induce cancer when administered repeatedly to the respiratory tracts of hamsters by intratracheal instillation. This work can be divided into three distinct phases: collection of foundry mold emissions, analysis of samples collected, and the carcinogen bioassay (Figure 1).

EXPERIMENTAL APPROACH

Phase I. Collection of Foundry Mold Emissions

a. Foundry Selection

The important criterion in the selection of foundries was that there were no emissions present from sand binders other than the particular binder being studied. Therefore, it was essential to collect samples from molds in which cores, when present, were composed of the same chemical binders as the molds. Also, reclaimed sand could only be taken from pouring lines using the same chemical binders as the molds being sampled in order to avoid contamination from other resins remaining associated with the recycled sand.

Mold emissions were collected in foundries which poured gray iron into molds under conditions in which emissions from cooling molds could be collected in the absence of significant cross-contamination by airborne emissions from other processes, such as melting or shakeout. In an actual foundry operation, this is difficult to accomplish. To help achieve this, samples were collected in isolated areas, such as cooling tunnels, wherever possible. Thus, foundries were sought in which cross-contamination was minimized either by appropriate local ventilation of nearby processes or by the absence of other fume- or dust-emitting processes in the vicinity of the mold cooling area.

b. Sampler

The large quantities of concentrated materials required for the bioassays necessitated the use of a high volume sampler from which the samples could be retrieved in a concentrated form. Since the samples had to be administered to animals in an aqueous medium, a sampler which could trap mold emissions in water was desired.

Other requirements of the sampling method were that it efficiently collects particles between 0.5 and 10 μm in diameter and excludes particles larger than 10 μm in diameter; that collected particles be maintained as a fine dispersion rather than as agglomerates or a condensed tar; that the sampler had to be portable and compact to facilitate its use in the foundries; that it had to maintain the physical and chemical integrity of collected mold emissions; and that organic solvents could not be used to retrieve the sample from the matrix of the collection device. The latter criterion was established because several studies have indicated that the potency of carcinogenic PAH may be influenced by their adsorption or condensation on the surface of respirable particles(14,15). Some carcinogenic PAH and mutagenic compounds are known to be present in mold emissions as condensates on the surface of fine particles(13,10). The use of organic solvents could remove these compounds from the surface of particles and, thereby, alter the toxicity of the samples.

Established sampling methods that were considered included cold traps, solvent impingers, cyclones, electrostatic precipitators, and porous membrane filters. Of these, the first three were rejected since they cannot efficiently collect particles less than 1 μm in diameter. A high volume electrostatic precipitator was tested and rejected because ozone, which is known to interact with PAH and other organic compounds(16), was generated during its operation. Methods which trap particles on filters were also excluded since recovery of the particles from the matrix of the filters could be difficult.

Since established methods for the collection of large quantities of airborne materials which met the criteria for this study could not be identified, a sampler (based on the principle of the venturi scrubber used in industrial stacks) was designed. The sampler (shown in Figure 2) consisted of a train of three components. The first was a cyclone precleaner which removed particles greater than 10 μm in aerodynamic diameter; the second, a venturi scrubber which trapped respirable particles in water droplets; and the third, an entrainment separator in which trapped particles were removed from the airstream. The details of this sampling system are fully described by Palmer, Scott and Moorman(7).

This system afforded a means for collecting particles in the desired size range (0.5 to 10 μm) as well as water-soluble gases and vapors. Samples could be rapidly collected (up to 1 g per hour

could be collected under the sampling conditions used in some foundries) and could be retrieved from the sampler in a highly concentrated form. Furthermore, since the airborne materials were collected directly into water, samples were virtually ready for administration to animals as soon as the samples were removed from the collection device. This represented a considerable advantage both in terms of convenience and from the standpoint that virtually nothing was known about the stability of the materials being collected; therefore, it was essential to minimize the handling of samples after collection to reduce the possibility of denaturation.

Figure 1 shows a schematic drawing of the scrubber. Air enters a cyclone precleaner at the rate of 100 cfm. This cyclone eliminates particles with diameters in the range of 10 microns and higher. The rejected particles gather in the bottom of the cyclone precleaner. The air discharged from the cyclone passes into the top of the venturi scrubber where it impinges on a stream of water. When this occurs, the water is dispersed into a mist. Particles greater than 0.2 microns are captured by the water droplets. The airstream takes a downward spiraling path through the scrubber and is then directed into the entrainment separator. Here, by cyclonic action, the particle-laden mist coalesces into water droplets which collect in a sump at the bottom of the separator. Water is removed from the sump and is recirculated to the venturi scrubber, where it is reused for further sample collection. In this way, the concentration of collected materials in the water increases with time.

Phase II. Analytical Characterization Phase

The samples collected from the cooling molds consist of a water-soluble fraction and a particulate fraction that was relatively insoluble in water. From our work, as well as from data published by other authors, it is known that emissions from cooling molds contain a wide range of chemical and mineral components. Among the determinations performed were measurements of the concentrations of eight polycyclic aromatic hydrocarbons in both the water-soluble and insoluble fractions of the samples. The levels of some of the phenols in the aqueous phase and the concentrations of metals in the whole sample were also measured. The total amounts of effluent collected was determined by measuring the percentage of total dry weight of the samples.

The elemental analysis of a typical foundry sample is shown in Table 2. Sixteen elements were determined by atomic absorption. A separate determination of silica gave the value of 3.3%. A comparison between the metal analyses of the emissions from the four types of mold binders indicated that the mold binder had no apparent effect on the metal concentrations of the mold effluents. The concentrations of certain metals were consistently lower than what might be expected in the emissions on the basis of the metallurgical content of the poured iron. These data show that the metals that are most readily released into the mold emissions are those with the highest vapor pressure at the pouring temperature.

Hundreds of PAH's have been identified evolving from pyrolyzed organic material similar to the binder chemicals used in the four foundry mold types being studied. Eight PAH's were selected for analysis to characterize complex effluent mixtures by such features as number of arene rings, nitrogen content, and heterocyclic character.

The concentrations of the PAH's in the cyclohexane extracts of particulates from all four foundry samples are shown in Table 3. The data are presented in terms of μg PAH per gram particulate. Since two of the GC/MS peaks contained two PAH each, the analyses were also performed by HPLC (high pressure liquid chromatography). Although this technique tends to be more subject to interferences for PAH than is GC/MS, it can differentiate between certain combinations of compounds such as benzo(a)anthracene and chrysene, which were not distinguished by GC/MS. Table 3 shows the combined concentrations, as determined by GC/MS, of benzo(a)anthracene and chrysene. Using HPLC, it was found that chrysene represented a considerably greater portion of the total benzo(a)anthracene/chrysene GC/MS peak than did benzo(a)anthracene in three of the four foundry samples. Of these two compounds, only chrysene was detected in the furan sample; also, there was approximately four times as much chrysene as benzo(a)anthracene in the urethane and shell samples. The opposite situation occurred in the green sand sample which had about five times as much benzo(a)anthracene as chrysene. There were no detectable quantities of either acridine, carbazole, or dibenz(a,h)anthracene in any of the samples. Naphthalene was found in only the

urethane sample and phenanthrene was present in all four samples. Measurable quantities of benzo(a)pyrene were only present in the shell and green sand samples.

The last series of compounds that were determined were the phenols. They were studied because of their ability to promote tumors in some animal bioassay systems. These are all highly soluble in water and, hence, only the aqueous phase was examined. Of these compounds, phenol itself was the most prevalent and was found in all four foundry samples. 4-Nitrophenol, the second most prevalent phenol, was present in all but the green sand sample. 2-Nitrophenol was found in the shell sample only, and 2,4-dimethylphenol was found in the shell and urethane samples (Table 4).

Phase III. The Carcinogen Bioassay

The bioassay was performed in two parts. First, acute range-finding studies were completed to determine maximum tolerated doses (MTD) and second, the life-long studies were conducted. This approach is similar to that described by Saffiotti et al(14).

Golden Syrian hamsters (7 weeks of age) of both sexes were purchased from Charles River Breeding Laboratories (Wilmington, MA) following serological screening. Prior to placement on study, all animals were quarantined for a period of two weeks. All animals were individually

housed in solid polycarbonate cages with sterile bedding and suspended from stainless steel racks equipped with nonwoven filter bonnets. Animals were kept under optimum hygienic conditions in environmentally controlled rooms.

In the acute range-finding study, four different doses of the foundry samples were administered to groups of 20 animals (10 males and 10 females) once a week for five weeks. The doses used for this study were 20, 10, 5, and 2.5 mg per instillation. Prior to dosing, the animals were anesthetized with an intraperitoneal injection of 0.5 mL of a 1% solution of Brevital[®] Sodium. The test articles were administered intratracheally through a 0.25 mL tuberculin syringe fitted with a blunt, bent 19-gauge needle. A dosing volume of 0.2 mL was used for all groups.

Throughout the study, the animals were routinely examined for signs of ill health or clinical symptoms of toxicity such as moribundity, mortality, failure to gain weight, lethargy, diarrhea, blood in the excreta, or other signs of obvious distress. One week after the fifth and final dose was administered, the animals were killed and the external and internal organs inspected for signs of gross abnormalities. These pathological and clinical observations served as the basis for determining doses for the chronic bioassays.

For the chronic bioassays, the maximum tolerated dose and one-half the maximum tolerated dose of each foundry sample were administered to two groups of 100 animals (50 males and 50 females per group) for 18 weeks. The doses for the green sand, furan, and shell mold emissions were 10 and 5 mg per instillation; those for the urethane emissions were 5 and 2.5 mg per instillation. The animals were held until 98% died. At that time, the remaining animals were sacrificed and examined by a qualified pathologist who evaluated the tissues of the respiratory tract and the other major internal organs for the presence of tumors, the tumor type, and the frequency of occurrence of each type of tumor.

All samples were titrated to a pH of 7.0 prior to instillation with 0.1 normal NaOH. Also a positive and negative control treatment was used. The positive control group received 0.2 mL of a mixture of 3 mg benzo-(a)pyrene and 3 mg of ferric oxide; the negative control group received 0.2 mL of distilled water. All treatment groups for the chronic bioassay consisted of 50 male and 50 female hamsters.

BIOASSAY RESULTS (Preliminary findings)

The scheduled final report will not be completed until May 1984; therefore, only some of the findings are available at this time. Most of the summaries of data have not been statistically evaluated and therefore are subject to review.

I. Survival

Table 5 presents the mean survival time in weeks for each treatment. A general observation is that the male hamsters outlived the females with 98% termination point occurring at 117 weeks in males and 103 weeks in females.

II Pathology

A. Respiratory System

No induced neoplasia was recognized in the treated hamsters other than in positive controls. Alveolar/bronchiolar cell hyperplasia was present to some degree in nearly all the treated hamsters. These reactions were intimately associated with aggregates of brownish-black pigment, presumably the induced compound. In a few of the hamsters, this cellular proliferation was extensive enough to classify as adenomatosis. Inflammatory changes were common in all groups, presumably enhanced by the treatment. Similar inflammatory changes and deposition of pigment was present in the tracheal/bronchial lymph nodes. Carcinomas were readily induced in the lung with the positive control having occasional metastases to the regional lymph nodes.

B. Endocrine System

Most of the neoplasms observed in this study occurred in this organ system. There was a fairly high incidence of cortical tumors in the adrenal of both sexes with a slightly higher trend in the males. Follicular cell tumors of the thyroid were present in most groups with a slightly higher incidence in the females receiving 20 mg of green sand. Parathyroid tumors and hyperplasia were scattered through most all groups with a slightly higher incidence in males receiving 10 mg of green sand and females dosed with 10 mg of shell. Moderate to severe amyloidosis was common to the adrenal and thyroids of both sexes. The above lesions are frequently present in aging hamsters and are probably not significant.

C. Gastrointestinal System

Nearly all of the lesions of any consequence in this system occurred in the liver. Other than an occasional bile duct tumor, no primary tumors were present in this organ. Degenerative and inflammatory lesions, when present, were not considered to be treatment related.

D. Cardiovascular System

Atrial thrombosis was a frequent finding in all groups, as were the secondary effects of this in the liver and lungs. Atrial thrombosis is a common spontaneous lesion in older hamsters.

E. Urogenital System

The kidney was the only organ in either sex that presented any lesions of consequence. Amyloidosis, glomerular degenerative changes, and nephritis were seen in varying degree of severity and incidence in both sexes with a higher incidence and severity in the males. Again, these changes were anticipated in aging hamsters and do not appear to be influenced by treatment.

F. Lymphatic/Hematopoietic System

Lymphomas and plasma cell tumors were present in small numbers with what appears to be an increased incidence in the males treated with 10 mg of green sand. No significant lesions were observed in the integument, nervous, or musculo-skeletal systems.

DISCUSSION AND CONCLUSIONS

The primary objective of this project is to evaluate the potential carcinogenicity of complex mixtures in foundry mold effluents. In pursuit of this objective, a number of accomplishments have occurred. A custom-designed, high volume, wet venturi scrubber was developed; this will provide technology for future investigations requiring large quantities of complex airborne emissions for bioassays. Additionally, analytical characterization of emissions from cooling molds of the four commonly used types has been completed.

These analyses showed that more PAH are present in the emissions collected from shell and green sand molds than in those from urethane and furan molds. Phenols, which were present in the emissions from all four mold types, were most prevalent in those from urethane molds. Certain toxic or biologically active metals such as lead and zinc, although present in only trace or undetectable amounts in the poured metal, were present in readily detectable quantities in some mold emissions as a result of their high vapor pressures.

One could speculate that if the particulate fraction alone from the shell and green sand emissions were bioassayed that a significant incidence of lung tumors would have resulted. This approach, however, would not have included the relevant mixtures to which the respiratory tract is exposed. Certainly, the effects of the aqueous fraction which includes promoters and other toxic substances must be considered. As a result of the relatively high acute toxicity of the samples, considerably less of the suspected carcinogenic fraction was administered. This probably explains the low tumor incidence.

In conclusion, many factors contribute to the chronic toxicity or carcinogenicity of foundry atmospheres. The actual concentrations of substances that can act as chemical carcinogens, the presence of other compounds which can act as tumor promoters or cocarcinogens, and the characteristics of the particulates with which they are associated all play a crucial role in determining the health hazards associated with inhaled pollutants. Long-term animal tests are currently accepted as the best available method for assessing the combined potential toxicity; however, the distinct difference of acute toxicity from the long-term toxicity presents a formidable challenge to realistic bioassay designs.

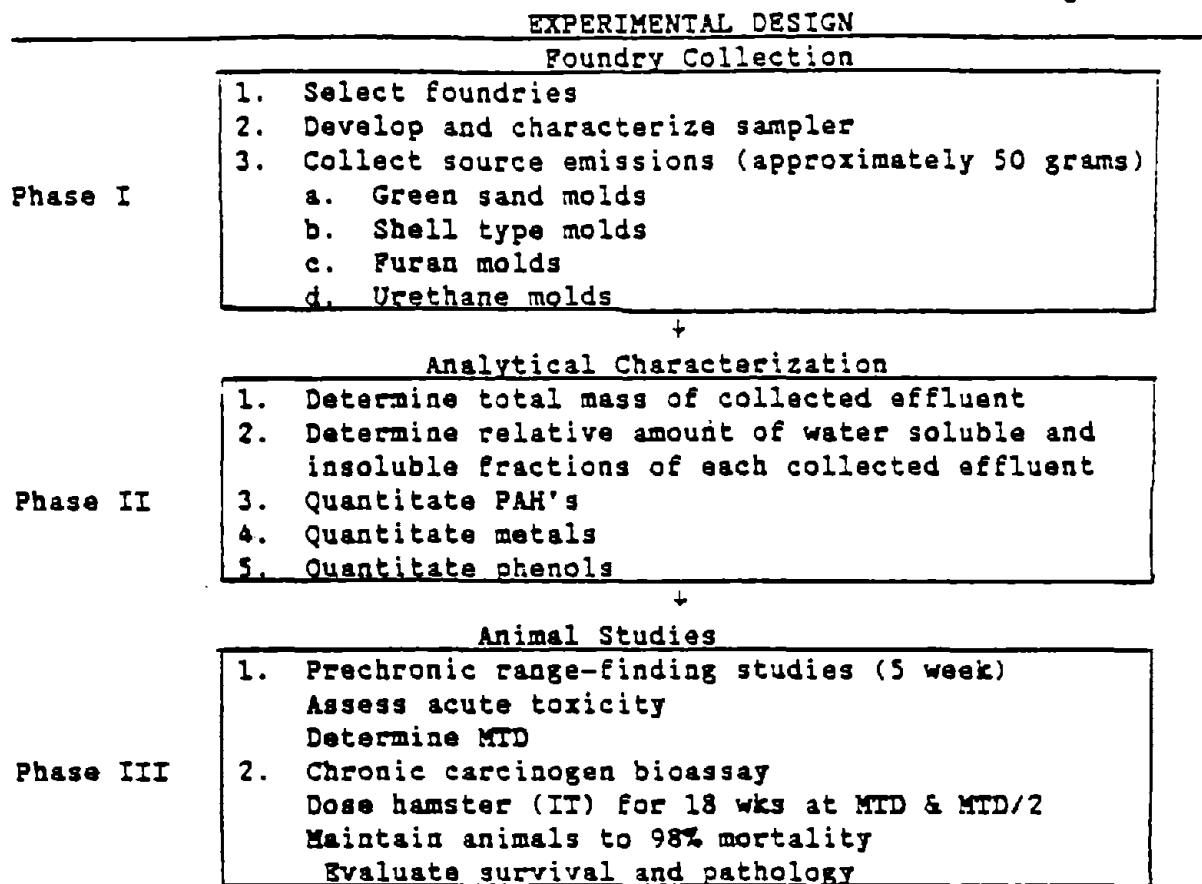
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Figure 1



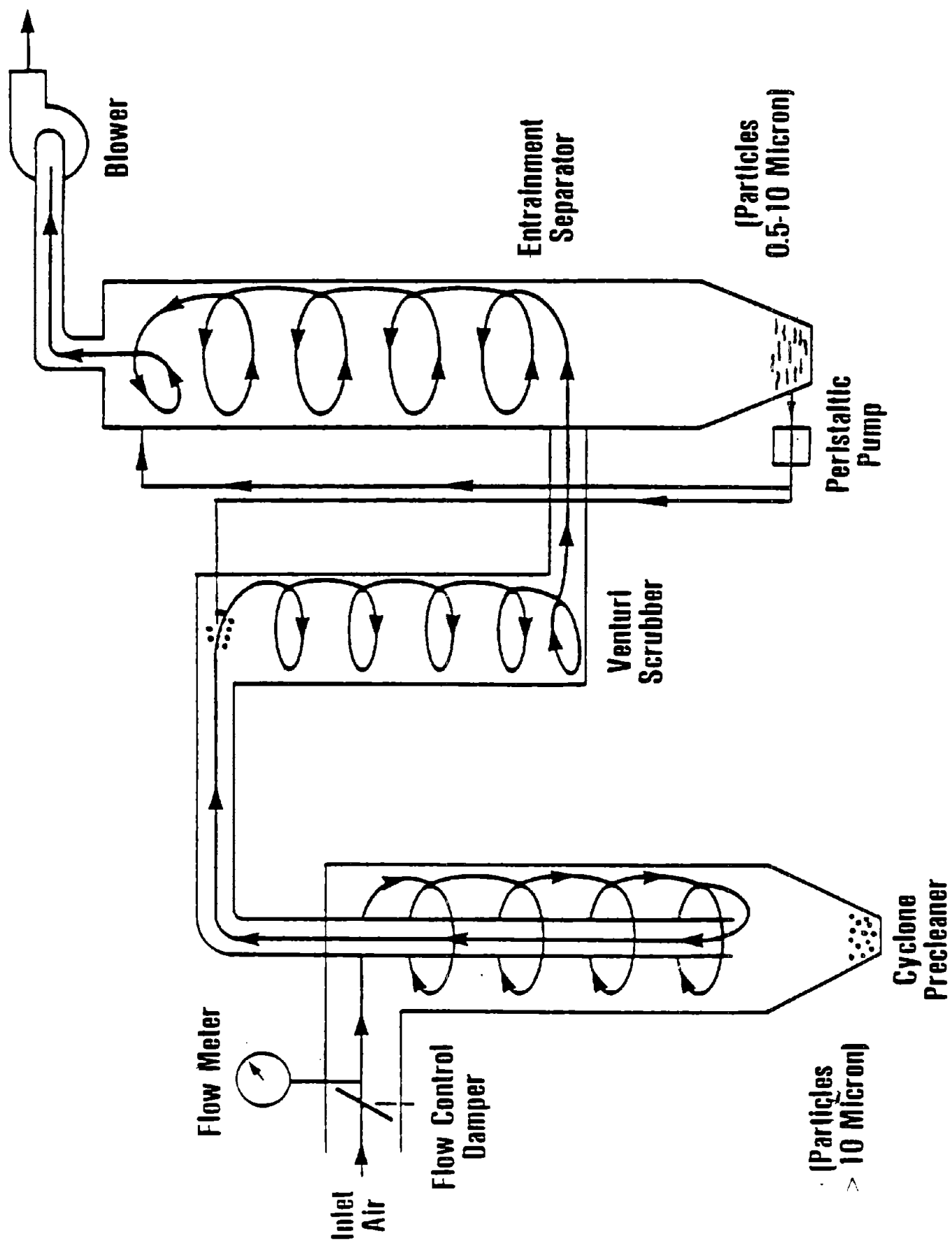


Figure 2

Table 1. REPORTS OF EXCESS LUNG CANCER AMONG IRON FOUNDRY WORKERS

Registrar General, Great Britain, 1939

Molders and pourers ranked 4th among occupations with high lung cancer rates

Turner and Grace, 1938

Foundry workers had highest lung cancer rates among 12 occupations in Sheffield, England

Milham, 1976

Molders had excess cases of lung cancer in Washington State

Egan et al., 1979

1.5-fold excess lung cancer among participants in Union Death Benefits Program

Koskela et al., 1976

>2-fold excess among molders with >5 years' exposure

Gibson et al., 1977

>2-fold excess among crane operators, molders, and finishers

Decoufle and Wood, 1979

>2-fold excess among all workers with >5 years' exposure

Table 2. Elemental Analysis of a Typical Sample of Mold Emissions

<u>Metal</u>	<u>Concentration</u> <u>(mg/gm sample)</u>
Aluminum	0.31
Arsenic	0.013
Beryllium	0.003
Cadmium	0.022
Chromium	<0.0055
Cobalt	0.055
Copper	3.9
Iron	8.08
Lead	0.36
Magnesium	0.023
Manganese	1.71
Mercury	<0.0001
Nickel	0.03
Silicon	1.6
Tin	0.114
Zinc	1.6

Table 3. CONCENTRATIONS OF PAH AND RELATED COMPOUNDS IN CYCLOHEXANE
EXTRACTS OF WATER-INSOLUBLE PARTICULATE ($\mu\text{g/g}$ PARTICULATE)

	COMPOUND	FURAN	URETHANE	SHELL	GREEN SAND
0	ACRIDINE	< 0.6	< 0.6	< 0.6	< 0.6
0	NAPHTHALENE	< 0.6	12	< 0.6	< 0.6
0	CARBAZOLE	< 0.6	< 0.6	< 0.6	< 0.6
0	PHENANTHRENE	31	230	1500	7200
+	BENZO(a)ANTHRACENE}	< 0.6	5.4	350	1100
+,0	CHRYSENE }				
++	BENZO(a)PYRENE	< 0.6	< 0.6	270	230
++	DIBENZO(a,h) ANTHRACENE	< 0.6	< 0.6	< 0.6	< 0.6

(1) 0.6 lower detectable level

(2) 0 noncarcinogenic

+ weakly carcinogenic

++ strongly carcinogenic

Table 4. CONCENTRATIONS OF PHENOLS IN AQUEOUS PHASE (ug/g SAMPLE)

COMPOUND	FURAN	URETHANE	SHELL	GREEN SAND
PHENOL	2000	50000	1600	1000
PENTACHLOROPHENOL	3	< 2*	< 2	< 2
4-NITROPHENOL	48	420	1800	< 2
2-NITROPHENOL	< 2	< 2	2300	< 2
2,4-DIMETHYLPHENOL	< 2	21	60	< 2

*Lower level of detection

Table 5. Mean Survival Time

Group	Treatment	Dose Level (mg/0.2 mL)	50% Survival (Week)	
			Male	Female
1	Vehicle Control	-	80	74
2	Ferric Oxide + Vehicle Control	3.0	87	70
3	B(a)P + Ferric Oxide	3.0 + 3.0	42	53
4	Shell	5.0	87	70
5	Shell	10.0	85	72
6	Green Sand	5.0	76	73
7	Green Sand	10.0	77	74
8	Furan	5.0	88	70
9	Furan	10.0	78	68
10	Urethane	2.5	82	72
	Urethane	5.0	80	68

98% Mortality occurred at 117 weeks in males and 103 week in females

Table 6.

Treatment		Dose		Organ Systems (Total Tumors)						
				Respiratory	Endocrine	Gastro- Intestinal	Uro- Genital	Lymphatic Hematopoietic	Total	
Vehicle	0	-		1	10	-	-	-	11	} 17
Control	0	-		1	4	-	1	-	6	
FeO ₃ +	0	3.0		5	8	-	1	1	15	} 25
Vehicle	0			1	7	-	1	1	10	
B(a)P +	0	3.0 +		34	2	3	-	2	42	} 83
FeO ₃	0	3.0		35	3	1	-	2	41	
Shell	0	5.0		--	11	-	-	-	11	} 20
	0			--	9	-	-	-	9	
Shell	0	10.0		--	12	1	-	-	13	} 22
	0			--	9	-	-	-	9	
Green	0	5.0		--	9	3	-	-	12	} 23
Sand	0			--	6	-	-	5	11	
Green	0	10.0		--	8	-	-	-	8	} 19
Sand	0			--	11	-	-	-	11	
Furan	0	5.0		--	4	-	1	-	5	} 8
				--	1	-	2	-	3	
Furan	0	10.0		--	7	-	-	-	7	} 13
	0			1	4	1	-	-	6	
Urethane	0	2.5		1	13	-	1	-	15	} 19
	0			--	4	-	-	-	4	
Urethane	0	5.0		-	18	-	-	-	18	} 36
	0			1	12	-	1	4	18	

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Cocarcinogenicity of Foundry Particulates in Hamsters

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COCARCINOGENICITY OF FOUNDRY PARTICULATES IN HAMSTERS

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ABSTRACT

Respirable size particulates, with more than 99 percent of each sample less than 10 μ m prepared from commercial samples of silica, olivine, zircon and chromite sands, aluminum silicates, ferric oxide, and a reference quartz (Minusil), and extensively characterized by chemical and physical methods, were administered in saline by intratracheal instillation at dose levels based on surface area standardized to Minusil. Groups of 50 male Syrian golden hamsters received 15 weekly administrations of either particulate plus 3 mg benzo(a)pyrene (B(a)P) or particulate alone. Hamsters were killed when moribund or at 92 weeks after the first treatment and tissues were examined by histopathologic techniques.

Survival was decreased ($p < 0.01$) in groups receiving Minusil without B(a)P and also significantly decreased in six of 12 particulate-B(a)P groups as compared to their respective particulate controls. Pulmonary tumors were observed in every group receiving B(a)P. Except for aluminum (60), corrected tumor incidences were significantly greater ($p < 0.006$) than the saline-B(a)P group, but were not different from the ferric oxide-B(a)P positive control group. The mean percent of lung tumor bearing animals ranged from 47 to 90, the former representing the saline-B(a)P group and the latter the Minusil-B(a)P group. The mean latency, calculated on the basis of necropsy date, ranged from 60 to 70 weeks, as compared to 73 weeks for the saline-B(a)P control. The most frequently observed tumor types were squamous cell and adenosquamous carcinomas and adenocarcinomas of the lungs and bronchi. No lung tumors were found in the saline control and very few groups receiving particulates alone developed lung tumors, i.e., ferric oxide (1/41), Minusil (1/50), zircon (F1) (2/42), and aluminum (70) (1/49). No pulmonary fibrosis was observed; however, significant increases ($p < 0.0001$) in granulomatous inflammation were observed in groups administered silica sand, Minusil and the aluminum (48) silicate.

Using a relative index of respiratory toxicity, developed from data including tumor bearing animals at risk, tumor types, total tumors, mean latency and granulomatous inflammation bearing animals, the following were ordered in decreasing toxicity when the particulate was administered with B(a)P: Minusil, Minusil + ferric oxide, olivine (Wa), zircon (Au), silica sand, aluminum (48), olivine (Nc), chromite, zircon (F1), ferric oxide, aluminum (70), aluminum (60), saline. It can be concluded from this study that: (1) neither of the crystalline quartz materials were carcinogenic in the hamster model; (2) silica sand and almost all substitute aggregates used in foundry mold production were cocarcinogenic; (3) the hamster is a poor model for studying fibrotic potential; and (4) granulomatous inflammation does not influence tumor development in this model.

INTRODUCTION

In the U.S. during 1978, there were over 400,000 production workers employed by more than 4,400 foundries. Ferrous type castings accounted for 85 percent of the total castings produced by these foundries; the remaining castings were aluminum (10 percent), zinc (2 percent), copper (2 percent) and magnesium (1 percent) (Palmer and Scott, 1981).

The foundry process basically involves the following steps: melting and alloying the metal; preparing the sand mold and cores; pouring of the molten metal into the mold; cooling of the mold and castings; shakeout, where the hot castings are separated from the molds; and finishing by blasting and grinding the castings with abrasives.

The molds used for metal casting are usually composed of silica sand. However, the tradition of using silica sand for metal casting is slowly being changed to the use of non-silica aggregates because of increased demands for improved cast surfaces and closer dimensional tolerances, and because of the physical, chemical and biological limitations of silica sand. The major non-silica aggregates presently used throughout the metal casting world include zircon sand, olivine sand, chromite sand and aluminum silicates (Kotzin, 1981). However, little is known about the biological effects of these materials.

The performance of some non-silica mold/core materials had been evaluated as early as the 1920s. However, their use was not established until casting designs became more complex and producers could supply aggregates of more uniform and consistent quality. Early use of these non-silica sands was investigated primarily for their characteristics and properties at elevated temperatures. Wettability by steel, thermal expansion, heat diffusivity, refractory characteristics and cost are the major factors when considering their use (Kotzin, 1981). Silica sand substitutes, such as olivine, may reduce some adverse biological effects such as silicosis, and routine use of olivine in place of silica sand was established in the 1940's in the Scandinavian countries (Tubich, 1967).

Recent epidemiological studies have concluded that there is a significant excess of lung cancer, as well as nonmalignant respiratory disease among foundry workers (Egan, et al., 1977). Palmer and Scott (1981) in their review article addressing the cancer problem in the foundry environment, discussed the possible agents responsible for contributing the hazard. These included various classes of carcinogens such as polynuclear aromatic hydrocarbons and other organic compounds, metals, cigarette smoking, and promoters/cocarcinogens, such as irritants and particulates.

The cocarcinogenic effect of particulates has been well established by use of the intratracheal instillation hamster model for studying lung cocarcinogenesis. Ferric oxide (Saffioti, 1968), carbon (Farrell and Davis, 1972), titanium dioxide (Stenback, et al., 1976), magnesium oxide (Stenback, et al., 1975), and amorphous silica (Stenback and Rowland, 1979) in combination with the ubiquitous carcinogen, benzo(a)pyrene (B(a)P) significantly increased the number of pulmonary tumors, compared to B(a)P alone. However, the results with aluminum oxide (Farrell and Davis, 1972; Stenback, et al., 1976) and manganese oxide (Stenback and Rowland, 1979) do not show a cocarcinogenic effect. Since the cocarcinogenic effect of particulates in combination with B(a)P administration cannot be generalized, it is necessary to study each material.

This study was designed to focus on two aspects of the pulmonary pathology experienced by foundry workers, silicosis and cancer as caused or modified by foundry aggregates. The purpose of this investigation was fourfold: first, to assess the chronic toxicity of foundry mold aggregates; secondly, to assess the cocarcinogenic potential of silica sand; thirdly, to assess in the animal model the cocarcinogenic potential of silica sand substitutes including, olivine from 2 geographical sources, chromite sand, zirconium silicates from 2 geographical sources and aluminum silicates of 3 types; and finally, to assess the relative fibrogenic activity of these materials as compared to a reference quartz (Minusil).

MATERIALS AND METHODS

Prior to initiating the animal phase of the study, selected physical and chemical parameters for the various particulates were evaluated. The goal was to introduce particles into the hamster in the respirable size range, i.e., less than 10 μ m. The analyses performed for each material included particle size, surface area, fiber counts, free silica and chemical content of bulk samples and of 1,000 individual particles. Radon daughter concentrations were determined on bulk samples of the coarse zircon sands.

A total of 11 materials were characterized (Stettler et al., 1981). Nine of these were aggregates used in foundries; the tenth, Minusil glass sand, was used as a positive control for fibrogenic response; ferric oxide was used as the positive cocarcinogen control. A list of the materials and their suppliers is given in Table 1. The nine foundry sands required further processing because they did not meet the criterion of being of respirable size.

A standard 325 mesh, 30-inch stainless steel screen was used to eliminate the oversize (+44 mm) fraction of the coarse materials. Because air classification required a minimum of 5 kg of the -325 mesh starting material, air classification (Donaldson Co., Model B-18 Acucut) was performed only for the aluminum silicate sands. The -325 mesh fraction for the other sands was much less than 5 kg. Consequently, where possible, commercial "flour" samples (-325 mesh) were used instead of the bulk samples to prepare the respirable fractions. Commercial flour samples were available for the silica sand and the two zirconium sands. Respirable fractions of these three materials were also prepared with the air classifier. No flour samples were commercially available for the olivine samples and the chromite sand. Samples of the coarse material for each of these sands were micronized in a Trost mill (Garlock, Inc., Model Gem T) to prepare the respirable particulates. The mill used in this operation had a polyurethane liner to minimize metal contamination.

The results of the physical and chemical analyses were previously described by Stettler, et al. (1981) and are summarized here for the convenience of the reader. Aliquots from stock suspensions of the respirable fractions were used to prepare duplicate filters for particle and fiber analyses. Samples were also prepared from the bulk coarse sands and bulk flours so that any changes in chemical composition imparted to the processed respirable particulates would be known. For particle sizing and individual particle chemical analysis of the processed materials, one of the filters was examined directly with a scanning electron microscope equipped with an energy dispersive x-ray

spectrometer system and an image analysis system using the backscattered electron image. The second set of filters, plus the blank filters and filters for the bulk coarse and flour sands, were used for fiber analysis using a transmission electron microscope equipped with an energy dispersive spectrometer system. Fibers from each sample were counted, sized, and then analyzed by energy dispersive x-ray analysis (EDXRA) and selected area electron diffraction (SAED). Surface area determinations for each of the respirable sand fractions were performed by nitrogen adsorption using an automatic surface area analyzer (Model 2200, Micromeritics Co., Norcross, GA).

Bulk chemical analyses were performed on aqua regia digests of each of the nine respirable dusts plus the Minusil sample. Analysis by atomic absorption spectrometry (equipped with a graphite furnace) was performed on each sample for beryllium, sodium, magnesium, and aluminum. Analysis for all elements heavier than aluminum (silicon through uranium) was performed simultaneously by proton-induced x-ray emission spectrometry (PIXE). All analyses were performed in duplicate.

The respirable fractions of the nonsiliceous foundry sands were analyzed for crystalline silica using x-ray powder diffraction (XRD). Interferences were encountered with the three aluminum silicate samples. These samples were treated with a phosphoric acid digestion technique to eliminate the interferences and then reanalyzed by XRD.

The B(a)P was prepared by dry grinding in a mullite mortar (Fisher Scientific Model No. 155) at 100 rpm for 24 hours. Fresh normal saline (sterile, nonpyrogenic, 0.9 percent NaCl in water--Abbott Laboratories) suspensions of this ground material were prepared weekly by ball milling for 7 days under optimum sterile conditions using a Fisher Model No. 753 Jar Mill at 290 rpm with porcelain jars fitted with teflon coated sealed tops and stainless steel balls. The jar mill apparatus was steam sterilized prior to each use. The suspension was stored in the dark at 50 C during the week of dosing. Any remaining suspensions were discarded at the end of each week. A light microscopy method (Coulter Counter-biaperture technique) was used to estimate particle size distribution of the B(a)P/saline suspension. This distribution was identified as the following:

Size (µm)	10	9	8	7	6	5	4	3	2	1
Percent, less than	80	75	70	64	58	50	40	30	19	7

To prepare the B(a)P + particulate suspensions an appropriate volume of B(a)P in saline suspension was pipetted from the weekly stock into a sterile flask. The correct amount of particulate was weighed and added to the above. To prepare the particulate suspensions, the appropriate amount of particulate was weighed and placed in a sterile flask and the correct volume of saline was added. The mixtures were stirred for 10 minutes on a magnetic stirrer. All suspensions were prepared daily just prior to instillation for the scheduled treatment group.

A total of 1520 out bred male Syrian golden hamsters, 4-6 weeks of age, were received in one shipment from Charles River Breeding Laboratories (Newfield, NJ) and were examined for general health and weighed. After a 26 day

quarantine period, acceptable 7 to 9 week old hamsters were randomly assigned to the treatment groups by computer generated random numbers and each animal was uniquely identified. The mean body weights for all groups were similar, ranging from 78 to 82 g.

Hamsters were housed individually in polycarbonate cages with sterile San-i-Cel bedding. The cages were suspended from stainless steel racks equipped with filter sheets. The cages and bedding were changed weekly and filter sheets changed biweekly. Feeders and fresh water bottles were provided weekly. Purina Lab Chow No. 5001 (certified) and water via water bottles were available ad libitum. Treatment groups, initially consisting of 50 hamsters each, were given either the particulate alone or in combination with 3 mg B(a)P (Table 2). The suspensions were administered by intratracheal instillation according to the method of Saffiotti, et al. (1968). An instillation volume of 0.2 mL was used and the suspensions were kept homogeneous by continuous magnetic stirring during the course of the instillation. The dose of each material was determined from the surface area data (Table 1) based upon the estimated maximum tolerated dose of Minusil taken as unity (0.7 mg X 15 instillations). For example, the estimated tolerated dose of Minusil (0.7 mg) had a total surface area of 0.0021 m² (0.0007g x 2.99 m²/g), and a comparable dose of chromite sand with a surface area of 1.01 m²/g was 0.0021 m² divided by 1.01 m²/g = 2.1 mg. The treatment schedule involved 15 weekly instillations. Hamsters were anesthetized with an intraperitoneal injection of 0.4 mL of one percent Sodium Brevital solution prior to instillation. For positive controls, ferric oxide + B(a)P were used for cocarcinogenesis and Minusil for fibrogenesis. The ferric oxide + Minusil group was included to test the combined effect. In addition, a ferric oxide alone and saline alone were used as negative controls and B(a)P in saline was used as an additional control.

Mortality and morbidity observations were made twice daily throughout the study and clinical signs of illness were made once weekly. Body weights were taken weekly during the first 15 weeks of treatment and at two-week intervals thereafter.

All surviving hamsters were killed 92 weeks after the first instillation by an overdose of Sodium Brevital. A terminal body weight was recorded prior to sacrifice. Complete necropsies were performed on those animals that died, were killed when moribund or killed at study termination and the lungs were weighed. Each necropsy consisted of an examination of the external body surfaces and orifices. The carcass was then opened and the following organs and tissues were examined in situ, dissected from the carcass, reexamined (including cut surfaces and mucosal linings), and preserved in 10 percent neutral buffered formalin: adrenals, brain, costochondral junction, esophagus, eyes, gallbladder, heart, kidney, large intestine, larynx, liver, lungs and bronchi, mammary gland, mandibular lymph nodes, mesenteric lymph nodes, muscle (thigh), nasal cavity, pancreas, pituitary, prostate, salivary gland, sciatic nerve, seminal vesicles, skin, small intestine, spinal cord, spleen, sternbrae (plus marrow), stomach, testes (with epididymises), thymus, thyroids and parathyroids, trachea, urinary bladder, tissue masses, and gross lesions.

The trachea and lungs were removed en bloc, weighed and infused with 10 percent neutral buffered formalin until the lungs were filled to normal inspiratory volume and then submerged in formalin for fixation. At the time of necropsy, location, size, number, shape, color, and texture of all gross lesions were recorded. Location, size, shape, and texture of tumors observed in the respiratory tract were also recorded.

Following a fixation period of no less than 48 hours, tissues were trimmed in preparation for complete histologic processing. Tissues were automatically processed using a Tissue-Tek III V.I.P. automated tissue processor (LAB-TEK Division, Miles Laboratory, Inc., Elkhart, Indiana). Processed tissues were embedded in paraffin using a Tissue-Tek II embedding system (Miles Laboratories, Inc.), sectioned at approximately 5 microns on a Lietz Rotary Microtome (Scientific Products, Columbia, Maryland), and the sections mounted on glass slides. The slides were stained with hematoxylin and eosin. Eyes were examined only if grossly abnormal and the spinal cord was examined in animals where neurologic signs had been present.

The following statistical analyses were performed on data derived from this study: (1) survival was statistically compared using a chi-square analysis; (2) the mean body weights of particulate + B(a)P groups were statistically compared to the particulate control groups at all recorded intervals, using a two-tailed Student's t-test; (3) mean lung weights were statistically compared using a Student's t-test; (4) tumor incidences were statistically analyzed using a two-tailed Fisher's Exact test; (5) the incidences of pulmonary granulomatous inflammation in the lung for each particulate control was compared to that of the saline control using a two-tailed Fisher's Exact test; (6) the numbers of nonrespiratory tract tumor bearing animals with hyperplasia of the larynx, trachea, lungs and the total respiratory system were compared, using a two-tailed Fisher's Exact test (The analyses compared each particulate control to its respective particulate + B(a)P counterpart); and (7) a two-tailed Fisher's Exact test, was used to analyze the number of hamsters with hyperplasia, tumors and hyperplasia with tumors in the stomach. Each particulate control was compared to its respective particulate + B(a)P counterpart.

In conducting the Fisher's analyses, a ratio of number of tumor bearing animals (numerator) to the effective number at risk (denominator) was performed for each set of groups compared. The same procedure was followed for the groups where a particular type of sand was compared either with another type of sand or with the addition of B(a)P. The granulomatous inflammation, hyperplasia of the respiratory tract and stomach tumors were also analyzed using the same procedure; i.e., a ratio of number affected (numerator) to the effective number at risk (denominator). The number at risk was determined as the number of survivors at the time of death of the first animal in either of the two groups which was found (upon histological confirmation) to have a tumor in the respiratory system. This procedure was used since the diagnosed tumors were nonobservable during the in-life phase of the study and therefore, the time of appearance could not be determined.

Only primary lung tumor bearing animals were used for the life table analyses. Only primary lung tumors were used for tumor incidences and tumor types. Animals with metastasized lung tumors were not used. Malignant lymphoma in the lung was not counted as a lung tumor since this tumor does not originate from lung tissue; therefore, hamsters which had malignant lymphoma as the only diagnosed lung tumor were not considered as tumor bearing animals for the purpose of lung tumor analyses. Animals with leukemia in the lung were also not considered.

RESULTS

Survival Analyses

Comparisons between the various particulate groups alone and the saline control revealed significantly decreased survival ($p < 0.05$) for the silica sand and the Minusil + ferric oxide groups. The survival of all other particulate groups was comparable to that of the saline control group.

Significantly decreased ($p < 0.01$) survival was also observed for hamsters treated with Minusil + ferric oxide + B(a)P as compared to the saline + B(a)P group. The survival rates of hamsters treated with chromite sand + B(a)P and olivine (Wa) + B(a)P were significantly lower ($p < 0.05$) than the saline + B(a)P groups. All other particulate + B(a)P groups showed survival similar to the saline + B(a)P group.

Comparison between each particulate control group and its respective particulate + B(a)P counterpart revealed significantly lower ($p < 0.01$) survival for hamsters treated with Minusil, zirconium (Au), chromite sand, and olivine (Nc) in combination with B(a)P as compared to hamsters treated with their matching particulate alone. Hamsters treated with zirconium (Fl) + B(a)P and aluminum (60) + B(a)P had decreased ($p < 0.05$) survival as compared to their respective particulate alone group. Hamsters treated with saline, ferric oxide, silica sand, Minusil + ferric oxide, olivine (Wa), aluminum (48) and aluminum (70) in combination with B(a)P survived comparably to that of their respective particulate alone group. Significantly decreased ($p < 0.01$) survival was noted for hamsters treated with silica sand or Minusil + ferric oxide without B(a)P as compared to that of the Minusil control.

No differences in survival were noted between the two zirconium silicates, the two olivine sands or among the three aluminum silicates. No differences in survival were noted among groups of hamsters treated with ferric oxide, Minusil or Minusil + ferric oxide in combination with B(a)P. In addition, no differences in survival were noted for hamsters treated with silica sand, Minusil, aluminum (48), aluminum (60) or aluminum (70) in combination with B(a)P.

Body Weights

Figure 1 displays those intervals where groups treated with particulate plus B(a)P were significantly lower ($p < 0.05$) than their counterparts receiving particulates alone. Nine of the groups, excluding the aluminum (48), aluminum (60), olivine (Wa) and saline plus B(a)P groups, showed prolonged significant

($p < 0.05$) weight loss during the 15 week treatment period. This weight loss continued beyond the instillation period in some particulate groups. However, there appeared to be no general correlation between prolonged lifetime weight loss and survival.

Lung Weights

The mean lung weights obtained at necropsy for each group with their corresponding standard deviations are presented in Figure 2. In the absence of B(a)P, significantly increased ($p < 0.05$) mean lung weights were observed for the silica sand, Minusil, and Minusil + ferric oxide particulate treated hamsters as compared to that of the saline control group. The saline plus B(a)P group also showed a significantly increased ($p < 0.05$) mean lung weight when compared to the saline control group. The mean lung weights of all other particulate groups were comparable to the saline control lung weights.

Mean lung weights for the ferric oxide + B(a)P, silica sand + B(a)P, Minusil + B(a)P, Zirconium (Au) + B(a)P groups were significantly increased ($P < 0.05$) when compared to the saline + B(a)P group. The mean lung weights of all other particulate + B(a)P groups were comparable to the saline + B(a)P group.

Significantly increased ($p < 0.05$) mean lung weights were observed for all particulate + B(a)P groups compared with their respective control (particulate alone). The only exception was the silica sand group which had similar average lung weights with and without B(a)P.

Clinical Observations

Wheezing, dyspnea, polypnea were the most frequently observed clinical signs. However, these findings appeared not to correlate with either survival or pathologic findings. Sporadic incidences of other clinical signs such as thin and hunched appearance, alopecia, rough hair coat, piloerection, sores on the skin, desquamation, opaque eyes, squinted eyes, ataxia, tremors, enlarged abdomen and soft feces were also noted. These sporadic incidences were observed throughout the study and did not follow any set pattern.

Pathology--General

Principal pathologic changes attributed to the compound administration were observed in the larynx, trachea, and lungs and bronchi. The total absolute respiratory tract and nonrespiratory tract tumor bearing animals along with the total tumor bearing animals are portrayed in Figure 3. These values are not corrected for the number of animals at risk. Tumors of the respiratory system were present in the hamsters instilled with the particulates + B(a)P as well as in the saline + B(a)P control group. A low incidence of respiratory tumors was seen in four of the particulate groups when instilled alone, i.e., ferric oxide, Minusil, zirconium (F1) and aluminum (70). The tumors seen were both benign and malignant. Other pathologic changes observed in the respiratory system included pulmonary granulomatous inflammation, hyperplasia, crystalline deposits and aspiration pneumonia. Pathologic changes including tumors were also seen in the other organ systems. These changes included: hyperplasia and tumors of the stomach; amyloidosis of the kidneys, adrenals,

liver and thyroid; chronic nephropathy; heart thrombosis; cystic structures in the liver; hyperplasia of the spleen, bone, bone marrow, and thyroid; atrophy of the thymus and testes; subcutaneous edema; adrenal cortex adenomas and carcinomas; and other types of tumors of a sporadic nature.

Morphologic Classifications of Respiratory Tract Tumors

The morphologic classification of the respiratory tract tumors seen in this study are analogous to those previously described and illustrated in the literature with other studies of respiratory tract carcinogenesis in hamsters. A description of the specific types of tumors seen is given below:

Epithelial polyp was observed as a excrescence of epithelial cells in the form of a mound or papilliferous projection into the lumen of the larynx, trachea, bronchus or bronchiole. Basement membranes were not violated and the epithelium grew in an outward or luminal direction. Epithelium was generally well differentiated; however, foci of disorganized intraepithelial growth was sometimes found.

Squamous cell carcinoma did not differ greatly from that which occurs in other organs. Branching cords or glandular formations of layered epithelium with squamous differentiation near the epithelial surface were commonly observed. Keratinization was common and lamellated pearls were sometimes formed. Intercellular bridges were not always visible suggesting a moderate degree of differentiation.

Adenosquamous carcinoma was a combined neoplasm having features of both adenocarcinoma and squamous cell carcinoma. Either prominent areas of squamous differentiation were found or the neoplastic epithelium had a transitional morphology between glandular and squamous epithelium. Traditional properties of malignancy were also employed to classify these neoplasms.

Adenoma was characterized by proliferation of fairly well differentiated, regular to swollen epithelial cells that lined or filled the alveolus. Nuclei were usually enlarged and cell polarity was retained with respect to basement membranes. Compression of surrounding normal tissue was usually observed at some point on the periphery of the mass.

Adenocarcinoma appeared as a solid, glandular, or combined proliferation of hyperchromatic epithelium. The masses occurred as isolated neoplasms in the lung parenchyma and near major airways. Occasional tumors were cystic and the large masses often had foci of necrosis. Varying degrees of cytologic atypia and an increased incidence of mitoses were found in the more anaplastic areas of the neoplasms. Anaplasia, local aggregation, and sometimes vascular invasion attested to the malignant nature of the lesions.

Sarcoma was classified as histiocytic, reticulum cell, fibrosarcoma, undifferentiated, and sarcoma NOS. The incidence of these neoplasms was minimal.

The incidences of tumors observed in the particulate + B(A)P groups, according to their segmental distribution in the respiratory system and morphologic classification are presented in Table 3. Metastatic tumors of the above morphologic types, melanomas, and neoplasms not otherwise specified (NOS), were also observed.

- A. Larynx--Of the 130 laryngeal tumors, 90 were epithelial polyps. The incidences were similar among the groups. In addition, 28 squamous cell carcinomas, 5 carcinomas, 5 adenocarcinomas, 1 papilloma and 1 sarcoma were also observed. No malignant neoplasm was seen in the saline + B(a)P group. The first laryngeal tumor appeared at the 14th week in the aluminum (60) + B(a)P group.
- B. Trachea--The tracheal tumors predominantly were also epithelial polyps (62/103). Except for the Minusil + B(a)P group, where only a single polyp was seen, the distribution was similar among the groups. In addition, 16 squamous cell carcinomas, 6 adenosquamous carcinomas, 3 carcinomas, 13 adenocarcinomas, 1 carcinosarcoma, 1 sarcoma and 1 neoplasm (NOS) were observed. The incidences of tracheal neoplasia observed in all the particulate + B(a)P groups were higher than the incidence in the saline + B(a)P control group, with the exception of the Minusil + B(a)P group which had only two tumors. The first tracheal tumor appeared at the 28th week in the olivine (Wa) group.
- C. Lungs and Bronchi--In the lung a total of 968 tumors was found and adenocarcinomas were observed most frequently with a total of 372. Adenosquamous carcinomas (222), squamous cell carcinomas (176) and adenomas (103) were the next most frequently observed. In the lungs and bronchi, in contrast to the large number of polyps observed in the larynx and trachea, only 21 bronchiolar epithelial polyps were detected. The incidences of tumors among the particulates + B(a)P groups were considerably higher than the saline + B(a)P control group, but comparable to the ferric oxide + B(a)P positive control group. The highest number of squamous cell carcinomas (35) was observed in the zirconium (Au) and the lowest number (6) in the aluminum (60) group. The highest number of adenosquamous cell carcinoma (25) was seen in the ferric oxide positive control group and the lowest number (9) in the olivine (Nc) group. Of the adenocarcinomas, the highest number (61) was seen in the Minusil group and the lowest (15) in the aluminum (60) group. The first lung tumor was seen in the Minusil group at the 23rd week.

The total number of tumors (all types) were higher in all particulate + B(a)P groups compared to the saline + B(a)P group and were comparable to the ferric oxide + B(a)P positive control group. The greatest total neoplastic response occurred in the Minusil and zirconium (Au) groups with 123 and 126 tumors, respectively. The least neoplastic incidences occurred in the saline + B(a)P group and aluminum (60) groups exhibiting 40 and 71 tumors, respectively.

The tumors were distributed throughout the lung; however, the neoplastic changes most frequently involved the left lobe. The second most frequently affected lobe was the right diaphragmatic lobe. The cardiac lobe appeared to have the lowest number of tumors (data not shown).

Respiratory Tract Tumor Incidences and Induction Time

Incidences of respiratory tract tumors, total numbers and distribution by site, the number of tumor bearing animals, the time of appearance of the first tumor, and the mean latency time (the latter two were calculated on the basis of autopsy date) are presented in Table 4 and Figure 3. The distribution of tumors by gross anatomical location in the respiratory tract is presented in Figure 4. The distribution of tumor bearing animals according to the types of tumors seen (classified as polyps, benign, and malignant) is presented in Figure 5. No respiratory tract tumors were observed in the saline vehicle control group.

A. Particulate Control Groups

A total of five (5) respiratory tract tumor bearing animals was observed in the particulate control groups. In the ferric oxide control there was one animal (3 percent) with one benign tumor in the larynx which appeared at week 62. In the Minusil control there was one animal (3 percent) with a single malignant tumor in the lung appearing at week 68. In the zirconium (Fl) group there were two tumor bearing animals (10 percent). The time of appearance of the first tumor was 86 weeks with a mean latency of 89.0 weeks. There were 2 benign and 2 malignant tumors for a total of 4 tumors. In the aluminum (70) group there was one animal (8 percent) with 3 tumors in the lungs and bronchi appearing at study week 92.

In the zirconium (Fl) group there was one animal with a metastatic osteosarcoma and one animal with a leukemia. In the olivine (Wa) group, a metastatic osteosarcoma in the lung of one animal was observed. These were not considered as respiratory tract tumor bearing animals since the tumors did not originate from the lung and leukemia is not a lung tumor. All other particulate control groups were free of respiratory tract tumors.

B. Particulate + B(a)P groups

1. Saline + B(a)P--This group responded the least of all carcinogen treated groups with 22 tumor bearing animals (47 percent) found and the average latency was the longest (72.0 ± 18.8 weeks) of all carcinogen treated groups. The distribution of these animals by tumor site, expressed as a percentage of the animals at risk, was: larynx and trachea, 13 percent; lungs and bronchi, 30 percent; and an overlap of the two or three sites in the entire respiratory system, 4 percent. Of the 40 respiratory tract tumors diagnosed 11 tumors were classified as polyps, one as benign, and 28 as malignant.
2. Ferric Oxide + B(a)P--The distribution of these 35 tumor bearing animals by tumor site expressed as a percentage of the animals at risk was: larynx and trachea, 7 percent; lungs and bronchi, 52 percent, and an overlap of 17 percent. A total of 80 respiratory tract tumors were diagnosed: 10 polyps, 5 benign, and 65 malignant.

3. Silica Sand + B(a)P--Thirty-six tumor bearing animals were present in this group and the distribution of these animals by tumor site expressed as a percentage of the animals at risk was: larynx and trachea, 13 percent; lungs and bronchi, 28 percent; and an overlap of 36 percent. A total of 98 respiratory tract tumors were diagnosed: 17 polyps, 24 benign, and 57 malignant.
4. Minusil + B(a)P--The 44 tumor bearing animals in this group represented the most intense response to the administration of a particulate and B(a)P. The distribution of these animals by site expressed as a percentage of the animals at risk was: larynx and trachea, 2 percent; lungs and bronchi, 67 percent; and an overlap of 20 percent. A total of 123 respiratory tract tumors were diagnosed: 9 polyps, 23 benign, and 91 malignant.
5. Zirconium (Au) + B(a)P--Forty-three tumor bearing animals were present in this group. The distribution of these animals by site expressed as a percentage of the animals at risk was: larynx and trachea, 4 percent; lungs and bronchi, 61 percent; and an overlap of 22 percent. A total of 126 respiratory tract tumors, the maximum number of tumors found in any group, were evaluated: 13 were classified as polyps, 5 as benign, and 108 as malignant.
6. Zirconium (Fl) + B(a)P--The distribution of the 36 tumor bearing animals by tumor site expressed as a percentage of the animals at risk was: larynx and trachea, 4 percent; lungs and bronchi, 45 percent; and an overlap of 28 percent. A total of 95 respiratory tract tumors were found: 12 polyps, 4 benign, and 79 malignant.
7. Chromite Sand + B(a)P--Thirty-seven tumor bearing animals were distributed by site, expressed as a percentage of the animals at risk: larynx and trachea, 9 percent; lungs and bronchi, 33 percent; and an overlap of 39 percent. A total of 87 respiratory tract tumors were diagnosed as follows: 16 were classified as polyps, 7 as benign, and 64 as malignant.
8. Minusil + Ferric Oxide + B(a)P--Thirty-eight tumor bearing animals were present in this group. The distribution of these animals by site was: larynx and trachea, 4 percent; lungs and bronchi, 55 percent; and an overlap of 21 percent. Of the 95 respiratory tract tumors found 9 were classified as polyps, 13 as benign, and 73 as malignant.
9. Olivine (Nc) + B(a)P--The distribution of the 40 tumor bearing animals by site expressed as a percentage of the animals at risk was: larynx and trachea, 4 percent; lungs and bronchi, 47 percent; and an overlap of 34 percent. A total of 107 respiratory tract tumors were diagnosed. Seventeen tumors were classified as polyps, 4 as benign, and 86 as malignant.

10. Olivine (Wa) + B(a)P--Thirty-nine tumor bearing animals were found and their distribution by site expressed as a percentage of the animals at risk was: larynx and trachea, 13 percent; lungs and bronchi, 46 percent; and an overlap of 26 percent. A total of 83 respiratory tract tumors were diagnosed: 12 polyps, 1 benign, and 70 malignant.
11. Aluminum (48) + B(a)P--Thirty-eight tumor bearing animals were distributed by site, expressed as a percentage of the animals at risk, as follows: larynx and trachea, 6 percent; lungs and bronchi, 34 percent; and an overlap of 36 percent. A total of 108 respiratory tract diagnosed were classified as 16 polyps, 6 benign, and 86 malignant.
12. Aluminum (60) + B(a)P--The distribution of the 31 tumor bearing animals by site expressed as a percentage of the animals at risk was: larynx and trachea, 10 percent; lungs and bronchi, 27 percent; and an overlap of 27 percent. Of the total of 71 respiratory tract tumors, 16 tumors were classified as polyps, 5 as benign, and 50 as malignant. This group had the earliest time of appearance, 14 weeks, of a hamster with a respiratory tumor.
13. Aluminum (70) + B(a)P--Thirty-seven tumor bearing animals were found and their distribution by tumor site expressed as a percentage of the animals at risk was: larynx and trachea, 4 percent; lungs and bronchi, 42 percent; and an overlap of 36 percent. A total of 80 respiratory tract tumors were diagnosed and 15 tumors were classified as polyps, 10 as benign, and 55 as malignant.

Respiratory Tract Hyperplasia

Hyperplasia was seen in animals with or without respiratory tract tumors. Alveolar/bronchiolar hyperplasia was characterized mainly by proliferation of well differentiated, low cuboidal epithelium along the alveolar septa. Compression of normal lung parenchyma at the periphery of the lesion was lacking. The distinction between large foci of alveolar/bronchiolar hyperplasia and adenoma was not always clear cut. Small amounts of alveolar bronchiolar hyperplasia were most commonly seen in the particulate + B(a)P groups and only occasionally found in the particulate control group. The distribution tended to parallel that of tumor development.

Although a distinct transitional state, e.g., foci of atypia or malignancy within an otherwise benign process, between hyperplasia and neoplasia was not observed; it is, however, reasonable to assume that such progression might occur, especially if one espouses the concept that nonneoplastic proliferative lesions have the ability to transform to an autonomous neoplasm.

Pulmonary Granulomatous Inflammation

The incidences of hamsters with pulmonary granulomatous inflammation are presented in Figure 6. The lesion consisted principally of numerous alveolar macrophages, hypertrophy of the alveolar lining cells and variable amounts of

neutrophils and lymphocytes. Alveolar septa were often thickened within the areas of inflammation. In the lung parenchyma phagocytosis of particulate material was prominent. This response was associated with the particulate deposits in the lung. Granulomatous inflammation was detected in all groups. However, the greatest incidences occurred when silica sand, Minusil, and Minusil + ferric oxide and aluminum (48) were instilled alone or in combination with B(a)P. The incidences were significantly increased ($p < 0.00001$) when compared to the saline control group.

Other Respiratory Tract Pathology

In addition to the tumors, granulomatous inflammation and hyperplasia, the other common findings in the lungs were crystalline or granular deposits in the distal airways, aspiration pneumonia, squamous metaplasia, and congestion. Crystalline or granular deposits were seen in all the dust instilled groups. The deposits were present in the region of the terminal bronchioles, respiratory bronchioles and alveoli. The particulate material appeared dark brown or black at low magnification, but consisted of angular, slightly refractile, yellow-brown bodies at higher magnification.

Low incidences of aspiration pneumonia, characterized by acute inflammation and the presence of foreign material, generally food or bedding origin, were noted in a few animals in the particulate + B(a)P groups except for Minusil + ferric oxide groups. The incidences of pneumonia did not have an effect either on the survival or clinical signs observed. Pneumonia is a common occurrence in dust instilled animals in this model system.

Other pathological findings of the respiratory system were sporadic, nonspecific and are commonly seen in the Syrian golden hamsters.

Hyperplasia and Tumors of the Stomach

The number of hamsters observed with hyperplasia, tumors, and hyperplasia with tumors of the stomach are presented in Table 5. Hyperplasia of the stomach involved the nonglandular portion (fore-stomach) and occurred as focal or diffuse areas of acanthosis with or without hyperkeratosis. The most frequently observed were fore-stomach papillomas, a total of 35 predominantly occurring in the B(a)P groups. These fore-stomach papillomas projected from the mucosal surface into the gastric lumen and consisted of a core of fibrovascular connective tissue lined by keratinizing squamous epithelium. Two squamous cell carcinomas and one adenocarcinoma were also observed in the stomach.

Hyperplasia alone, was seen in all the groups except in the Minusil, zirconium (F1), olivine (Nc), aluminum (48), and aluminum (70) particulate alone groups. Stomach tumors appeared to be more prevalent in the particulate + B(a)P groups than the particulate control groups. When the incidences of hyperplasia were combined with the tumor incidences, the effect of B(a)P administration appeared to be dramatic. However, this is not an unusual finding for this type of study since similar observations have been reported in the literature.

Fisher's analyses showed a statistically significantly increased ($p < 0.01$) number of hamsters with hyperplasia alone, when Minusil, zirconium (Fl), olivine (Nc) or aluminum (70) were administered with B(a)P as compared to the saline + B(a)P control group. Statistically significantly increased ($p < 0.01$) number of hamsters with stomach tumors alone only were seen when zirconium (Au) and chromite sand were instilled with B(a)P. The number of hamsters with hyperplasia and stomach tumors was higher than the saline + B(a)P control when Minusil, zirconium (Fl), both the olivine sands, and all three aluminum silicates were instilled with B(a)P. These findings were statistically significant ($p < 0.01$).

Other Non-Respiratory Tract Lesions and Tumors

Amyloidosis, a common, naturally occurring degenerative disease of aging hamsters, was a frequent observation among all groups. The number of animals with amyloidosis in the kidney, and adrenals, were higher than those in the liver and thyroids. In this study, the incidences and distribution of amyloidosis seen throughout the organ systems were not different from that reported for male hamsters in the literature.

In the kidneys, chronic nephropathy was observed in all the treated as well as the saline control groups. No pattern or treatment related effects were observed. This lesion was characterized by diffuse tubular changes consisting of dilatation, cast formation and atrophy. In addition, interstitial fibrosis and chronic inflammation were common. Occasionally, sclerotic changes of the glomerule and Bowman's capsule were associated with the tubular and interstitial lesions. Like amyloidosis, chronic nephropathy is also related to the aging phenomenon in hamsters.

In the heart, thrombosis was the most frequent finding, occurring with comparable incidence for all the groups except for the ferric oxide + B(a)P positive control and olivine (Wa) + B(a)P groups, where none were observed. Heart thrombi were usually found in the atrium and were generally organizing. The thrombi were associated with an inflammatory lesion in the myocardium. This lesion is also commonly found in aging hamsters.

In the liver, multiple cystic structures lined by flattened bile duct epithelium and filled with a greenish fluid were frequently observed as liver cysts.

In the spleen, hyperplasia was generally observed only in the particulate + B(a)P hamsters. This response might represent a primary effect of B(a)P on the spleen or a systemic immunologic response to neoplasia which occurred commonly with B(a)P treatment.

In the sternum and rib junction, as well as in the bone marrow and femur, low incidences of hyperplasia were observed in all groups, except the zirconium (Au) + B(a)P group. Six observations of hyperplasia in the sternum and rib junction and five incidences in the bone marrow and femur were observed in this group. These effects may be related to the elevated radionuclide concentrations found in the zirconium sands, which were associated with radon and radon daughter decay schemes, of 202.7 and 163.1 pCi/g for the Florida and Australian sands, respectively.

Other lesions such as hyperplasia of the thyroid, atrophy of the thymus and testes and subcutaneous edema were also observed. The incidences of such lesions were sporadic and not treatment related.

In evaluating the incidences, distribution, and types of tumors observed in the other organs, no significant differences were seen as compared to the historic data available on spontaneous tumors in hamsters. In this study, as reported in previous studies, the most frequent tumors were the adrenal cortex adenomas and carcinomas. Other types of tumors were sporadic and appeared in a comparable manner among all the groups. None of the tumors seen in the other organs indicated a treatment related effect.

Summary of Comparisons Among Groups

Laryngeal Tumor Bearing Animals--The particulate control vs particulate + B(a)P treated groups were compared for laryngeal tumor bearing animals. No statistical significance was observed for laryngeal tumor bearing animals between the saline control (0/38) and saline + B(a)P control group (5/44) or the ferric oxide (1/35) and the ferric oxide + B(a)P (5/32) groups. The number of laryngeal tumor bearing animals in all other particulate + B(a)P groups was significantly higher ($p < 0.01$) than their respective control.

Tracheal Tumor Bearing Animals--The particulate control vs particulate + B(a)P groups were compared for tracheal tumor bearing animals. No difference was observed between the saline control and the saline + B(a)P groups nor were any differences seen between the Minusil + B(a)P or Minusil + ferric oxide + B(a)P groups when compared to their respective controls. All other particulate + B(a)P groups showed a significantly increased ($P < 0.01$) number of tracheal tumor bearing animals when compared to their respective control.

Lung Tumor Bearing Animals--The particulate control vs particulate + B(a)P groups were compared for lung tumor bearing animals. The number of lung tumor bearing animals was significantly higher ($p < 0.01$) in each particulate + B(a)P group as compared to their own particulate control group.

Total Respiratory Tract Tumor Bearing Animals--The number of total respiratory tract (larynx + trachea + bronchi and lung) tumor bearing animals were compared for the following sets. A significantly ($p < 0.00001$) higher number was observed in the particulate + B(a)P groups as compared to their particulate control group. Except for the aluminum (60) + B(a)P group, higher ($p < 0.01$) incidences were found between all other particulate + B(a)P groups and the saline + B(a)P group. The number of tumor bearing animals in the ferric oxide positive control and other particulate + B(a)P groups were similar. No significant differences were observed for this set of comparisons.

In addition the following groups were compared for total respiratory tract tumor bearing animals:

- silica sand + B(a)P vs Minusil + B(a)P
- zirconium (Au) + B(a)P vs zirconium (Fl) + B(a)P
- olivine (Nc) + B(a)P vs olivine (Wa) + B(a)P

Minusil + ferric oxide + B(A)P vs ferric oxide + B(a)P
Minusil + ferric oxide + B(a)P vs Minusil + B(a)P
ferric oxide + B(a)P vs Minusil + B(a)P

aluminum (48) + B(a)P vs silica sand + B(A)P
aluminum (48) + B(a)P vs Minusil + B(a)P
aluminum (48) + B(a)P vs aluminum (60) + B(a)P
aluminum (48) + B(a)P vs aluminum (70) + B(a)P

aluminum (60) + B(a)P vs silica sand + B(a)P
aluminum (60) + B(a)P vs Minusil + B(a)P
aluminum (60) + B(a)P vs aluminum (70) + B(a)P

aluminum (70) + B(a)P vs silica sand + B(a)P
aluminum (70) + B(a)P vs Minusil + B(a)P

No significant differences were observed between these comparisons, except when the Aluminum (60) + B(a)P (31/49) tumor bearing animals were compared with the Minusil + B(a)P group (44/49) where $p < 0.002$.

DISCUSSION AND SUMMARY

In this study, silica sand and silica sand substitutes (olivine sands, chromite sand, zirconium silicates, and aluminum silicates) were evaluated for their cocarcinogenic effect in combination with B(a)P. Ferric oxide was used as a positive cocarcinogenic control. In general, survival was excellent during the study duration of 92 weeks and survival at termination was higher than usually reported. In hamsters treated with the B(a)P and particulate, suffocation due to neoplastic involvement of the respiratory system is a frequent occurrence and significantly decreases survival. In this study, the survival of the particulate + B(a)P groups, in general, was somewhat lower than the particulate control but only significantly so for the Minusil, both zirconium groups, the chromite, olivine (Nc) and the aluminum (60) groups. This is attributed to tumor related mortality. Compared to the saline control, particulate exposure without the carcinogen decreased survival in the silica sand and Minusil + ferric oxide groups.

The average body weights of hamsters in the particulate + B(a)P groups were generally lower than their respective particulate groups. These differences, particularly noted during the first 15 weeks and sporadically thereafter, may be attributed to the combined effects of treatment, survival and tumor burden.

The significant increases in average lung weights of the silica sand, Minusil + ferric oxide and Minusil groups compared to the saline control are likely related to the crystalline silica content of these materials. The incidence of hamsters with granulomatous inflammation also appears on first inspection to be related to increased crystalline silica content; however, no increase in mean lung weight was noted in the aluminum (48) group even though a significant increase in granulomatous inflammation was observed. As reported by Stettler and coworkers (1981), except for the silica sand and Minusil which are essentially pure quartz, all substitute foundry sands contained less than one percent crystalline silica. It is interesting to note that the aluminum

silicates 48, 60 and 70 contained 0.7, 0.8 and 0.4 percent crystalline silica, respectively, while all other sands contained less than 0.5 percent. Yet, the advertised mineralogy for aluminum silicates is listed as 62 percent mullite plus 38 percent cristobalite and glass for aluminum (48), 80 percent mullite and 20 percent glass for aluminum (60), and 87 percent mullite, 3 percent alumina and 10 percent glass for aluminum (70). One might expect to obtain a linear dose response relationship for the incidence of granulomatous inflammation with these aluminum silicates based on the cristobalite/glass content; however, this was not observed in spite of similar amounts of each particulate administered. Nolan et al. (1981) cited a number of reasons why the single mineral species quartz may possess a wide range of biological activities including: "blunted activity" in the presence of measurable concentrations of iron or aluminum and changes in activity due to mechanical methods used to reduce the size of the particle. The aluminum silicates used in this study contained the following percentages of particles containing iron using the individual particle analysis counting technique: aluminum (48) 17.1; aluminum (60) - 38.9; and aluminum (70) - 48.9. Bulk chemical analysis of these particulates reflected an increasing trend in iron content, i.e., 5.9 ± 0.1 , 6.2 ± 0.2 and 7.7 ± 0.4 mg/g, respectively; however, the content was lower than expected. Dauber et al. (1980) considered iron contamination in quartz important in that they took special precautions to remove it before using the quartz in their experimental silicosis-intratracheal guinea pig model. These steps were not taken in the current study. Bulk analysis also revealed increased nickel, copper, zinc and lead contents for the aluminum (48) and decreased concentrations of zirconium, calcium and barium when compared to the other aluminum silicates. The question of iron, aluminum and freshly generated quartz surfaces obviously requires further mechanistic investigations to determine which, if any, markedly affect the biological activity. That aluminum silicates may be linked to human pulmonary fibrosis has been reviewed by Golden and coworkers (1982).

The types of tumors seen throughout the respiratory system and their histopathological features were essentially analogous to those previously described for this model (Saffiotti et al., 1968). Histopathological evaluation of all organs revealed the respiratory system to be the primary target for pathological alterations with changes attributable to compound administration occurring in the larynx, trachea, bronchi and lung. Benign and malignant tumors were seen in all segments of the respiratory system. Treatment induced tumors were always of respiratory tract origin as opposed to metastases from other organs. In addition to tumors, other pathologic changes such as pulmonary granulomatous inflammation, hyperplasia, crystalline (dust) deposits in the distal airways and aspiration pneumonia were common findings. Pathological alterations observed in other organ systems (nonrespiratory tract lesions and tumors), except for gastric hyperplasia and tumors, were not directly related to treatment. That these findings occur spontaneously in hamsters is evident from historic data.

Silica sand when instilled alone did not induce any tumors in the respiratory tract or to any significant degree in any other organ. This is in contrast to the report of Wagner (1976), noting the development of malignant histocytic lymphoma in rats, and of Holland et al., (1983) who found lung adenomas and carcinomas in F-344 rats when given Minusil by intratracheal instillation or

inhalation exposure. The current study confirms the lack of response in the hamster reported by Holland and coworkers (1983). The number of tumor bearing animals and morphologic types seen in silica sand + B(a)P group were similar to that of the positive control group, ferric oxide + B(a)P; the number of tumors was increased and the average latent period decreased, but not significantly when compared to the positive control. One striking difference from the ferric oxide group was the increase in the number of pulmonary adenomas observed in the silica sand group; this difference was also observed in the Minusil groups. No fibrosis or hyperplasia of the respiratory system was observed when silica sand was instilled alone. However, a statistically significantly higher incidence (88.0 percent) of pulmonary granulomatous inflammation was seen in this group as compared to the saline control group (4.2 percent). This finding was comparable to the Minusil control group (84.0 percent). The lack of fibrotic response in hamsters compared to that found in rats may be an interesting basis for development of a model for this prevalent disease. No other significant findings were observed in the hamster lungs treated with silica sand.

A significant cocarcinogenic response was observed for all the silica sand substitutes except aluminum (60). Aluminum (60), compared to the other particulates produced a weak cocarcinogenic response, but the number of tumor bearing animals was not statistically different from that of the saline + B(a)P group.

The morphologic characteristics of the tumors seen in the silica sand substitutes + B(a)P groups were similar to those seen in the ferric oxide positive control and silica sand + B(a)P groups. No statistical differences in the number of tumor bearing animals were found between the zirconium sands, olivine sands, or the aluminum silicates when instilled with B(a)P. The number of tumor bearing animals in the Minusil + ferric oxide + B(a)P group was similar to those seen in the Minusil + B(a)P and ferric oxide + B(a)P groups. The morphologic characteristics and the segmental distribution of the tumors were comparable for all silica sand substitutes when instilled with B(a)P. The upper respiratory tract (larynx and trachea) lesions were predominantly epithelial polyps. The lung and bronchial tumors were predominantly malignant. Although tumors occurred in all lobes of the lung, the left lobe and the diaphragmatic lobe were most frequently affected. A possible explanation for this finding is that exposure to those lobes is greater since they are the largest lobes morphologically, and histologically have the greatest bronchiolar diameter. Stenback and Rowland (1978) in their studies with talc and B(a)P instillation also observed a greater number of tumors in the left and diaphragmatic lobes.

The incidence of respiratory tract hyperplasia was statistically evaluated only for nonrespiratory tract tumor bearing animals. The number of hamsters with hyperplasia in the aluminum (60) + B(a)P group was significantly higher than that seen in the aluminum (60) control. It is interesting to note that while aluminum (60) was not an effective cocarcinogen with B(a)P, this treatment did produce a higher incidence of hyperplasia. The survival of the aluminum (60) + B(a)P group was significantly lower than that of its particulate control. If these hamsters had survived longer, it is possible that a transition from hyperplasia to neoplasia may have occurred, thereby

increasing the cocarcinogenic response. The remaining silica sand substitutes when instilled alone were not carcinogenic in the hamster respiratory tract. The low incidence of tumors seen in the zirconium (Au) and aluminum (70) groups was not statistically significant. Hyperplasia of the respiratory system was seen sporadically but was not treatment related. Hyperplasia is a common finding in dust instilled animals (Rowland et al., 1980).

The silica sand substitutes failed to induce fibrosis, but statistically significant occurrences of pulmonary granulomatous inflammation were seen in the silica sand, Minusil, Minusil + ferric oxide and aluminum (48) groups when compared to the saline control.

Hyperplasia and tumors of the stomach were seen in hamsters instilled with the particulates with or without B(a)P. These lesions were more prominent in animals instilled with B(a)P. This may be related to the pulmonary clearance of the materials leading to ingestion of the instilled compound and a subsequent tumor enhancing effect.

In an attempt to determine the relative severity of respiratory toxicity for silica sand and its substitute aggregates, an index was derived and is presented in Figure 7. The factors for the index included: corrected percent of tumor bearing animals (TBA/Number at Risk); weighting of the tumor response by using a relative measure for seriousness of the tumor morphology, i.e., multiplying by one for polyps (P(1)), two for benign tumors (P(2)) and three for malignant tumors (P(3)); a weighting factor for average tumor latency (mean latency/total weeks observed); and the addition of another weighting factor for granulomatous inflammation response (number with granulomatous inflammation/number at risk). Using this relative index, only zirconium (Au), olivine (Wa), Minusil + ferric oxide and Minusil were more "toxic" than silica sand, the most commonly used foundry aggregate. All groups except aluminum (70), aluminum (60) and saline (+ B(a)P) were more "toxic" than ferric oxide. All particulate + B(a)P groups are listed in order to decreasing "toxicity" as follows: Minusil, Minusil + ferric oxide, olivine (Wa), zirconium (Au), silica sand, aluminum (48), olivine (Nc), chromite, zirconium (Fl), ferric oxide, aluminum (70), aluminum (60) and saline.

CONCLUSIONS

In this study, silica sand and silica sand substitutes instilled intratracheally to male Syrian golden hamsters were found not to be carcinogenic in the hamster respiratory tract. When instilled with the carcinogen B(a)P, silica sand and silica sand substitutes exhibited a definite cocarcinogenic response. However, one of the silica sand substitutes, aluminum (60), was not as effective as compared to the other sands in producing this response. No fibrogenic response was induced by silica sand and silica sand substitutes evaluated in this study, but granulomatous inflammation was produced and this may be indicative of a fibrotic response in other species. The hamster appears to be a poor model for studying fibrotic response.

Relying solely on the data from this study, there is an indication that only one silica sand substitute, aluminum (60), does not offer the same risks as

silica sand, i.e., cocarcinogenic and granulomatous inflammation (fibrosis). The practicality of substituting this material for silica sand is not known, but it is probably limited. Except for aluminum (48), all sand substitutes offer similar risks to silica sand for cocarcinogenic effects when coupled with carcinogen exposure of the PNA type; however, the potential risk of fibrotic response may be reduced with the substitutes. The use of zirconium sands must be evaluated carefully; the implications of the abnormally high radon daughter levels have been discussed by Boothe et al. (1980). The independent findings of Boothe and coworkers has resulted in the Oregon State Health Division imposing limited controls on the radiological aspects of foundry operations where major foundries have been required to obtain radioactive materials licenses to use and dispose of zircon sands.

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Table 1. Materials, Their Sources, MMAD^a and Surface Areas

Materials and Sources	Study Identification Name	MMAD ^b (μm)	Surface ^b Area (m^2/g)
Ottawa Silica Sand, Sil-Co-Sil 395, -325 GFN ^c - Ottawa Silica Co., Ottawa, IL	Silica Sand	7.23	1.88
Control Silica Sand, Min-U-Sil ^d , 5 μm -- Pennsylvania Glass Sand Corp., Pittsburgh, PA	Minusil	5.06	2.99
Zirconium Silicate Sand (Australia), -325 GFN, noncalcined--Continental Mining Processing Co., Cincinnati, OH	Zirconium (Au)	7.16	2.41
Zirconium Silicate Sand (Florida) -325 GFN, Calcined--Continental Mineral Processing Co.	Zirconium (Fl)	6.70	2.26
Chromite Sand, GFN 55/60 Manufacturer: American Colloid Co.-- Carpenter Brothers, Inc., Milwaukee, WI	Chromite	8.57	1.01
Olivine Sand (North Carolina), GFN 70-- IMC Chemicals Group, Inc., Terre Haute, IN	Olivine (NC)	9.92	1.02
Olivine Sand (Washington), GFN 70-- IMC Chemicals Group, Inc.	Olivine (Wa)	7.36	1.71
Aluminum Silicate Sand Remasil 48 RP 200C--Remet Corp., Chadwick, NY	Aluminum (48)	7.35	1.47
Aluminum Silicate Sand Remasil 60 RP 200-C--Remet Corp.	Aluminum (60)	6.72	1.46
Aluminum Silicate Sand Remasil 70 RP 325, Coarse Grind-- Remet Corp.	Aluminum (70)	5.84	1.11
Ferric Oxide (certified grade, red anhydrous--Lot No. 785156)-- Fisher Scientific Co.	Ferric Oxide	1.37	6.90
Benzo(a)Pyrene (99 + percent pure-- gold label lot No. 02087) Aldrich Chemical Co.	B(a)P		

a Mass Median Aerodynamic Diameter

b From Stettler et al. (1981)

c GFN = Grain fineness number

d A registered trademark

Table 2. Treatment Groups and Dosage^a of Particulates

<u>Group No.^c</u>	<u>Treatment</u>	<u>Dosage of Particulates per Instillation</u>
1 ^e	Saline (Vehicle Control)	none
2 ^f	Saline + B(a)P ^b	none
3	Ferric Oxide	3
4 ^e	Ferric Oxide + B(a)P	3
5	Silica Sand	1.1
6	Silica Sand + B(a)P	1.1
7	Minusil	0.7
8	Minusil + B(a)P	0.7
9	Zirconium (Au)	0.9
10	Zircon (Au) + B(a)P	0.9
11	Zircon (Fl)	0.9
12	Zircon + B(a)P	0.9
13	Chromite	2.1
14 ^e	Chromite + B(a)P	2.1
15 ^d	Minusil + Ferric Oxide	0.7 + 3
16	Minusil + Ferric Oxide + B(a)P	0.7 + 3
17	Olivine (Nc)	2.1
18	Olivine (Nc) + B(a)P	2.1
19	Olivine (Wa)	1.2
20	Olivine (Wa) + B(a)P	1.2
21	Aluminum (48)	1.4
22	Aluminum (48) + B(a)P	1.4
23	Aluminum (60)	1.4
24	Aluminum (60) + B(a)P	1.4
25	Aluminum (70)	1.9
26	Aluminum (70) + B(A)P	1.9

a All doses instilled in 0.2 ml saline weekly for 15 weeks

b All B(a)P groups instilled with 3 mg B(a)P plus amount of particulate shown

c Initial group size = 50 hamsters

d Final group size = 49 hamsters due to mis-sexed or escaped animal

e Final group size = 48 hamsters due to mis-sexed and/or escaped animals

f Final group size = 47 hamsters due to mis-sexed and/or escaped animals

TABLE 3
INCIDENCE OF TUMORS BY TYPE AND LOCATION IN PARTICULATE PLUS B(a)P GROUPS: LARYNX

Tumor Type	Group No.: 2	Saline	4	Ferric Oxide	6	Silica Sand	8	Minusil	10	Zirconium (Au)	12	Zirconium (Fl)	14	Chromite
Polyp	5	5	3	3	7	7	8	8	6	6	6	6	5	5
Papilloma	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Carcinoma, squamous cell	0	0	2	2	2	2	2	2	1	1	1	1	5	5
Carcinoma	0	0	0	0	1	1	0	0	1	1	1	1	1	1
Adenocarcinoma	0	0	0	0	3	3	0	0	0	0	1	1	0	0
Sarcoma	0	0	0	0	0	0	0	0	0	0	0	0	1	1

Tumor Type	Group No.: 16	Minusil + Ferric Oxide	18	Olivine (Hc)	20	Olivine (Wa)	22	Aluminum (48)	24	Aluminum (60)	26	Aluminum (%)
Polyp	4	4	12	12	5	5	8	8	11	11	10	10
Papilloma	0	0	0	0	1	1	0	0	0	0	0	0
Carcinoma, squamous cell	5	5	2	2	2	2	3	3	2	2	1	1
Carcinoma	1	1	0	0	0	0	0	0	0	0	0	0
Adenocarcinoma	0	0	0	0	0	0	1	1	0	0	0	0
Sarcoma	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 3
INCIDENCE OF TUMORS BY TYPE AND LOCATION IN PARTICULATE PLUS B(a)P GROUPS: LARYNX

Tumor Type	Group No.: 2	Saline	4 Ferric Oxide	6 Silica Sand	8 Minustl	10 Zirconium (Au)	12 Zirconium (F1)	14 Chromite Sand
Polyp	5	3	7	8	6	6	5	
Papilloma	0	0	0	0	0	0	0	0
Carcinoma, squamous cell	0	2	2	2	1	1	1	5
Carcinoma	0	0	1	1	0	1	1	1
Adenocarcinoma	0	0	3	0	0	1	1	0
Sarcoma	0	0	0	0	0	0	0	1

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Tumor Type	Group No.:	16 Minustl + Ferric Oxide	18 Olivine (IIC)	20 Olivine (Ila)	22 Aluminum (48)	24 Aluminum (60)	26 Aluminum (70)
Polyp	4	12	5	8	11	10	
Papilloma	0	0	1	0	0	0	0
Carcinoma, squamous cell	5	2	2	3	2	1	1
Carcinoma	1	0	0	0	0	0	0
Adenocarcinoma	0	0	0	1	0	0	0
Sarcoma	0	0	0	0	0	0	0

TABLE 3 (continued)
INCIDENCE OF TUMORS BY TYPE AND LOCATION IN PARTICULATE PLUS B(a) GROUPS: TRACHEA

Tumor Type	Group No.:	2	4	6	8	10	12	14
	Saline	Ferric Oxide	Silica Sand	Minasil	Zirconium (Au)	Zirconium (F1)	Chromite Sand	
Polyp	3	4	10	1	3	6	9	
Carcinoma, squamous cell	0	0	2	0	1	3	3	
Carcinoma, adenosquamous	0	1	0	0	1	0	0	
Carcinoma	0	0	0	0	0	0	0	
Carcinosarcoma	0	0	0	0	1	0	0	
Adenocarcinoma	0	1	1	1	2	0	1	
Sarcoma, undifferentiated	0	0	0	0	0	0	0	
Neoplasms, HOS	0	0	0	0	0	0	0	

Tumor Type	Group No.:	16	18	20	22	24	26
	Minasil + Ferric Oxide	Olivine (Nc)	Olivine (Va)	Aluminum (43)	Aluminum (60)	Aluminum (70)	
Polyp	3	4	5	6	3	5	
Carcinoma, squamous cell	1	1	0	3	1	1	
Carcinoma, adenosquamous	0	1	1	1	1	0	
Carcinoma	0	0	2	0	0	1	
Carcinosarcoma	0	0	0	0	0	0	
Adenocarcinoma	0	0	2	2	2	1	
Sarcoma, undifferentiated	0	0	1	0	0	0	
Neoplasms, HOS	0	1	0	0	0	0	

TABLE 3 (continued)
INCIDENCE OF TUMORS BY TYPE AND LOCATION IN PARTICULATE PLUS B(a)P GROUPS: LUNGS AND BRONCHI

Tumor Type	Group No.: 2	Saline	4	Ferric Oxide	6	Silica Sand	8	Minusil	10	Zirconium (Au)	12	Zirconium (F1)	14	Chronite Sanc
Polyp	3	3	3	0	0	0	0	0	4	0	0	0	2	
Carcinoma, squamous cell	4	4	5	10	10	8	8	35	13	17	17	17	17	
Carcinoma, adenosquamous	7	7	25	17	17	18	18	17	22	22	22	22	9	
Carcinoma	0	0	0	0	0	1	1	2	0	0	0	0	0	
Carcinoma, squamous cell metastatic	0	0	0	0	0	0	0	6	0	0	0	0	0	
Carcinoma, adenosquamous, metastatic	1	1	0	0	0	0	0	0	0	0	0	0	0	
Carcinoma, metastatic	0	0	0	0	0	0	0	0	0	1	1	0	0	
Adenoma	1	1	5	24	23	23	23	5	3	7	7	7	7	
Adenocarcinoma	9	9	29	22	61	61	61	33	37	22	22	22	22	
Adenocarcinoma, metastatic	0	0	0	0	0	0	0	6	0	0	0	0	0	
Sarcoma	0	0	2	0	0	0	0	1	0	5	5	5	5	
Sarcoma, metastatic	0	0	0	0	0	0	0	1	0	0	0	0	0	
Melanoma, metastatic	5	5	0	0	0	0	0	0	0	0	0	0	0	
Neoplasms, IUS	2	2	0	0	0	0	0	0	1	1	1	1	0	

INCIDENCE OF TUMORS BY TYPE AND LOCATION IN PARTICULATE PLUS B(a)P GROUPS: LUNGS AND BRONCHI

TABLE 3 (continued)

Tumor Type	Group No.:	16 Minusil + Ferric Oxide	18 Olivine (Hc)	20 Olivine (Wa)	22 Aluminum (48)	24 Aluminum (60)	26 Aluminum (70)
Polyp		2	1	2	2	2	0
Carcinoma, squamous cell		16	30	15	10	7	6
Carcinoma, adenosquamous		12	25	12	23	18	17
Carcinoma		0	1	5	2	1	0
Carcinoma, squamous cell, metastatic		0	0	0	0	0	0
Carcinoma, adenosquamous, metastatic		0	0	0	0	1	0
Carcinoma, metastatic		0	0	0	0	2	0
Adenoma		12	3	0	6	5	9
Adenocarcinoma		33	22	29	36	15	25
Adenocarcinoma, metastatic		0	3	1	0	0	2
Sarcoma		5	1	0	0	0	1
Sarcoma, metastatic		0	0	0	5	0	0
Melanoma, metastatic		0	0	0	0	0	0
Neoplasms, NOS		1	0	0	0	0	1

TABLE 4
RESPIRATORY TRACT TUMORS

GROUP NO.	TREATMENT	NUMBER ^a	RESP. TBA ^b	NO. OF TUMORS BY SITE			MEAN LATENCY ^c
				LARYNX	TRACHEA	BRONCHI + LUIG	
1	SALINE CONTROL	48 (0)	0 (0)	0	0	0	--
2	SALINE + B(a)P	47 (47)	22 (47)	5	3	32	72.6+18.8
3	FERRIC OXIDE	50 (34)	1 (3)	1	0	0	62
4	FERRIC OXIDE + B(a)P	48 (46)	35 (76)	5	6	69	70.2+15.0
5	SILICA SAND	50 (0)	0 (0)	0	0	0	--
6	SILICA SAND + B(a)P	50 (47)	36 (77)	13	13	72	56.5+17.3
7	MUSIL	50 (35)	1 (3)	0	0	1	68
8	MUSIL + B(a)P	50 (49)	44 (90)	10	2	111	68.5+16.3
9	ZIRCONIUM (Au)	50 (0)	0 (0)	0	0	0	--
10	ZIRCONIUM (Au) + B(a)P	50 (49)	43 (88)	8	8	110	62.8+18.3
11	ZIRCONIUM (F1)	50 (21)	2 (10)	0	0	4	89.0+4.2
12	ZIRCONIUM (F1) + B(a)P	50 (47)	36 (77)	9	9	77	68.3+16.5
13	CHROMITE SAND	50 (0)	0 (0)	0	0	0	--
14	CHROMITE SAND + B(a)P	48 (46)	37 (80)	12	13	62	62.3+16.8
15	MUSIL + FERRIC OXIDE	49 (0)	0 (0)	0	0	0	--
16	MUSIL + FERRIC OXIDE + B(a)P	50 (47)	38 (81)	10	4	81	66.7+15.4
17	OLIVINE (Hc)	50 (0)	0 (0)	0	0	0	--
18	OLIVINE (Hc) + B(a)P	50 (47)	40 (85)	14	7	86	65.7+17.7
19	OLIVINE (Wa)	50 (0)	0 (0)	0	0	0	--
20	OLIVINE (Wa) + B(a)P	50 (46)	39 (85)	8	11	64	60.5+21.8
21	ALUMINUM (48)	50 (0)	0 (0)	0	0	0	--
22	ALUMINUM (48) + B(a)P	50 (50)	38 (76)	12	12	84	67.8+15.7
23	ALUMINUM (60)	49 (0)	0 (0)	0	0	0	--
24	ALUMINUM (60) + B(a)P	50 (49)	31 (63)	13	7	51	69.6+19.1
25	ALUMINUM (70)	50 (13)	1 (8)	0	0	3	92
26	ALUMINUM (70) + B(a)P	50 (45)	37 (82)	11	8	61	66.9+19.5

a = Number of hamsters histopathologically examined (number of hamsters at risk at the time of appearance of the first tumor).

b = RESP. TBA: Number of Respiratory Tract Tumor Bearing Animals (Percent of RESP. TBA: Based on the number of hamsters at risk at the time of appearance of the first tumor)

c = mean latency in weeks + standard deviation and (week of appearance of first tumor)

TABLE 5. Number of Hamsters with Hyperplasia and Tumors of the Stomach

Group No.	Treatment	Hyperplasia	Tumors	Hyperplasia ^a and Tumors
1	Saline Control	3	0	3
2	Saline + B(a)P	3	1	4
3	Ferric Oxide	1	1	2
4	Ferric Oxide + B(a)P	3	4	5
5	Silica Sand	2	1	3
6	Silica Sand + B(a)P	5	1	6
7	Minusil	0	0	0
8	Minusil + B(a)P	5 ^b	2	6 ^b
9	Zirconium (Au)	3	0	3
10	Zirconium (Au) + B(a)P	1	3 ^b	4
11	Zirconium (Fl)	0	1	1
12	Zirconium (Fl) + B(a)P	11 ^b	3	13 ^b
13	Chromite Sand	1	0	1
14	Chromite Sand + B(a)P	4	4 ^b	8
15	Minusil + Ferric Oxide	3	0	3
16	Minusil + Ferric Oxide + B(a)P	5	4	9
17	Olivine (nc)	0	0	0
18	Olivine (Nc) + B(a)P	5 ^b	1	6 ^b
19	Olivine (Wa)	1	0	1
20	Olivine (Wa) + B(a)P	6	2	8 ^b
21	Aluminum (48)	0	0	0
22	Aluminum (48) + B(a)P	3	3	6 ^b
23	Aluminum (60)	1	0	1
24	Aluminum (60) + B(a)P	6	3	9 ^b
25	Aluminum (70)	0	0	0
26	Aluminum (70) + B(a)P	7 ^b	4	11 ^b

^a The number of hamsters in this category may not equal the sum of the categories because animals may have both attributes.

^b Significantly different from its particulate control ($p < 0.01$).

FIGURE 1

FREQUENCY OF SIGNIFICANT WEIGHT LOSS WITH VARIOUS PARTICULATES + B(a)P

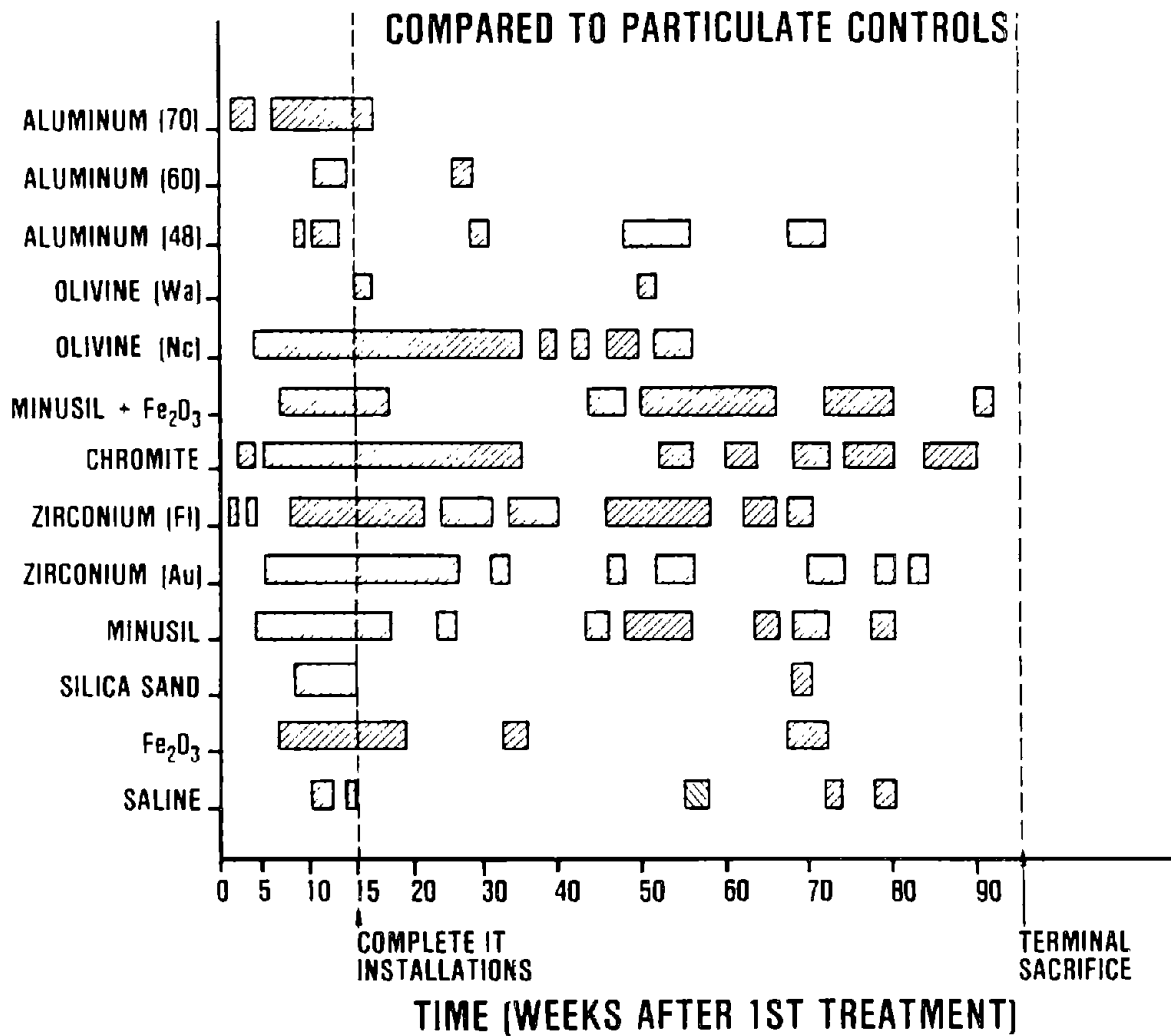


FIGURE 2

MEAN LUNG WEIGHTS AT NECROPSY

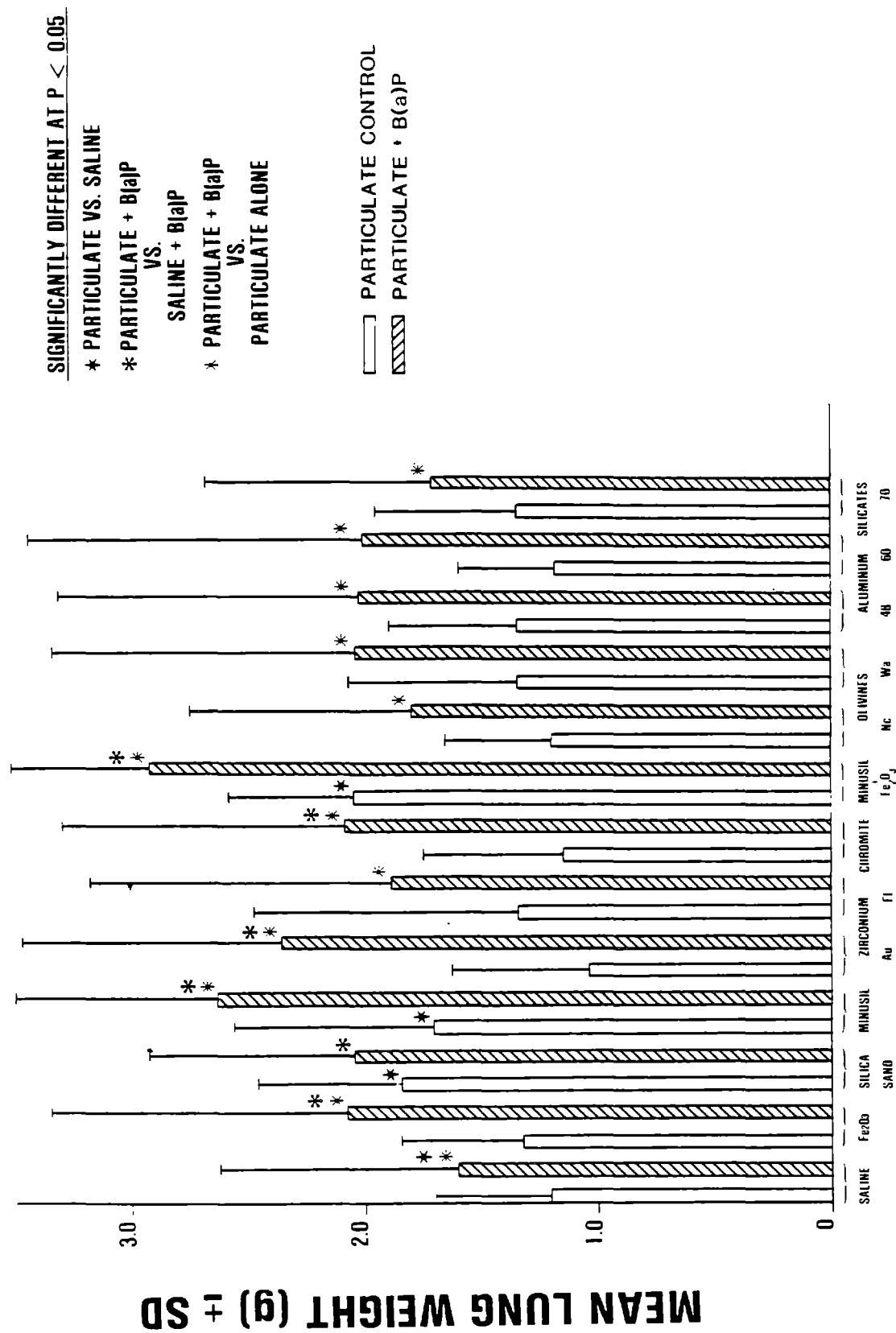


FIGURE 3

NUMBER OF TUMOR BEARING HAMSTERS

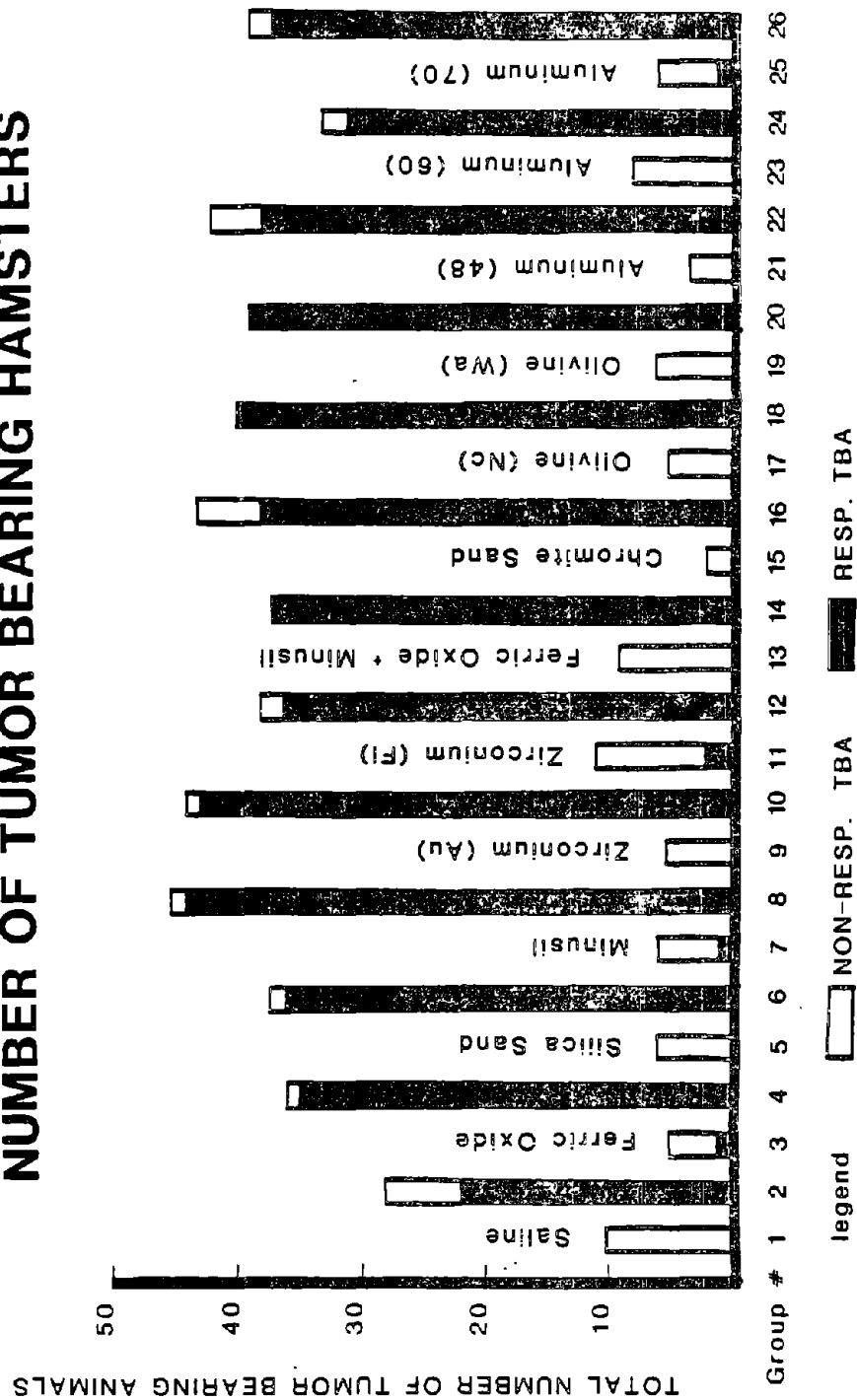


FIGURE 4

LOCATION OF RESPIRATORY TRACT TUMORS

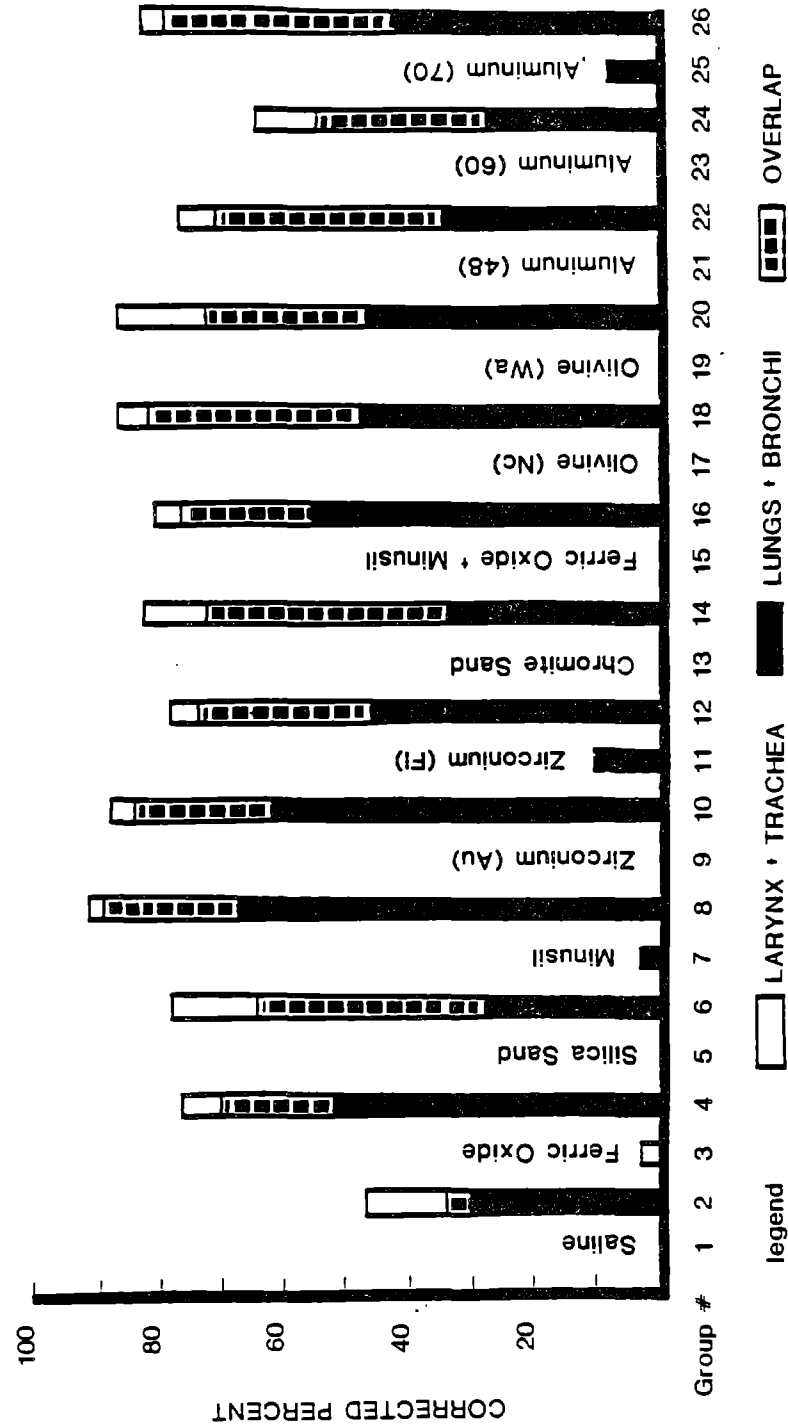


FIGURE 5

DISTRIBUTION OF RESPIRATORY TUMOR TYPES AND CORRECTED % TBA

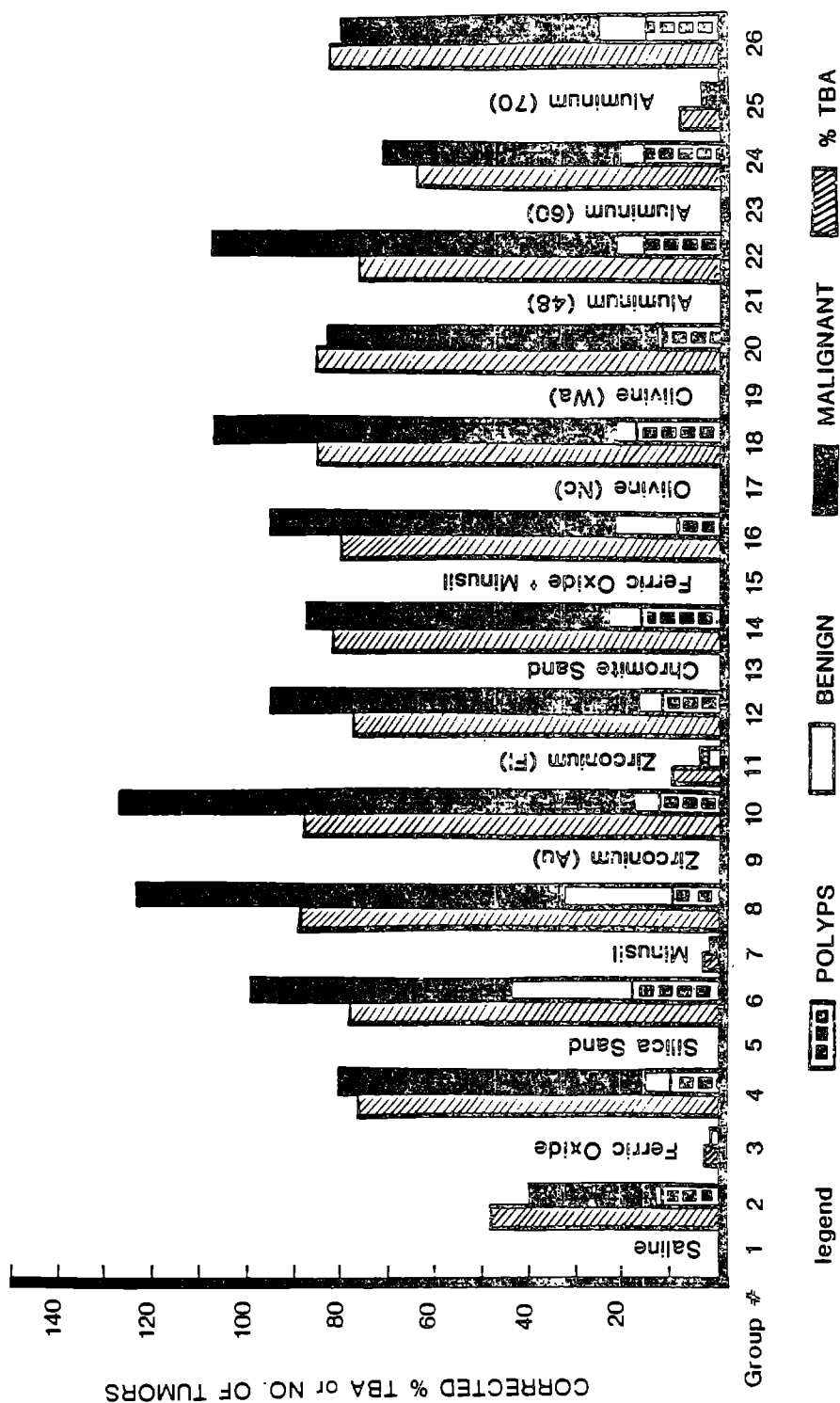


FIGURE 6

INCIDENCE OF PULMONARY GRANULOMATOUS INFLAMMATION

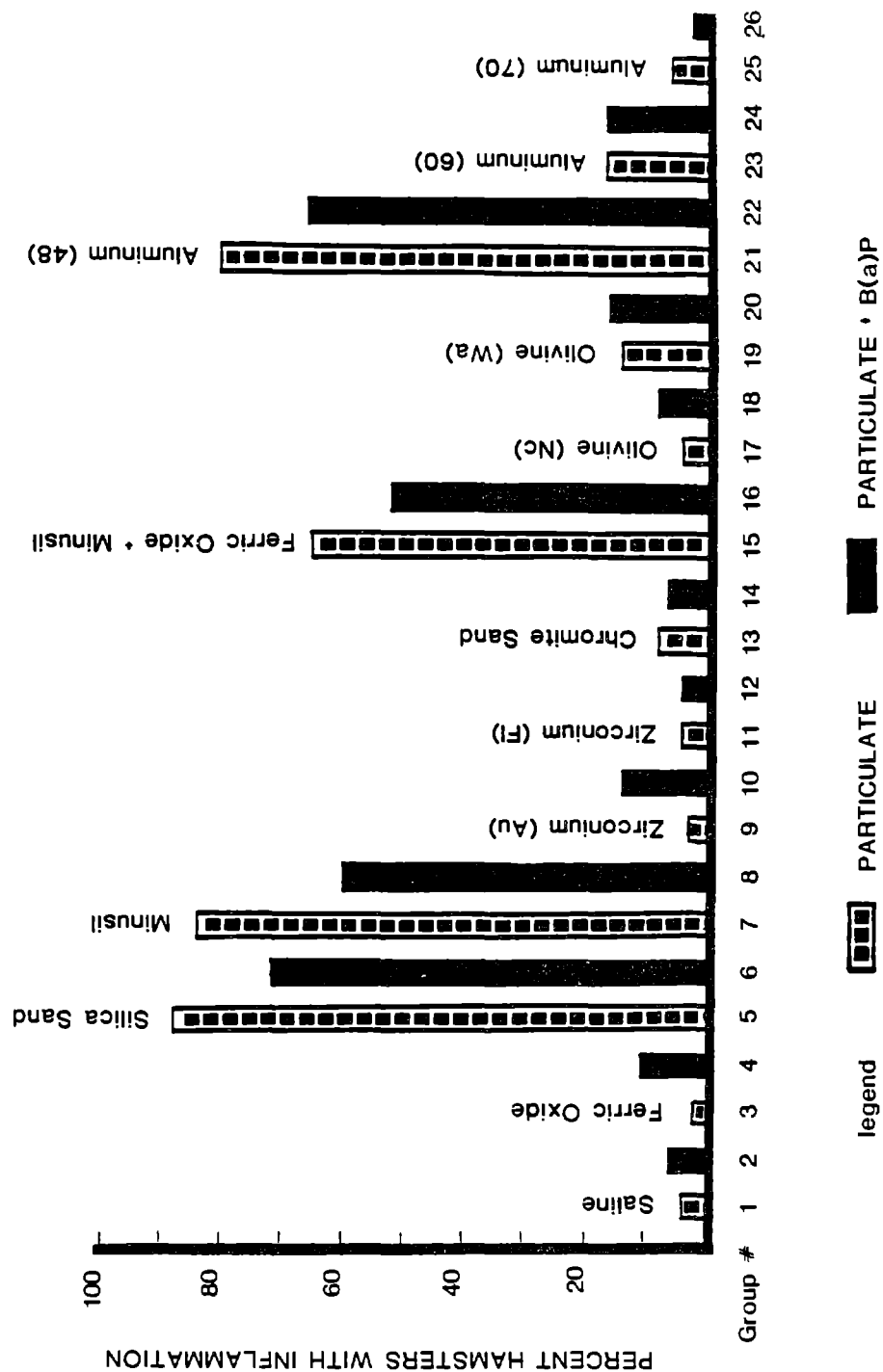
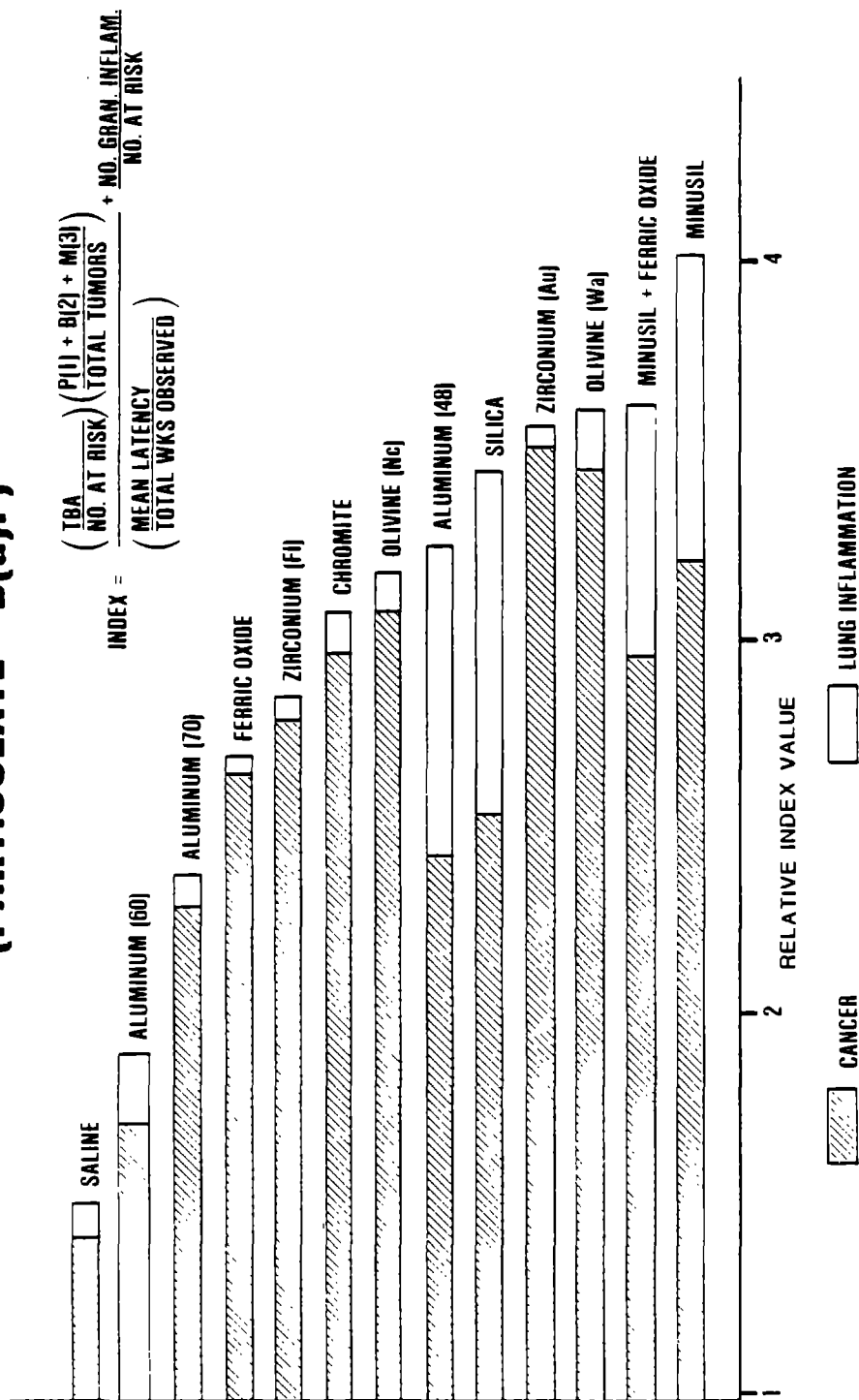


FIGURE 7

RELATIVE INDEX OF RESPIRATORY TOXICITY (PARTICULATE + B(a)P)



PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

SIRA Project -
Dosimetry Studies in Selected Locations
Where Epidemiology Studies Are Planned

Dr. Herbert Wiser
Environmental Protection Agency

**SIRA Project - Dosimetry Studies in Selected Locations
Where Epidemiology Studies Are Planned**

Dr. Herbert Wiser, EPA

The purpose of this study of the dosimetry studies was to develop and utilize a personal dosimeter that will determine the actual exposure of humans to UVB radiation, even UVA radiation, and to correlate this with the NCI's epidemiology studies that have been conducted by Joe Scotto and his staff.

Those epidemiology studies, which have been completed and were very fruitful as far as data is concerned, related the general level of ultraviolet radiation coming down through the ozone layer in different localities around the country.

However, the measurements were taken at single sites in given cities or a given state, which was supposed to be representative of that radiation flux. And even though the correlation of that average flux with the actual incidence of the non-melanoma skin cancer in the different localities was good, and even though we know people are exposed over many years to different flux levels of radiation to build up the dose necessary to initiate the tumors, still it was very desirable to have personal dosimeters that people can use. In this way, we can have cohorts use dosimeters in their daily life to find out the actual flux of a typical or total exposure.

The actual UV radiation meter that has been used in the labs is either a very large spectrophotometer which measures individual wavelengths and integrates them, or the one that is used in the field, which is the Robertson-Berger meter. This integrates over the range of most of the UVB up through part of the UVA, and gives a much heavier weight to the UVA than, say, the action spectrum for that weight radiation band. However, it is very cumbersome, and one couldn't just carry it around during everyday activities.

So we were to develop a small, portable dosimeter. This was done under a separate contract, or part of the study, to package things, based on those requirements, and perhaps by also looking at some other type of photometer.

The end result was, that even though there were exposure studies done in San Antonio to determine this with various controlled exposures, it turned out the instruments were not adequate for the experiment. They were what might be called quasi-quantitative. There were problems with the calibration curve. They tended to be satisfactory at low exposures and at high exposures for integrated dose, but for in-between values, the scattering was just too large to be of real use, if we were to try to commercialize the item.

The studies of exposures to humans were carried out, and the results showed that these instruments could not provide quantitative data, but could provide qualitative data. They can provide information on the differences among various exposures, either with regard to localities or people, from which it can be determined that exposure differences in general are inadequate. That was the final conclusion of the experiment.

As an aside, though it was not funded through this project, EPA also funded, with Mr. Berger of Robertson-Berger fame and Professor Urbach's laboratory at Temple University in Philadelphia, an attempt to develop a small film badge, say, the size of a wristwatch, using film very sensitive to ultraviolet light. One of the film types was the type used by astronomers. Yet, there too, even though the response was, for the most part, linear with dose, one could not determine the zero level, so that one could not know whether the integrated dose was really that dose or whether it was something added on to the background. So it showed that one could not simply integrate the dose per repeated exposures and come up with a quantitative reading. That project also was dropped. At the present time, except for the large laboratory instrument and the Robertson-Berger meter, which is about the size of a shoe box, we have not been able to come up with any personal dosimeters that are really practical and useful.

I have some reprints here about the exposures and the readings, etc., for those of you who are interested. This was done by Dr. Arthur Sober up at Massachusetts General.

We also, though we don't have it here, have a full report of approximately 100 pages. For those of you who wish to receive it, I would be glad to send it to you or have Dr. Sober send you his reports.

In a nutshell, that's what this study showed. We are sorry it was not fruitful. On the other hand, it did show that there is a big difference between instruments that can provide semi-quantitative data and those that can provide the quantitative data that are really necessary to do a credible job of determining people's exposure.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Effect of Varying Doses of UV on Mammalian Skin:
Simulation of Decreasing Stratospheric Ozone

Julian M. Menter
Isaac Willis

Morehouse School of Medicine
Atlanta, GA

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I. TITLE:

Effect of Varying Doses of UV on Mammalian Skin: Simulation
of Decreasing Stratospheric Ozone:

Julian M. Menter and Isaac Willis
Morehouse School of Medicine, Atlanta, Georgia 30310

II Date Initiated - 1979

III. Abstract

The overall objectives of these studies have been (1) to determine the carcinogenic potentialities of varying increments of UVB (290-320 nm) in combination with UVA (320-400 nm) in a manner which simulates ozone depletion, (2) to determine the photobiological and possible immunological bases for the results, and (3) to conduct pilot studies aimed at assessing the potential effectiveness and photochemical stability of certain sunscreens under similar conditions. We irradiated albino SK-1 hairless mice with solar-simulating radiation (SSR, 290-400 nm) or with "monochromatic" bands at selected UVA and UVB regions, starting with 0.5 or 0.9 MED (minimal erythema dose) followed by 20% incremental increases (of the original dose) every two weeks for 4-14 consecutive weeks. Under these conditions, tumors can be produced in as little as 4 weeks. Clinical and histological responses as functions of total dose, dose fractionation, spectral distribution and light intensity (fluence rate) showed that photocarcinogenesis is a dynamic process, in which tumorigenesis is a complicated function of the above photobiological parameters. Erythema and tumorigenic responses are not simply related. Action spectra for early precancerous responses (benign hyperplasia) were similar to that for mild erythema; later chronic responses (actinic keratic-like lesions through frank tumors) were progressively shifted to longer wavelengths. Measurements of UV-induced epidermis-stratum corneum thickening (UVT) by absorption-forward scattering spectroscopy showed that UVT could account for a significant amount of dose-dependent UV attenuation, especially at $\lambda < 300$ nm. This effect probably accounts for most of the observed dose-dependent shifts in action spectra, although other factors may play a role, too. We propose a dose-response model in which UV-excitation of target chromophore(s) initiates events which lead to UVT. The radiation reaching such targets is attenuated by a wavelength - and dose-dependent inner filter effect. This model predicts an approximately linear relationship between $\log \Delta OD_{320}$ and the "cumulative dose" seen by the target, with slope proportional to $\epsilon \phi$ of the reactive chromophore(s). The "cumulative dose" was estimated from the total delivered dose and the skin absorption. For $\lambda \geq 290$ nm, our data was in good agreement with this model for the case where the targets were assumed to be in the basal layer, but not for the case where they were assumed to be in the stratum corneum. These results suggest that the receptor(s) for UVT lie near the basal layer. Preliminary *in vivo* experiments with certain "combination" sunscreens (SPF = 6, 8, 15 respectively) showed that these preparations significantly, but not completely, retard the development of clinical and histological precancerous lesions. Both PABA and oxybenzone appear to be stable to SSR, by absorption spectrophotometric criteria, although other interactions cannot yet be ruled out. Preliminary experiments with adult-thymectomized δ -irradiated (ATX) mice indicate that early keratotic verrucoid lesions are cancerous, and that they may also be highly antigenic. UV-induced suppression of contact hypersensitivity has been demonstrated in hairless mice. These results indicate a UV-induced systemic (probably immunologic) alteration. Further mechanistic studies will be concerned with a more, complete characterization of the phenomena underlying the above results, with special attention to conditions which simulate ozone changes.

IV. Introduction

The malignancies and premalignancies unequivocally associated with sun exposure include solar keratoses, basal cell epitheliomas, squamous cell carcinomas and keratoacanthomas. The evidence that such tumors might be associated with solar radiation includes their increased prevalence (a) on parts of the body habitually exposed to sunlight, (b) in lightly pigmented and, thereby, less protected individuals in areas of the world which have the greatest isolation, and (c) in individuals who spend more time out of doors.

The problem of ultraviolet carcinogenesis is complex. Among the photobiological factors which must be considered are (a) relative effectiveness of different wavelengths in carcinogenesis, (b) dose-response relationships, (c) time-dose effects, (d) repair processes including UV-induced skin thickening ("hardening") and (e) immunologic considerations. Closely related to the above are the environmental effects on UV carcinogenesis, which involve geographic, temporal, anatomical, and life-style-related considerations (e.g., modification of the stratospheric ozone layer by human activities). Sunscreens provide still another type of "environmental" modification, and it is of critical importance to know how effective a given sunscreen will be against cancer, both under existing solar conditions, and under ozone depletion. This work deals in some detail with the photobiological factors vis a vis the ozone depletion problem as explained below. In addition, preliminary experiments which are aimed at assessing the efficiency and stability of "typical" sunscreen preparations with respect to photocarcinogenesis are described.

Although it is generally agreed that the UVB portion of the solar spectrum (i.e., 290-320 nm) is most effective in causing skin cancer, knowledge of the relative effectiveness of these wavelengths is not well known (i.e., reliable action spectra do not exist). Moreover, the UVA region (320-400 nm), which was long thought to be unimportant in UV carcinogenesis is now known to augment UVB sunburn response and also appears to augment the UVB carcinogenesis. In preliminary experiments, we observed that the photoaugmentation effect for sunburn response is time-dependent; the time interval between exposure must not exceed six hours for the effect to be observed. The observation of UVA involvement is a great practical (as well as mechanistic) importance because of the predominance of UVA in the total UV solar spectrum.

Because of the complexities brought on by the above-mentioned photo-augmentation phenomenon, as well as by competing repair-, biochemical, and immunologic mechanism, the dose-response or time-dose effects may not be straight forward. Indeed, time-dose reciprocity does not exist for UV-induced tumors (Forbes et al, 1979; Willis et al, 1981; 1983). The detailed knowledge of time-dose-response characteristics are not known, but are required for explanation and prediction of observed carcinogenic effects.

In recent years, concerns have been raised that environmental pollution may affect skin cancer incidence by affecting the stratospheric ozone shield. The ozone shield removes more than 99% of ultraviolet radiation of wave-lengths less than 320 nm from the incoming sun's rays. (Emmett, 1979). The ozone, formed by the interaction of oxygen and wavelengths shorter than 240 nm, then screens out longer ultraviolet wave-lengths up to about 320 nm. The ozone is naturally destroyed by various mechanisms including combination with OH ions from water and destruction catalyzed by certain gases such as NO. The stratosphere differs from the lower atmosphere (troposphere) in that it has a very low turnover rate, so that any pollution accumulates and is very slowly removed.

Man could, theoretically, reduce the stratospheric ozone concentration by a variety of ways including thermonuclear explosions, exhaust gases from SST's, and halomethanes used as spray propellants. Predictions on the degree to which these activities will increase the actual frequency of carcinogenesis are difficult to make because they require estimation of several relationships which are not at present well known, namely the degree to which these man-made activities will increase the amount of ambient UV radiation, and the degree to which these increases will be effective in producing cancer. (We already have a glimpse of the problems posed in elucidating the degree to which UVB increases will result in skin cancer, for an appreciation of the formidable problems associated with assessing the degree of ozone depletion, see Fox, 1979). An earlier estimate (Maugh, 1980) projects a 16% reduction in stratospheric ozone and consequent 44% increase in UVB radiation reaching the earth's surface. More recent estimates (Urbach, 1982) predict that steady state reduction in ozone due to continued production of chlorofluorocarbons could be between 5% and 9%. Epidermiological models predict that for 9% decrease in ozone, basal cell cancer incidence will rise 2-5% and approximately 4-10% for squamous cell carcinoma (Urbach, 1982).

V. Objectives of this Work

The overall objectives addressed by these studies are to:

- 1) Determine the carcinogenic potentialities of varying increments of UVB (290-320 nm) in combination with varying increments of UVA (320-400 nm) in a manner which simulates ozone depletion.
- 2) Determine the photobiological basis for the results obtained above.
- 3) Assess the potential effectiveness and photochemical stability of certain sunscreen agents vis a vis ozone depletion.
- 4) Conduct pilot studies to assess the relative role of systemic and antigenic effects in UV-carcinogenesis under simulated conditions of ozone depletion.

These objectives are concerned with the effect of (a) superimposing a constant or varying band of UVA on the action of UVB radiation ("photo augmentation effect") and (b) ozone depletion.

VI. Methods

A. Photobiological Experiments:

To accomplish the above goals, we irradiated albino hairless mice with "solar-simulating" ultraviolet light and with "monochromatic" light at selected wavelengths in the UVA and UVB regions. Solar-simulating UV (SSR) was obtained from a 1600 watt ozone-free Xenon arc, whose emission was passed through a 45° dichroic mirror and a 2 mm Corning #9863 filter. This combination limits the spectral output to the range 290-400 nm. The #9863 filter does not significantly affect the short wavelength cutoff, which is limited to ~290 nm by the impure quartz used for ozone-free emission.

"Monochromatic" light (band-width 10 nm) is isolated from a 200 watt Xenon-mercury arc (Canrad Hanovia) by means of a Bausch and Lomb high intensity grating monochromator. To simulate varying thicknesses of stratospheric ozone, we vary the amount of UVB while keeping that of UVA essentially constant. This is accomplished by filtering SSR through a series of Schott WG-320 filters ranging from 0.5 to 4.0 mm. These are sharp cut-off filters whose characteristics are given in Table 1. UVA is obtained by means of filtering SSR through a Schott WG-345 filter, which cuts off essentially all light below 324 nm (Table 1). Absorption data was obtained using a Cary 219 or a GCA-McPherson Absorption Spectrophotometer.

Table 1

Characteristics of Schott Filters used to Vary
Amount of UVB Output from Solar Simulating Lamp *

<u>Filter</u>	<u>Thickness (mm)</u>	<u>λ_{cut}</u>	<u>$\lambda_{50\%}$</u>	<u>$\lambda_{90\%}$</u>	<u>$\lambda_{99\%}$</u>
WG-320	0.5	310	305	296	292
"	1.0	313	308	300	295
"	2.0	318	313	305	300
"	3.0	321	316	307	303
"	4.0	323	318	309	304
WG-345	2.0	346	341	330	324

* All wavelengths in nm. λ_{cut} refers to approximate wavelength where filter starts to cut off. $\lambda_{50\%}$, $\lambda_{90\%}$ and $\lambda_{99\%}$ refer to wavelengths where 50, 90, and 99% of the incident light is absorbed.

Light intensities (fluence rates) were varied by means of neutral density metal screens constructed and calibrated in this laboratory. The attenuation afforded by these screens is essentially constant throughout the entire UV-visible range.

Sk-1 hairless mice were irradiated on the back with either the solar-simulator on the monochromator. Minimal erythematous doses (MED) were established for each wavelength band. Subsequently, mice were irradiated 5 days per week starting with either 0.5 MED or 0.9 MED via the rapid tumor induction technique described by Willis et al (1981). Essentially, this involves incrementally increasing the dosage by 20% of the original dose every tenth irradiation day. Clinical and histological responses were monitored as follows:

Table 2

Responses of SK-1 Hairless Mice
to UV Radiation

E-1	Mild to moderate macular erythema
E-2	Intense macular erythema
1+	Light scaling accompanying erythema
2+	Firm scaling, palpable keratosis
3+	Raised palpable keratotic plaque corresponding to early malignant development as defined by Epstein et al (1969)
4+	Papilloma or tumor corresponding to extensive malignant development.

Histology. Biopsy specimens taken at various times during the experiment were formalin-fixed and prepared for routine H and E staining.

B. Measurement of UV-induced Epidermal-Stratum Corneum
Thickening (UVT):

Inbred SK-1 albino hairless mice were irradiated 5 days per week with the grating monochromator at 280,290,300,307, and 313 nm, (starting at 0.9 MED followed by 20% incremental increases of the original dose) every 2 weeks for 4-14 consecutive weeks. Irradiated and control full-thickness epidermis including stratum corneum (separated from dermis by treatment at 60° C for 30 s) were examined histologically and by forward scattering - absorption spectroscopy in the 250-400 nm range. Instrumentation for these essential experiments were made available by Dr. R.M. Sayre, Memphis, TN. This instrumentation consists of a Beckman Acta MBI Spectrophotometer with diffuse reflectance sphere optics interfaced with a digital MINC - 11 mini-computer (Digital Equipment Corp). For quantitative work, UVT was most conveniently expressed as increased absorbance at 320 nm, Δ OD 320.

C. Efficiency and Stability of Sunscreens Preparations
Under Irradiation with UVA and UVB

For these preliminary studies, commercial sunscreen preparations containing Padimate O and "benzophenones" were used, with SPF values of 6,8, and 15 respectively. Groups of 10 mice were delineated as follows: A no sunscreen, B,C,D,- treatment with the SPF 6,8, and 15 preparations respectively. Each sunscreen was applied via an Eppendorf pipette, and evenly spread over a 2 cm square on the back at 2 μ l/cm². After the sunscreen was allowed to dry for 15 minutes, all groups were irradiated with SSR as described above.

Solutions of PABA and of oxybenzone in isopropanol were extensively photolized with solar-simulating radiation (630 J/cm² for PABA and 3200 J/cm² for oxybenzone). Changes in composition were monitored by absorption spectrophotometry.

D. Systemic and Antigenic Effects involved in in UV Photocarcinogenesis with Increases in UVA and UVB

These experiments were carried out in conjunction with Dr's. E.C. DeFabo and M.S. Fisher.

Adult thymectomized SK-1 mice were prepared as follows: Four week-old mice are anesthetized with metofane (methoxyflurane), followed by removal of thymuses via vacuum suction through a mediastinal incision. After a recovery period of 2-4 weeks, these mice were γ -irradiated with 450 R, to produce ATX mice (we thank Dr. T.F. Craft, Georgia Tech, Atlanta, GA for these irradiations).

Twenty-four hours after γ -irradiation, these ATX mice, along with normal control, mice were challenged with 1 mm³ tumor tissue fragments prepared from hairless mice which has been irradiated with solar-simulating radiation. These mice were scored at weekly intervals for tumor growth.

Induction and measurement of contact hypersensitivity (CHS) was carried out as described by DeFabo and Noonan (1983). Briefly, 100 μ l of a 1% or 5% solution of 2-chloro-1,3,5-trinitrobenzene (TNCB) in acetone was used as sensitizing dose. Five days later, a challenge dose of 5 μ l TNCB was applied to the surface of the ear. Ear thickness was measured with an engineer's micrometer before and 24 hours after application of the challenge dose. Percent of control response was determined as described in De Fabo and Noonan (1983).

VII. Results and Discussion

A. Photobiological Studies

Much of the initial results have been published in the previous workshop (Menter and Willis, 1981) as well as in the open literature (Willis and Menter, 1983; Menter et al, 1982). The results of these studies show that:

- 1) Photocarcinogenesis is a dynamic process
- 2) Decreasing filter thickness (simulating decreasing ozone) lowers the threshold dose required for tumor production.
- 3) Because of the dynamic nature of tumor production, the rate of tumorigenesis is a complicated function of radiation dose-delivery and spectral distribution.
- 4) Early precancerous responses have action spectra similar to that for mild erythema (figs. 1,2) ($\lambda_{\text{max}} \approx 300$ nm). Later chronic responses show an apparent dose-dependent shift to larger wavelengths. Fig. 3 shows that result for a 2+ ("keratotic-like") lesion, with an apparent λ_{max} at 307nm. For more severe responses, this effect is much more exaggerated. We were unable to obtain tumors for $\lambda < 300$ nm.

- 5) Despite early similarities in action spectra, there is not a simple relationship between erythema and tumorigenic responses.

In additional on-going studies, the effect of light intensity (fluence rate) on UVA photo augmentation of (a) UVB erythema and (b) UVB carcinogenesis is under investigation. These studies, conducted at different light intensities, are different from previous dose fractionation studies, in which the doses were given at a single light intensity, but at different schedules. UVB erythema is well known to obey Bunsen-Roscoe reciprocity; the response depends only on the total dose, and not on the light intensity, (and therefore time necessary to deliver the dose). We find that UVA photoaugmentation of UVB erythema also obeys Bunsen-Roscoe reciprocity over an approximate 50-fold range.

On the other hand, the development of precancerous lesions under SSR does not obey Bunsen-Roscoe reciprocity. Even over only a 5-fold difference (45 mw/cm^2 - 225 mw/cm^2), it can clearly be shown that such lesions are produced more efficiently with the higher intensity radiation. The effect of fluence rate on UVA photoaugmentation of UVB carcinogenesis is also currently under investigation.

The above results further underscore the mechanistic dissimilarity between erythema and photocarcinogenesis. The lack of dependence on fluence rate on production of UVB erythema as well as its augmentation by UVA suggests a relatively "simple" photochemistry, in which the events leading to UVB erythema and its augmentation by UVA are much more rapid than competing "dark" events which occur at their expense.

On the other hand, the marked intensity dependence of precancerous development suggests a far different situation, with, perhaps, the involvement of a photostationary state. One may speculate in this case, that "dark" processes may successfully prevent a reactive photochemical intermediate or product from building up, even over the short term. These latter findings are in accord with our previous results on dose fractionation, and they seem to indicate the importance of light intensity in photocarcinogenesis and, perhaps, photoaugmentation of carcinogenesis.

B. Measurement of Epidermis-Stratum Corneum Absorption; Dose-Response Relationships for UVT

Previous experiments indicate the dynamic nature of tumorigenesis (i.e., the apparent action spectrum seems to shift towards longer wavelengths as photocarcinogenesis ensues). Such a shift could result, at least in part, from dynamic UV-induced stratum corneum-epidermal thickening (UVT), whereby a thicker skin would preferentially absorb shorter UV ($\lambda < 300 \text{ nm}$). We therefore sought to quantitatively measure dose- and wavelength-dependent changes occurring on UVT, and to seek a dose-response relationship which might (1) allow an estimate of the importance of the latter in the apparent "shift" of "the" photocarcinogenesis action spectrum and (2) provide a basis for explanation and prediction of dose-response curves for photocarcinogenesis under both monochromatic radiation, as well as polychromatic UVA and UVB, as occurs under ordinary environmental conditions.

Irradiated and control full-thickness spidermis, including stratum corneum, were prepared and examined by forward scattering-absorption spectroscopy as described above (We thank Drs. R.M. Sayre and P.P. Agin for these measurements). At all wavelengths, there was a general UV-induced increase in optical spectra. Under chronic conditions moderate doses (7.2 MED) caused similar increases over the range 280-320 nm (fig. 4) At higher doses, thickening progressed markedly at $\lambda \geq 300 \text{ nm}$, but progressed only slowly, if at all, for 280 nm and 290 nm.

Results for long term irradiation (≈ 100 MED) are shown in fig. 5. These increases in epidermis-stratum corneum absorbance correlated well with clinical and histological evidence of skin thickening.

We proposed a dose response model in which UV excitation of target chromophore(s) initiates events which lead to thickening. At any time, the radiation reaching such a target is attenuated by a wavelength and dose-dependent inner filter effect. For quantitative purposes, UVT could be conveniently expressed as the increase in absorbance at 320 nm, ΔOD_{320} . Our model predicts an approximately linear relationship between $\log \Delta OD_{320}$ and the "cumulative dose" seen by a target, as shown in equation (1): These derivations of equation (1) will be presented in a later publication.

$$\log \Delta OD_{320} \approx N'' \cdot \text{Dose} + \log (M/N'')$$

(1) Where N'' , and M are constants and

Dose = intensity X time.

The "cumulative dose" was estimated from the total delivered dose and the skin absorption data. For wavelengths equal to or greater than 290 nm, our data was in good agreement for this model for the case where the targets were assumed to be in the stratum corneum. Figs. 6-10 show the results (assuming a basal layer target) for irradiation at 313, 307, 300, 290, and 280 nm.

From our data it appears that UVT can account for a significant amount of UV attenuation, especially at ≤ 300 nm. Equation (1) is obeyed for $290 \leq \lambda \leq 313$ nm, at 280 nm, more complicated behavior is observed, and the data depart from linearity at high doses. This indicates that additional factors play a significant role in the reduced tumorigenic efficiency in this region, and that further study is needed. It appears also that the magnitude of this effect is significant to explain most, if not all, of the apparent shifts in action spectra observed above (although other effects may well play a significant role in this regard).

C. Efficiency and Stability of Sunscreen Preparations Under Irradiation with UVA and UVB

Preliminary experiments are in progress to assess the efficacy of sunscreens in prevention of skin cancer under chronic conditions of solar simulating radiation. For groups of ten mice were delineated: A - no sunscreen, B, C and D are formulation of Padimate O and Benzophenones that give respective SPF values of 6, 8 and 15.

Sunscreen was applied and animals were irradiated as described above. After 12 weeks of irradiation, all animals in group A (no sunscreen) had developed 3+ and 4+ responses. Group B - D had developed erythema, sometimes followed by scaling.

Erythema may not be noticed or may be slight, and precancerous changes may not be readily apparent. However, even under these conditions, there may be important systemic/immunological alterations which may greatly enhance the probability of tumor development. This latter aspect is further discussed below.

In preliminary tests on the stability of certain sunscreen agents solutions in isopropanol of both PABA, and of oxybenzone were extensively photolyzed with solar simulating radiation. Changes in composition were monitored by absorption spectrophotometry. Under these conditions, no absorption changes in either solution could be observed. A thumbnail calculation indicated that if a photochemical reaction with a measurable change in absorption were to occur, its quantum yield would have to be

≤ 10⁻⁴. However, the following possibilities must also be considered: (1) photo-physical or photochemical interaction either between sunscreen and vehicle or between a PABA and a benzophenone derivative in a "combination" sunscreen (2) similar interactions in skin between sunscreen, vehicle, and/or skin components which could conceivably produce an altered chemical or immunological state.

D. Systemic and Antigenic Effects involved in
UV Photocarcinogenetics with Increases in UVA + UVB)

To answer all of the questions posed by these studies, it is necessary to prepare SK-1 hairless mice which have been thymectomized and subjected to sublethal X or radiation ("ATX mice"). Such mice are necessary to bear out the assumption, implicit in all of these experiments, that the UV-induced tumors are highly antigenic. In this case, they should be rejected by non-UV treated syngeneic recipients, but should grow progressively in syngeneic mice that have been immunosuppressed (ATX) or treated with intermittent doses of UVB radiation. Because of these properties, these ATX mice are important in studies involving tumor transplantation and growth of SSR - induced tumors.

ATX mice were prepared as above, and challenged with 1 mm³ fragments from early keratotic verrucoid lesions via trocar-implantation. These transplants developed into full-blown squamous cell carcinoma. Early results indicate that similar lesions are rejected in normal, syngeneic mice and may therefore be highly antigenic. These findings, though preliminary, are nonetheless of great interest, since the majority of previous cases of such behavior have involved fibrosarcomas.

UV induced suppression of contact hypersensitivity, which we have demonstrated to occur in other strains of mice, is being used as a marker for systemic and antigenic effects in cutaneous UV effects. Previous studies (DeFabo and Noonan, 1983) indicate that this phenomenon has many photobiologic and immunologic similarities with UV-mediated suppression of tumor rejection.

Initial results appear very encouraging. The first, and perhaps, most critical experiment has been successfully performed: We have elicited contact hypersensitivity in the SK-1 hairless mouse; the degree of hypersensitivity observed was comparable to that seen in other strains of mice. This finding was highly significant, because (1) the ability of our model to be contact hypersensitized is obviously fundamentally essential for all further experimentation; (2) it shows the feasibility of using the hairless mouse as an animal model for these studies. As a corollary, it verifies that mice with the unusual skin property of having little or no hair, can be contact hypersensitized in the same fashion as normal haired strains.

Next, we observed dose-dependent suppression of contact hypersensitivity in hairless mice by suberythral doses of UVB (FS-40 lamps). Mice were irradiated with fractionated UVB doses ranging from approximately 0.3 to 2.0 MED five days prior to sensitization and subsequent challenge with TNCB. Measurement of ear swelling as described in DeFabo and Noonan (1983) revealed a dose-dependent suppression of contact hypersensitivity by UVB, as indicated above.

The above finding is very significant since it also verifies and extends the important observation that UV can induce a systemic alteration (in this case suppression of contact hypersensitivity) such that antigenic specific suppressor cells are formed in the spleens and lymph nodes of UV irradiated animals. Although in these initial experiments, we did not specifically look for suppressor cells (this is the aim of future experiments), the response is very similar both in terms of kinetics of dose-response and suppression of ear swelling.

It is unlikely that some other form of suppression occurred, especially as we took great pains to record responses of suberythral doses. In addition to measuring suppressor cells, we will employ doses of UV which extend the range in both directions. The latter experiments are aimed at a further verification of our preliminary results.

VIII. Significance

Past and present investigations have shown that UVB radiation (290-320 nm) is the primary cause of cutaneous cancer, and that UVA (320-400 nm) though itself a poor carcinogen, can augment the effects of UVB. Our studies have been concerned with the relationship between skin cancer and stratospheric ozone layer thickness. It is common knowledge that even slight decreases in stratospheric ozone lead to significant detrimental effects in living matter on earth. Such a change is of significance in investigating the potential extent of such deleterious effects, as well as providing information concerning prophylaxis and adjustments necessary to prevent these effects. Our research investigations which have been carried out under the auspices of the EPA have been concerned with the deleterious effects of solar radiation reaching the earth's surface, with special emphasis on how such effects including, systemic, immunologic alterations would be amplified as a result of ongoing alterations in stratospheric ozone thickness. These effects on human skin are significant in that they might lead to a significant increase of serious skin cancer in particular at high altitudes, in "clean" air environment and/or in future space travel.

IX Proposed Course - Future Plans

In the mechanistic photobiological studies, we plan to complete the preliminary work in the fluence rate (intensity) studies, as well as in the UVT studies. These studies are of great importance for a fundamental understanding of the photochemical aspects of UV carcinogenesis. The UVT studies, however, will additionally allow a correction to be made for the dose- and wavelength-dependent distortions inherent in "raw" action spectra, which are caused by optical absorption and scattering of skin. Such a correction could allow a much better guess as to the nature of the photo-reactive chromophore(s); at present, such a guess is virtually impossible to hazard.

We will continue on course with the sunscreen experiments for, both in vitro and in vivo efficiency studies (under conditions of ozone depletion). These will dovetail with the immunological studies, in addressing the logical question as to whether or not sunscreens prevent or retard systemic immunological effects of sub-carcinogenic UV (again, as a function of conditions simulating ozone depletion).

As for the immunological studies; assuming that the original preliminary results are re-confirmed, we will address the following questions.

- 1) Can solar-simulating radiation produce a systemic defect in tumor rejection?
- 2) Is such an effect subject to photoaugmentation (or the reverse)?
- 3) What of the mechanism of such effects (if present) - are T-suppressor cells induced, etc?

Future experiments envisioned for the contact hypersensitivity (CHS) studies involve a further search for the photoreceptor, believed to be urocanic acid (DeFabo and Noonan, 1983).

These experiments involve (1) removal of the stratum corneum by tape stripping, in which case attenuation or loss of systemic suppression of CHS should occur if urocanic acid is the receptor, and (2) preliminary in vitro tests of "dark" and "light" binding of urocanic acid to stratum corneum components.

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Fig. 1

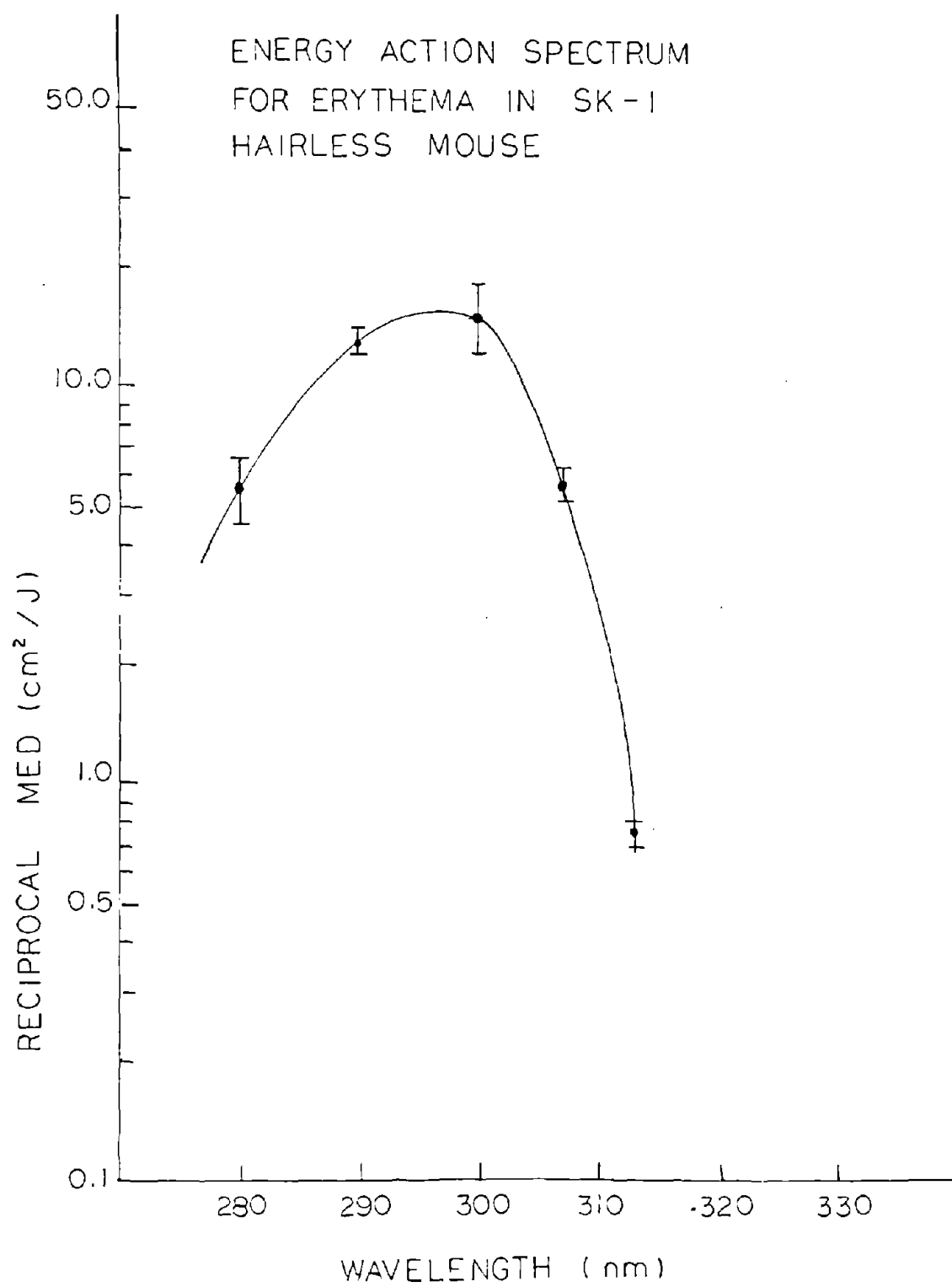


Fig. 2

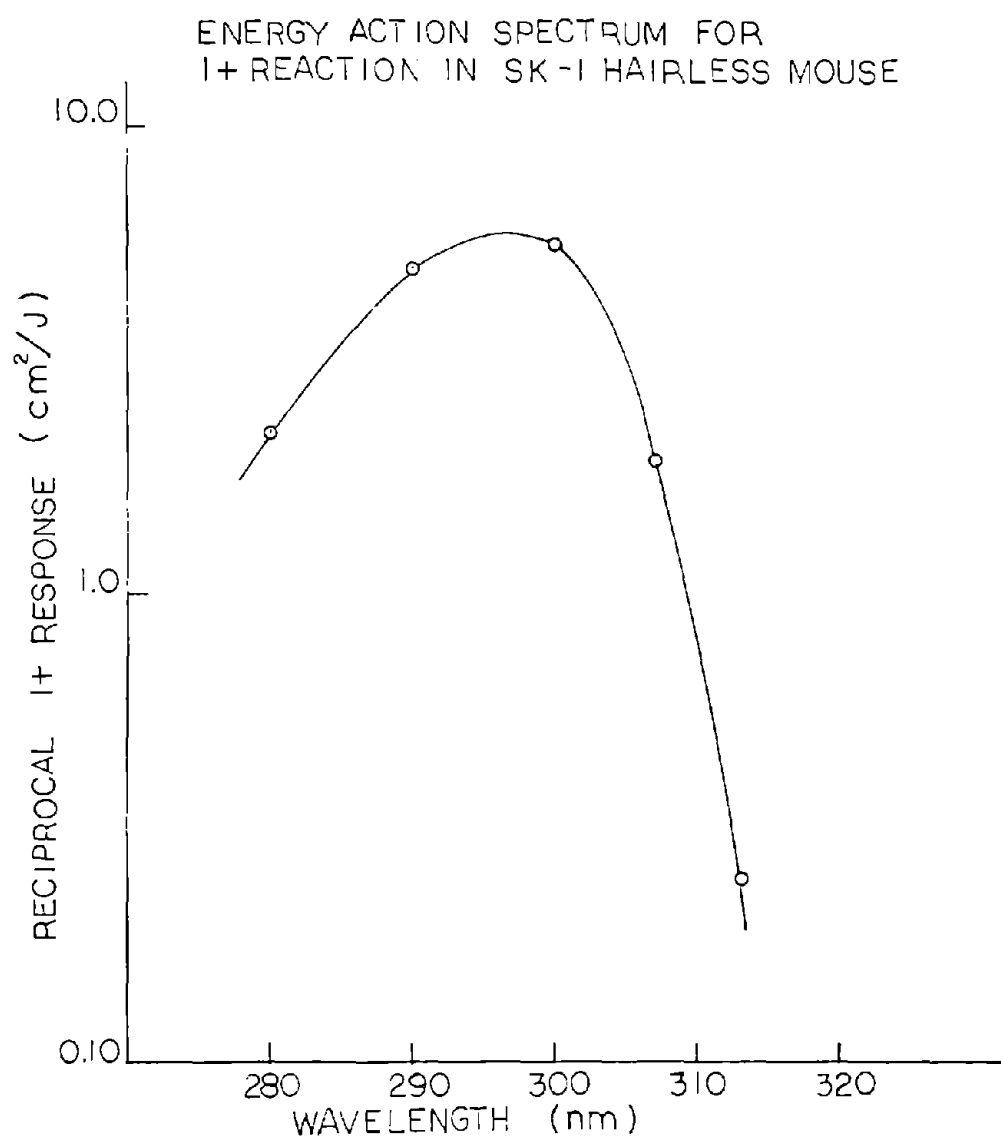


Fig. 3

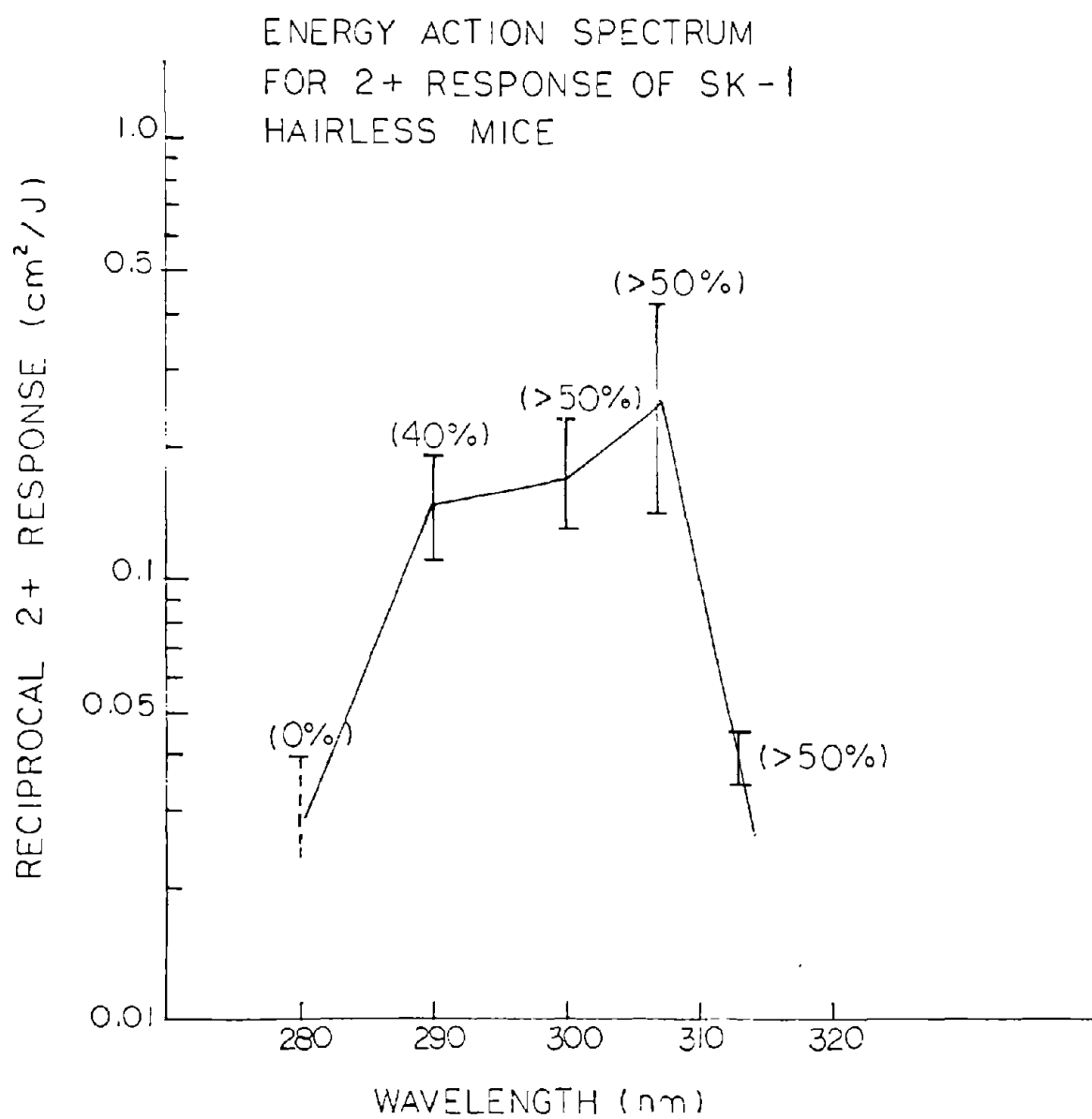


Fig. 4

FORWARD - SCATTERING ABSORPTION
OF UV - IRRADIATED HAIRLESS
MOUSE SKIN (1+ REACTION)
7.2 MED / WAVELENGTH

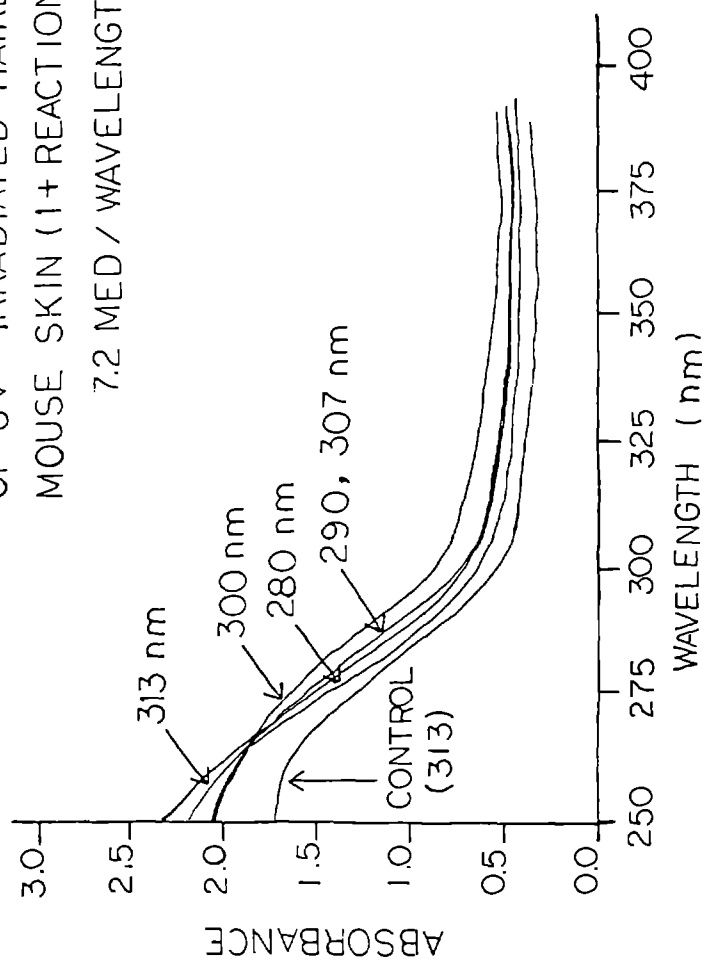


Fig. 5

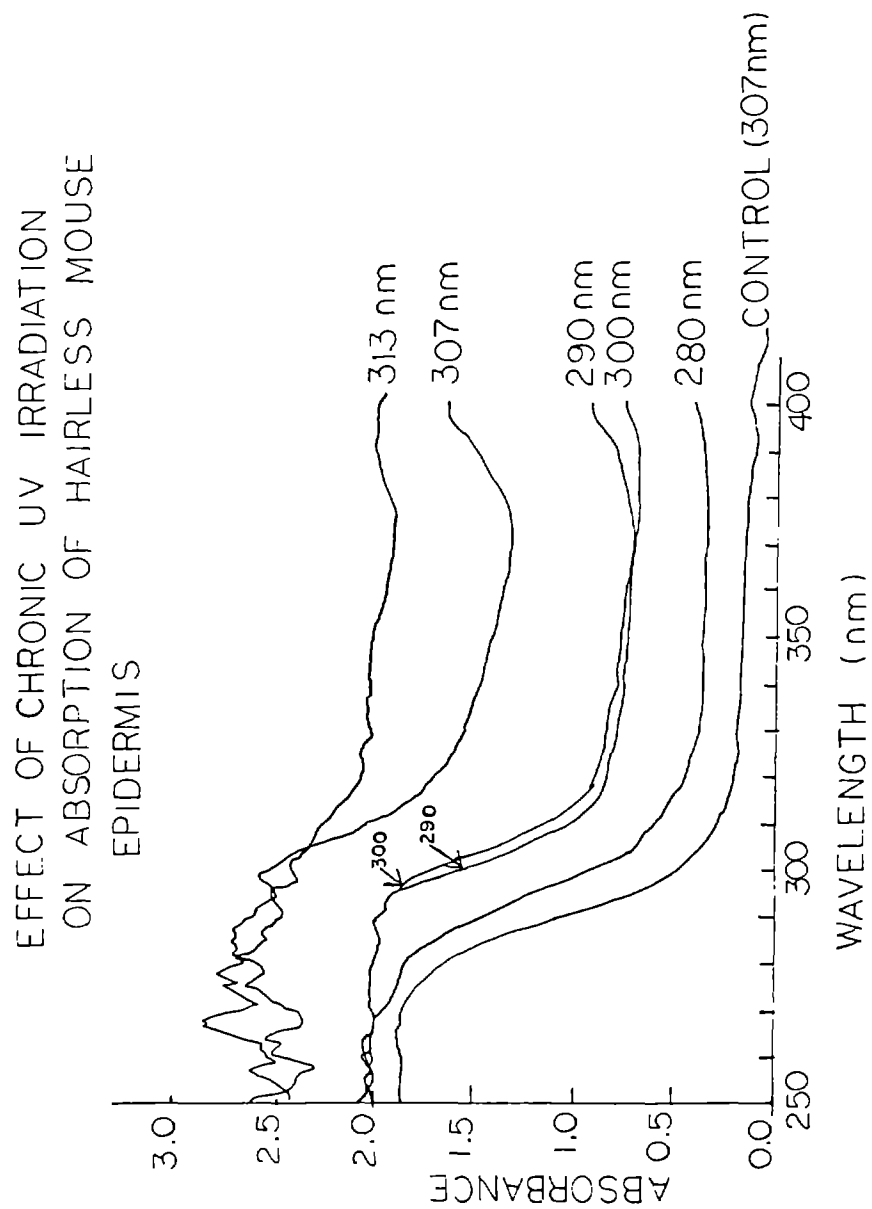


Fig. 6

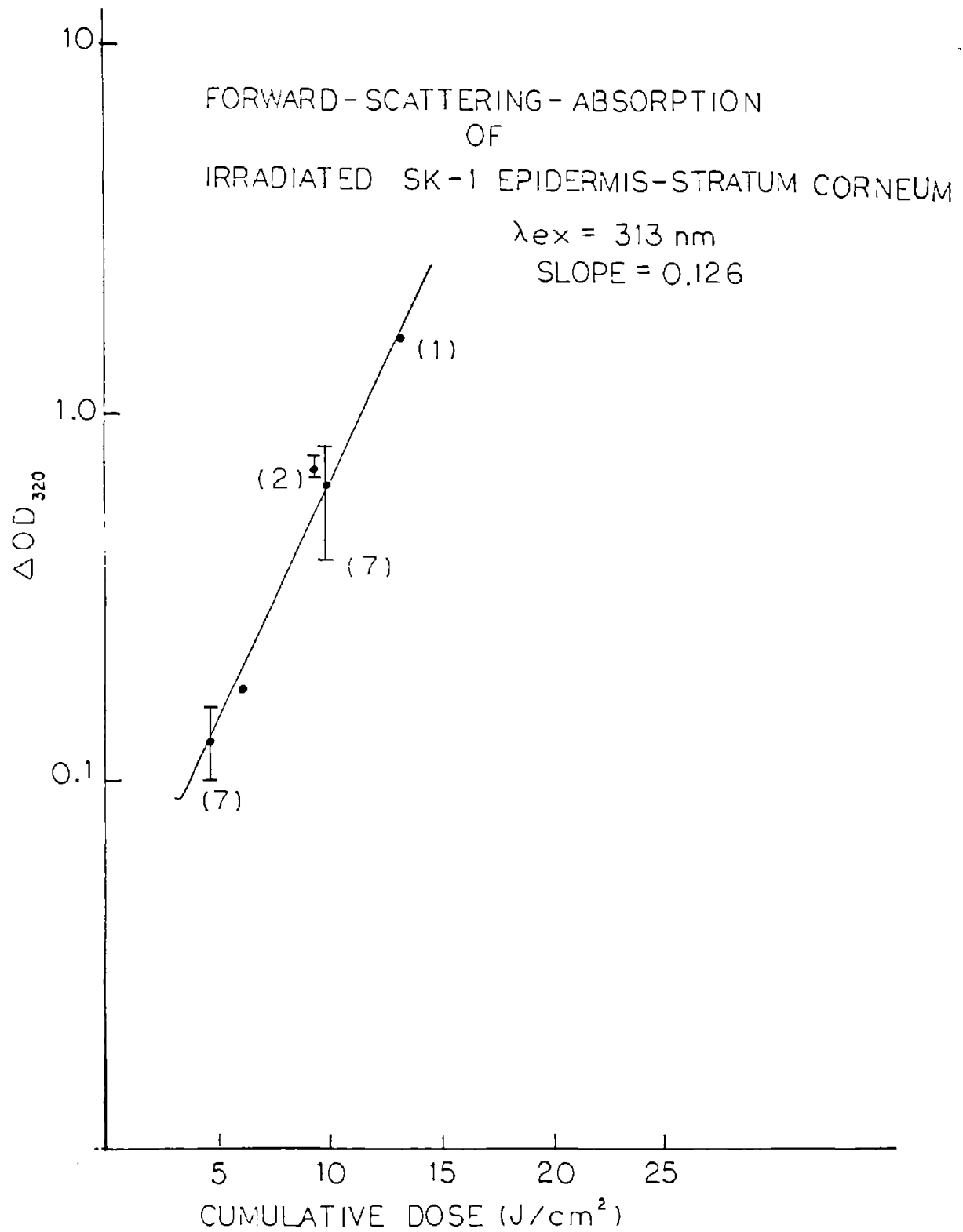


Fig. 7

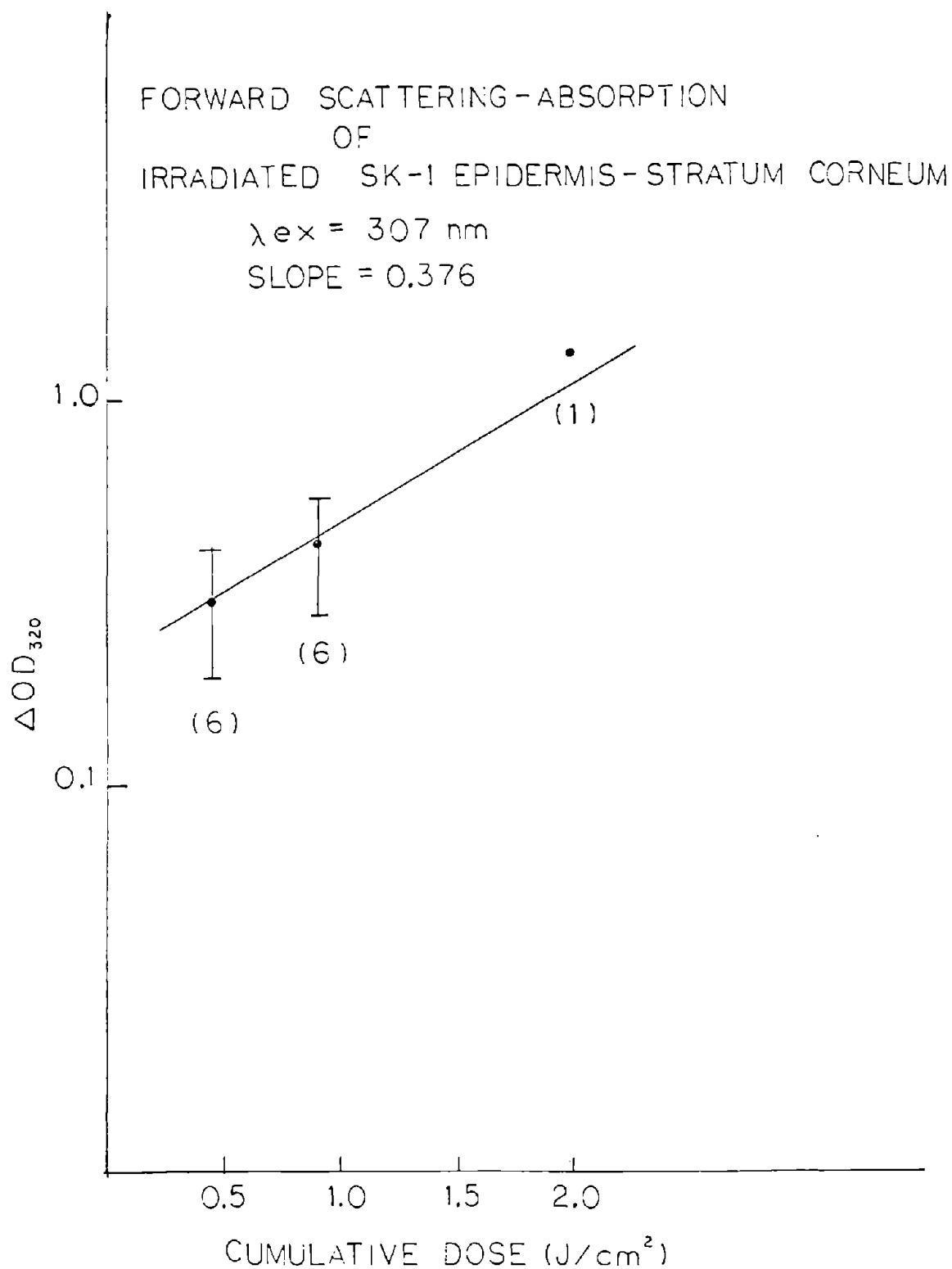


Fig. 8

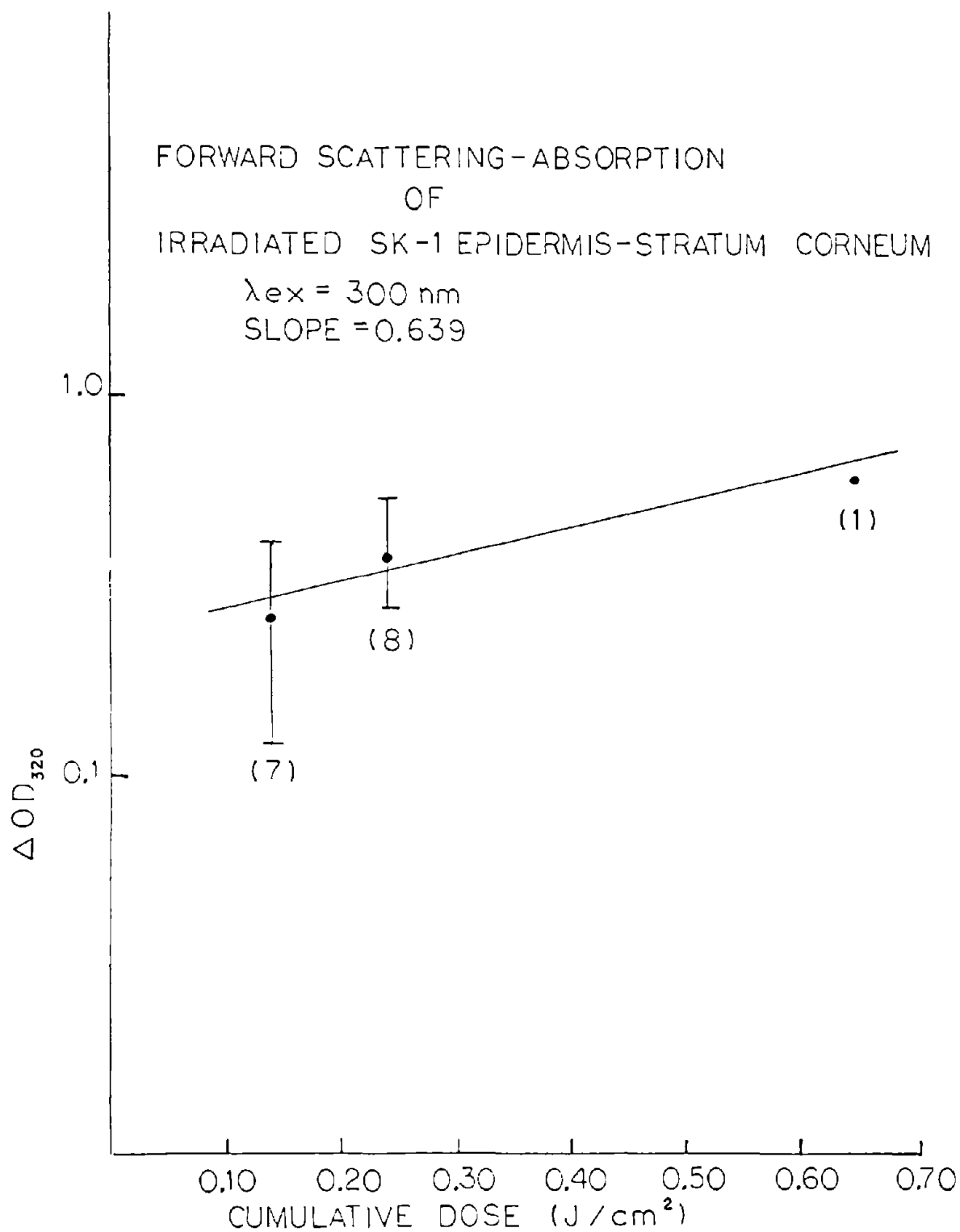


Fig. 9

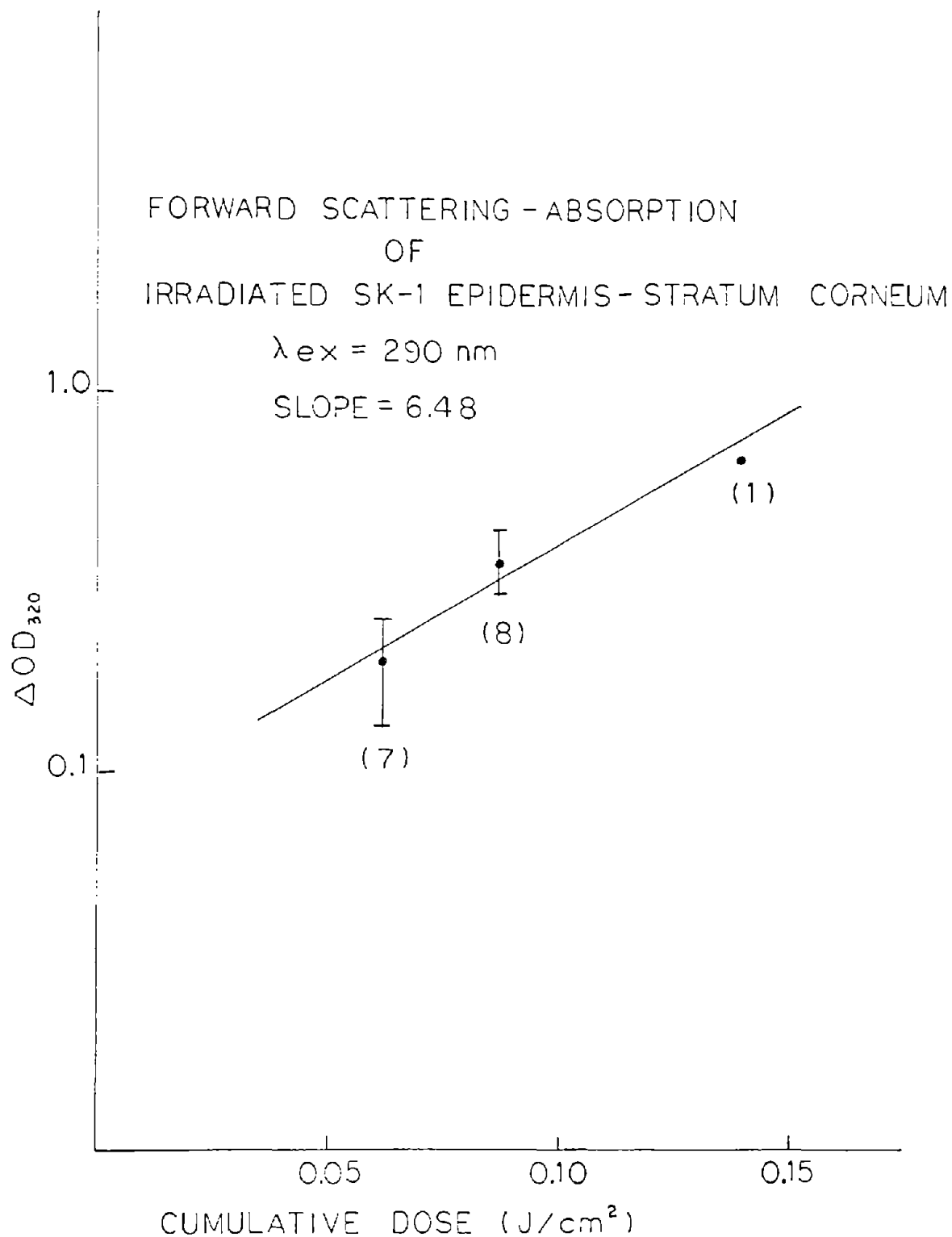
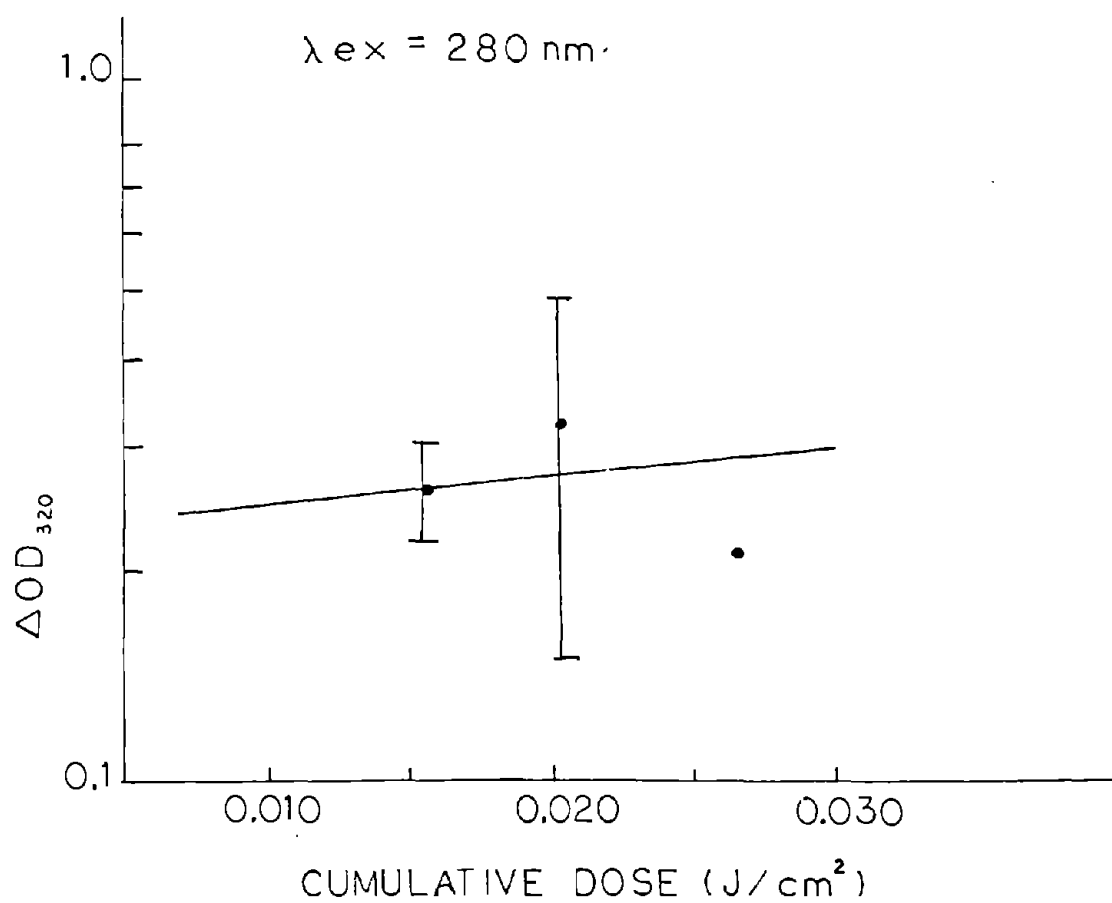


Fig. 10

EPIDERMAL - STRATUM CORNEUM FORWARD-SCATTERING-
ABSORPTION IN THE SK-1 HAIRLESS MOUSE
AS FUNCTION OF UV - IRRADIATION



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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

A Comparison of the Skin Carcinogenicity of
Condensed Roofing Asphalt and Coal Tar Pitch Fumes

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A COMPARISON OF THE SKIN CARCINOGENICITY OF
CONDENSED ROOFING ASPHALT AND COAL TAR PITCH FUMES

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Abstract

The relative tumorigenicity to mouse skin of condensed volatile components of roofing petroleum asphalts and coal tar pitches was investigated. The primary factorial experiment involved 32 groups of 50 mice each. The experimental variables were: two strains of male mice (CD-1 and C3H/HeJ); four initial roofing materials (asphalt, Types I and III; coal tar pitch, Types I and III); two temperatures of generation of volatile fume materials (232° and 316° C); and exposure to simulated sunlight vs. the absence thereof. Treatment with condensed volatiles and light was performed twice weekly for 18 months. Additional groups were a combination treatment (alternate weekly application of 316° C volatiles from Type III petroleum asphalt and Type I coal tar pitch); a positive control (benzo(a)pyrene, with and without sunlight); and negative controls for solvent and sunlight.

Analyses of the results have led to the following conclusions. Temperature used in preparation of the condensed fume material had a pronounced effect on tumorigenesis, the 316° C preparation being the more active. The C3H/HeJ strain was much more sensitive than the CD-1 strain to the tumorigenic activity of the fumes. Simulated sunlight, as used in this experiment, had an inhibitory effect on the rate of appearance of tumors and on the final tumor incidence. Ratios of tumor types, i.e., percent benign:malignant, were high for the C3H/HeJ groups (~ 30:60) and much lower for the CD-1 groups (~ 33:5). In comparison to benzo(a)pyrene (B(a)P) controls, the coal tar pitch fume materials showed effects that appeared to be consistent with their B(a)P content. The petroleum asphalt fume materials showed higher activity than would be expected based on their content of B(a)P or of total PAH, suggesting the presence of other factors contributing to the tumorigenic response.

Supplementary chemical analysis indicated that aliphatic hydrocarbons were major components of the petroleum asphalt fumes, while the coal tar pitch fume components were largely aromatic.

Introduction

Asphalt and coal tar pitch are bituminous materials which are similar in appearance and have been used interchangeably for roofing, paving and in other industrial applications^{1,2}. Whether exposure to asphalt represents significant health risk has been debated for years. This debate is further clouded by the confusion in the definition and usage of the terms asphalt, pitch, coal tar, bitumen, petroleum pitch, and coal tar pitch. Bingham and coworkers,² in a critical review of the literature described petroleum technology and included a glossary of terms, which should serve as a guide in elucidating this sometimes perplexing terminology.

Coal tar pitch, a residue in the partial evaporation or fractional distillation of coal tar produced by the destructive distillation of bituminous coal, was among the first substances detected as a human carcinogen and successfully tested for its carcinogenic effects in animals.³ Asphalt is a bitumen which occurs in nature or is obtained in the refining of crude petroleum. Several attempts^{2,4,5,6,7,8,9,10,11,12} to produce tumors in animals exposed to raw, solvent diluted or aerosolized asphalt were reviewed by NIOSH which concluded that "reliable reports associating malignant tumors with exposure to asphalt fumes have not been found in the literature" and "although available information has not clearly demonstrated that a direct carcinogenic hazard is associated with asphalt fumes, NIOSH is concerned that future investigations may suggest a greater occupational hazard from asphalt fumes than is currently documented in the literature."¹

Wallcave et al.¹¹ suggested that differences in bioassay results with coal tar pitches and asphalt were related to polynuclear aromatic hydrocarbon (PAH or PNA) content, reflecting their thermal histories. Asphalt production temperatures range from 350° to 400° C and those of coal tar pitches exceed 1000° C. PAH content of coal tar pitches may be several orders of magnitude greater than that of petroleum asphalts, with coal tar pitch containing more higher-molecular weight PAHs.¹³ Wallcave's suggestion is useful for the raw products, but under field conditions thermal histories may be much different from those of the raw materials. For example, Thomas and Mukai¹⁴ reported that heating in roofing kettle operations is poorly controlled and commonly the materials are heated as high as 638° C (1000° F), well above their recommended kettle temperatures of 204 to 273° C. This results in pyrolysis and increased production of PAHs. Some field operations such as the asphalt hot mix operation for road paving do maintain moderate temperatures, generally lower than 160° C.¹⁵

In a study of workers with at least 5 years and an average of 15.1 years exposure to asphalt in 25 oil refineries, Baylor and Weaver¹⁶ found a few cases of bronchitis, asthma, and emphysema but no other significant health effects compared to a control population. They cited further information on workers employed in highway construction, roofing manufacture and truck driving over asphalt highways that exposure to petroleum asphalt constituted no health hazard. Zeglio in 1950 (cited by NIOSH¹), reporting on Italian workers who used natural asphalt, possibly adulterated with coal tar pitch to insulate electrical cables and telegraph and telephone lines, concluded that characterizing bitumen vapors only as irritating did not recognize the

rhinitis, oropharyngitis, laryngitis, bronchitis, and X-ray and respiratory changes from rales to emphysema that they caused.

More recently Hammond et al.¹⁷ found that occupational exposures of roofing workers to asphalt and coal tar pitch fumes for more than 20 years were associated with increased mortality from cancer and other pulmonary diseases including emphysema, chronic bronchitis and asthma. Leukemia and cancer of the lung, upper respiratory tract, stomach, bladder, and skin had excess mortality. Smoking habits were not considered, and the causative factors could not be identified because of mixed exposure to coal tar pitch and asphalt fumes.

Lawther¹⁸ studied mean 8-hour shift exposures of roofing and gas retort workers to benzo(a)pyrene (B(a)P), a ubiquitous carcinogen often mentioned as an indicator of PAH exposure.^{19,20} He found for roofers, exposures of less than 1 and 13 μg for asphalt and hot pitch, respectively and for gas retort workers exposures averaged 27 μg . Bingham et al.² noted that a single carcinogen, such as B(a)P, can serve as a guide to carcinogenic potency, but the presence or absence of B(a)P does not always account for the observed potency, and roles of other carcinogens, cocarcinogens and inhibitors of carcinogenesis must be considered. The International Agency for Research on Cancer²¹ stated that predictions of human cancer risks cannot be made from simple knowledge of PAH levels.

In addition to the risks associated with exposure to the coal tar pitch and petroleum asphalt fumes in the roofers environment, the added risk of sunlight exposure in the out-of-doors environment must be considered. Various investigators working with experimental animals have found that ultraviolet and visible light augment the carcinogenicity of PAH exposure.^{22,23}

NIOSH estimates that 12,000 roofing contractors employ over 116,000 workers in the U.S.²⁴ Since these workers have combined exposures to asphalt, coal tar pitch and sunlight, an experiment was designed to study the relative importance of each and combinations of them. The purposes were to assess: (1) the carcinogenic potentials of condensed volatiles from two commonly used roofing asphalts and two commonly used coal tar pitches collected from fumes generated at recommended application temperatures; (2) the carcinogenic potentials of the condensed volatiles collected from fumes generated at temperatures in excess of their recommended application temperatures; (3) the effects of simulated sunlight on the carcinogenic outcome of the above materials; and (4) the responses in pigmented and nonpigmented mice.

Materials and Methods

Sample Identity, Collection, and Characterization

There are four types of asphalt and three types of coal tar pitch used on roofs. Two types of each, chosen for this study on the basis of common use and extremes of classification, were Type I and Type III asphalt, referred to in the industry as "dead level" and "steep," respectively, and Type I and Type III coal tar pitch often referred to as "regular roofing" and "low fuming" or

"low burn" pitch, respectively. They are produced by several manufacturers to physical specifications recommended by the American Society of Testing and Materials.²⁵

Approximately 270 kg of each of the materials were purchased: Type I and Type III asphalts, purchased from a distributor of Exxon, Inc., Roofing Products (Beacon Sales, Inc., Somerville, MA), were produced by distillation and air blowing of Arabian crude; Type I coal tar pitch, obtained from Reilly Tar and Chemical Corp. (Cleveland, OH), and Type III coal tar pitch, provided by Koppers Company, Inc. (Monroeville, PA). All materials were manufactured to ASTM specifications and were shipped from available inventory. Fumes were generated

from an easily controlled glass generation system and the condensed material was collected in a glass cryogenic system (Figure 1). Laboratory generation and collection was used instead of a roofing kettle to avoid problems of inadequate sample mixing, generation temperature extremes, exposure to sunlight and difficulty in collecting condensed field samples at a subambient temperature. The roofing material was made into small pieces, approximately 10L placed into a weighed 12L round bottom reaction flask, and warmed in a forced air oven at 150° C. When the the material was soft it was stirred with a stainless steel multifinned stirring rod at 250 to 300 rpm. The rod was driven by a compressed air motor and inserted through the neck of the flask lubricated with a teflon stirring gland. An electric heating mantle was used to attain the desired generation temperatures ($\pm 5^{\circ}$ C) of 232° C, the approximate recommended kettle temperature, and 316° C, just below the flashpoint of the materials.

The fume collection system consisted of 20 mm O.D. glass transfer tubes and 500 mL glass impingers placed in three cryotrap, one containing ice (0° C) and two dry ice/isopropanol (-77° C). An impinger containing a 50/50 mixture of cyclohexane/acetone was used after the cryotrap to provide additional collection through dissolution. Air, precleaned and dried using a high efficiency filter, silica gel and granular activated charcoal for removal of particulates, water, and organic vapors, respectively, and heated to 100° C was pulled through the system at a rate of 10 Lpm. The laboratory light was filtered through yellow cellulose acetate-butyrate filters to reduce ultraviolet light induced photooxidation and decomposition during generation and collection.

After the fumes were condensed and collected, the individual impingers and transfer lines were weighed and the material was quantitatively transferred from them with an excess of 50/50 cyclohexane/acetone solvent mixture to a large flask. The solvent was then removed at reduced pressure at 5° C, and analyzed by gas chromatography/mass spectrometry (GC/MS) to confirm that significant amounts of compounds of interest were not present. The collected condensates were weighed and dissolved in a 50/50 mixture of cyclohexane/acetone. One to two generations were needed to obtain the necessary amount of material with the coal tar pitches at either temperature; with the asphalts 34 to 59 generations were required at 232° C and 6 to 7 at 316° C. The materials collected from each generation were combined to produce about 4L each of eight skin painting solutions (4 materials x 2 temperatures).

The collections at 316° C compared to the collections at 232° C, yielded 9 to 16 times more volatile material from asphalts but only 2 to 7 times more from coal tar pitch.

For the final concentration of the skin painting solutions, the coal tar pitch condensates were diluted to attain a B(a)P concentration of 0.01 percent in order to produce an estimated 100 percent tumor incidence at approximately one year after the start of the experiment.²⁶ The final asphalt condensate solutions contained 50 percent total solids, which is a commonly used practice for assaying complex mixtures with low PAH concentrations. Concentrations of selected PAHs in skin painting solutions were determined by combined GC/MS (Finnigan Model 4023) analysis using a high resolution glass capillary column (25-m coated with SP-2250) for separation and quantitation of closely related isomers. A compound was considered as identified when the retention time and the mass spectrum of the PAH of interest matched those of a standard PAH. Table 1 shows the concentrations of 18 identified chemicals and total concentrations of condensed fumes in the 8 skin painting solutions.

Benzo(a)pyrene for the positive controls was obtained from the NCI Chemical Repository (IITRI, Chicago, IL). Cyclohexane and acetone were both HPLC grade (Fisher Scientific, Inc.). Cyclohexane/acetone was chosen as the solvent system for collection, rinsing and preparation of skin painting solutions because: (1) the mixture was a better solvent of the material than either alone; (2) each solvent had a low boiling point and was compatible with PAH stability; (3) the solvents did not absorb light in the simulated sunlight range; and (4) both solvents were inactive with respect to mouse skin bioassay.

Solar Simulator

The solar simulator (figure 2) used a 15-cm Atlas 6.5 kW Xenon arc, water cooled, quartz enveloped burner located about 53-cm above and midway between two turntables. Two additional turntables, completely shielded from light, were contained in the unit for sham irradiation. Wavelengths shorter than 290 nm were excluded by metal oxide coated, 3 mm Tempax glass, No. 114 Schott filters that also reflected infrared radiation (>790 nm) away from the mice. Light measurements were made to validate the geometric concept of the turntable arrangement using a silicon photodiode detector (EG&G) equipped with narrow-band optical interference filters (309 to 790 nm). An automatic integrating system adjusted exposure time for fluctuations and changes in intensity of the arc due to aging to maintain a constant exposure. The solar simulator was contained in an aluminum housing.

Stainless steel (3 x 3 mesh) cage units, specially designed so that two of them fit on each of the four round turntables, contained 50 4 x 9 x 3 cm cells to individually house all mice of a group for simultaneous exposure, and permitted simultaneous light exposure of four groups and sham exposure of four groups. During exposures the turntables were rotated at one rpm, so that on the average each mouse was exposed at the same distance from the source. Cooling air, was delivered to maintain the chamber temperature at about 24 to 28° C.

The goal of the experiment was to produce skin tumor onset in a light exposed control group at approximately 50 weeks. From Bingham and Nord²⁷ and Burns²⁸ it was estimated that the total UVB (280-320 nm) dose required was approximately $2 \times 10^5 \text{ W s/m}^2$ ($2 \times 10^8 \text{ ergs/cm}^2$). Since the mice were theoretically to be exposed twice weekly for a total of 100 exposure sessions, and the wattage at the exposure site was 0.68 W/m^2 , the required duration of exposure per session was approximately 49 minutes. More accurate spectral data revealed that the original integrated irradiance between 280 and 320 nm was too high and thus the actual exposure per session was 1480 W s/m^2 with the desired total dose delivered in 135 sessions.

Skin Carcinogenesis Tests

Nonpigmented Swiss CD-1 (Charles River) and pigmented C3H/HeJ (Jackson Laboratories) male mice were used. Upon arrival, at six weeks of age, they were quarantined for 6 to 9 weeks. They were housed individually in suspended stainless steel cages and provided food and water ad libitum except during the chemical and light exposures. Each of the 48 experimental groups consisted of 50 randomized mice of one strain, and each mouse was individually identified. The 48 experimental groups were:

- a. 32 groups for the primary factorial experiment, i.e., 2 strains x 4 materials x 2 generation temperatures x 2 light exposure conditions (presence or absence of simulated sunlight); each animal dosed twice weekly with 50 μL of the appropriate test material.
- b. 4 groups for the solvent control, i.e., 2 strains x 2 light exposure conditions; each animal dosed twice weekly with 50 μL of the cyclohexane/acetone (1:1) vehicle.
- c. 2 groups for cage control, i.e., 2 strains, dosed twice weekly with 50 μL solvent and not sham irradiated but always maintained in their individual cages.
- d. 4 groups for the positive control, i.e., 2 strains x 2 light exposure conditions dosed twice weekly with 50 μL of the solution of 0.01 percent B(a)P in cyclohexane:acetone (1:1).
- e. 4 groups for a combination treatment of asphalt and coal tar pitch fume condensate, i.e., 2 strains x 2 light exposure conditions; each animal dosed twice weekly with the high temperature condensate from Type III asphalt and Type I coal tar pitch, on alternate weeks.
- f. 2 groups for light exposure twice weekly with no skin painting treatment i.e., 2 strains.

These groups are indicated in Table 2 where the effective number of mice are presented.

Cage racks were regularly rotated within the animal quarters, and to minimize effects of fluorescent lights, lamps were enclosed in clear filter tubes (Crown Plastics Corp.). The light cycle was 12 hours light and 12 hours

dark. Each mouse was weighed prior to each first weekly application for 6 weeks, and biweekly thereafter.

Hair was clipped as needed from the interscapular region, using a separate No. 40 clipper head for each test material, and the test material was applied with disposable-tip automatic pipettes in a ventilated hood. After chemical treatment, each mouse was placed in a cell of a clean solar exposure cage unit and kept under a hood.

At 30 minutes following the application to the last mouse in an experimental group, the group was exposed to the light, or sham exposed. To insure uniformity of exposure, the mice were rotated weekly into different cells of the solar cage unit by a formal procedure. The treatments continued for 78 weeks (18 months).

Mice were observed daily for systemic toxicity, and gross appearance of tumors. Gross diagnosis of a skin carcinoma was based on a lesion that upon palpation was attached to underlying tissues. Mice found dead were necropsied. Those that were moribund were killed and necropsied and when groups were terminated at 78 weeks, those remaining were necropsied. Tissues were examined and preserved in 10 percent buffered formalin. Skin tumors were excised and fixed in buffered 10 percent formalin and prepared for microscopic examination. The distribution of tumors between dermal sarcomas, papillomas and epidermal carcinomas was noted particularly.

Additional details of the experimental methods are given elsewhere.²⁹

Statistical Methods

The survival curves were estimated using the product limit (Kaplan Meier) estimate³⁰ with day as the interval of time. The null hypothesis that the groups of interest had the same survival distributions was tested using the Breslow (generalized Wilcoxon) statistic. It should be noted that the Breslow test gives greater weight to the early events and is less sensitive to the events of interest that occur later in time when few animals remain in study. Animals sacrificed at termination of the study were considered censored, others as dying naturally. The mean, standard error of the mean, and 25, 50 and 75 quantiles were calculated. It should be noted that if the longest surviving animal is censored (as is usually the case in this study), the calculated mean value underestimates the true mean.

The time-to-tumor curves were calculated in the manner described previously for survival only the time of observation for the animal's first tumor (a tumor that was subsequently confirmed histopathologically) was used as the event rather than time of death.

To test for simple differences in the number of tumor bearing animals at risk, the Fisher-Irwin Exact Test was used. For tumor incidence, the heterogeneity of groups was tested, after allowing for differences in longevity, utilizing the "onset rate" analysis as suggested by Peto et al.³¹ This method assumes that the appearance of skin tumors are mortality-independent events. Only animals that had tumors that were observed

and histopathologically confirmed were considered tumor bearing animals. The time period used in calculations was one day and the number of animals at risk for a given group on a given day are those animals alive and without any previously observed tumors. The number of tumor bearing animals were compared, although there may have been multiple tumors per animal.

For all analyses, the significance level selected was $p=0.01$. It was recognized that a given group was included in multiple comparisons and the probability level of a given test may have been greater than that calculated; therefore, $p=0.01$ level was used to insure conservative conclusions for the study.

The following group comparisons were made:

- a. each group versus appropriate solvent group
- b. each group versus appropriate benzo(a)pyrene group
- c. light versus no light groups
- d. high temperature versus low temperature groups
- e. C₃H/HeJ species versus CD-1 species

The following comparisons were made for the groups:

- a. survival distribution
- b. time to tumor distribution
- c. number of tumor bearing animals
- d. onset rate analysis combining the above factors

Results

General Health and Survival

Body weights reflected no effect of the treatments. Weight gains occurred in the first 2 months; after that there were only minor oscillations. Killing moribund or cachectic mice was a factor in these oscillations.

Mean survival times in the treated C₃H/HeJ groups ranged from 44.3 to 68.7 weeks. Mean survival times ($\pm$ standard error of the mean) for the solvent controls were 65.6 ± 3.0 and 73.9 ± 2.2 weeks for non-solar and solar groups, respectively. In the CD-1 groups, mean survival for the treated groups ranged from 52.6 to 67.8 weeks with the mean $\pm$ S.E. for solvent controls for non-solar and solar groups, 63.9 ± 2.6 and 67.8 ± 2.1 weeks, respectively. The control groups had expected survival rates, although there were more deaths in the CD-1 mice due to endemic urinary tract infections.

Tumor Incidence

Tumors resulted from administration of condensed fumes from both types of asphalt and coal tar pitches. Their corrected incidence and mean latent periods are summarized in figures 3 and 4, respectively. Cumulative tumor incidence corrected for number of animals at risk at the time of appearance of the first tumor are displayed in the appendix figures 1-10. The average latent period ranged from 39.5 to 56.1 weeks among the C₃H/HeJ groups, and from 47.4 to 76.5 weeks among the CD-1 groups treated with the condensed fumes of the roofing materials.

The percentages of effective total mice bearing tumors are shown graphically in Figure 3 for benign (papillomas, kerato- acanthomas, fibromas and unclassified benign epitheliomas), malignant (squamous cell carcinoma and fibrosarcomas) and total tumors. Most of the benign tumors were papillomas and most malignant tumors were squamous cell carcinomas. The incidence of all malignant tumors was much lower in the CD-1 mice, about 5 percent versus about 60 in the C₃H/HeJ strain. Fibrosarcomas were seen more often in the C₃H/HeJ mice than in the CD-1 mice.

Comparisons to Solvent Controls

The solvent control groups did not develop tumors with the exception of one benign tumor found in the C₃H/HeJ strain exposed to light. Therefore all groups had significantly more tumors than the comparable solvent control group.

All C₃H/HeJ groups with two exceptions had significantly different survival curves from the appropriate solvent control groups. The two exceptions were Type I asphalt (232° C) with and without light exposure. In all cases the treated mice died earlier than the solvent control groups. Survival of CD-1 groups differed significantly from the appropriate solvent control groups with the following six exceptions: both asphalt types at high and low temperature of generation without light exposure; Type I pitch (316° C) with light exposure; and Type III pitch (316° C) without light exposure.

In the negative cage control groups only one CD-1 mouse developed a histologically confirmed benign tumor when treated with solvent only; the C₃H/HeJ strain remained tumor free. Exposure to the light source without solvent treatment caused development of one malignant tumor in the C₃H/HeJ strain but no tumors were observed in the CD-1 group. Regressed tumors not confirmed by histopathology, were observed in almost every group (Table 3).

Mouse Strain Comparisons

In comparing strains of mice, except for the solvent control groups, the survival time was in general shorter for the C₃H/HeJ strain. This difference was significant for: Type I asphalt (232° C) with light; Type III asphalt (316° C) without light; Type I pitch (232° C) with and without light; Type I pitch (316° C) without light; Type III pitch (232 and 316° C) without light; both combination exposure groups; and the C₃H/HeJ survived longer than CD-1 solvent control with light.

Comparing mouse strain differences in response to the various treatments, all C₃H/HeJ groups had a significantly higher incidence of tumor bearing animals (TBA). With two exceptions, i.e., Type I pitch (232°) and B(a)P groups without light exposure, the time to tumor curves were significantly different with the C₃H/HeJ groups developing tumors earlier.

It should be emphasized that a significantly large number of grossly observed tumors regressed in the CD-1 strain as compared to the C₃H/HeJ strain (Table 3). These data were not incorporated into the current analyses since histopathological confirmation was not available.

Effects of Temperature

For both asphalts the mean latency period was reduced an average of 9.5 ± 2.5 weeks (mean $\pm$ S.D.) when C₃H/HeJ mice were treated with the higher temperature material compared to the response from the lower temperature condensed fumes (Table 4). Similarly, mean survival was reduced 10.0 ± 3.0 weeks which corresponds to the reduced latency period. Groups not exposed to light showed no differences in percent TBA and those exposed to light were found to have ~9.5 percent increased, but statistically insignificant, tumor incidence by Fisher's Exact Test. The onset rate analysis determined that all groups exposed to the higher temperature materials were significantly more susceptible.

Those C₃H/HeJ mice exposed to coal tar pitch fumes exhibited very little change in latency or survival due to increased temperature of generation, except for the Type III groups exposed to light, where an inhibitory effect was noted on time to tumor development, but there was no effect on tumor incidence. Onset rate analysis revealed a significant difference in the light exposed Type III pitch groups which appears to be an effect based solely on latency differences. The groups not exposed to light got more tumors (~9 percent) with increased temperature of generation but these increases were not significant. Even though the B(a)P concentrations were normalized for the pitch groups, changes were observed in the measured responses.

The CD-1 groups exposed to asphalts without light generally exhibited a non-significant increase in percent TBA with increasing temperature, but no changes in survival or latency were observed. The CD-1 groups exposed to condensed pitch fumes generally responded less to the higher temperature materials but no significant differences were noted for any individual measured parameters, or using the onset rate analysis.

Effects of Light

The C₃H/HeJ asphalt groups exposed to light generally responded by developing less tumors (Table 5). This was particularly noted with the low temperature groups. Latency and survival increased with this decreased tumor response. Onset rate analysis showed significant differences for both low temperature materials. The pitch groups of this mouse strain showed little if any change in percent TBA by altering light exposure. The most prominent change in TBA ($p < 0.05$) was observed in the Type I pitch (232° C). Increased

latency and survival were the trends. Onset rate analysis showed all four materials to affect mice differently in the presence of light. These results are almost entirely attributable to latency differences. Light inhibited ($p < 0.05$) tumor formation in the B(a)P group with a corresponding increase in latency time and survival. Onset rate analysis showed a significant difference. The combination group responded in a fashion similar to the pitch group, with the overall effect being significantly different with light exposure.

The CD-1 mice exposed to asphalt fumes reacted erratically. The overall response in the higher temperature groups appeared to be decreased with light exposure, but these were not significant using the onset rate analysis method. There was an increased time to tumor noted in the higher temperature groups; however, these same groups showed decreased mean survival. The results of exposing the pitch fume groups to light were very dramatic; tumor development in all groups was inhibited by an average of 19.5 ± 5.8 percent. There was a corresponding significant increase in time to tumor development, an average of 11.7 ± 3.4 weeks. The survival time was not affected. Onset rate analysis showed significant differences in three of the four groups. As with the C₃/HeJ strain, tumor development caused by B(a)P in the CD-1 strain was severely inhibited by light exposure with a corresponding increase in latency, but there was no effect on survival. The combination group, as before, appeared to mimic the coal tar pitch fume effects. Both comparisons were statistically significant using an onset rate analysis.

Comparison to the B(a)P Response

Reviewing the responses in the C₃H/HeJ strain, fumes collected from Type I asphalt appeared to be more carcinogenic than the 0.01 percent B(a)P increasing the average percent TBA by approximately 11 ± 8 percent and decreasing the latency period by an average of 11.5 ± 8.5 weeks (Table 6). However, there was no consistent effect on survival; the largest decrease occurred with the Type I (316° C) receiving concomitant light exposure. The onset rate analysis showed significance for only the 316° C groups. The Type III asphalt fumes did not induce consistent change when compared with the corrected percent TBA from the B(a)P group; although, mean time to tumor was again significantly decreased an average of 12.8 ± 7.2 weeks and survival was generally decreased. As with the Type I asphalt, the Type III (316° C) group exposed to light showed the most change and only the 316° C groups were significantly different by the onset rate analysis.

Consistent trends were found in the C₃H/HeJ mice exposed to condensed pitch fumes as compared to B(a)P. The mice receiving the low temperature material without light showed no change in percent TBA but significant decreases in latency and survival. When the temperature of generation was increased, again with no light exposure, increases in percent TBA were observed with similar changes in latency and survival as noted with the low temperature material. Mice exposed to light and pitch fumes from both temperatures experienced significant increases in tumor development and decreases in latency when compared to the B(a)P group. Decreased mean survival times paralleled the time to tumor changes. All pitch groups were found to be significantly different by the onset rate method from the

corresponding B(a)P group. The groups receiving the combination treatment paralleled the effects noted above with the individual member component groups.

The CD-1 strain, in comparing their reaction to both types of condensed asphalt fumes and 0.01 percent B(a)P, had significantly decreased tumor incidences when exposed to the fumes and sham-irradiated. The average difference in reduced tumor response was 44.5 ± 9.9 percent TBA, with the largest reduction in the lower temperature groups. A corresponding significant increase in time to tumor was observed, i.e., approximately 14.5 ± 2.4 weeks with little reduction in survival as compared to the B(a)P group. All four groups were significantly different from the non-irradiated B(a)P group by the onset rate analysis method. The light exposed CD-1 mice as a subset of asphalt exposed groups showed a general slight but non-significant reduction in percent TBA, a significant decreased survival time and no change in mean latency time when compared to the B(a)P group. The overall effects analyzed by the onset rate method were not different from the B(a)P group.

The effects were reversed in the pitch exposed CD-1 groups. Slight reductions in percent TBA, significantly decreased survival and non-significant reductions in latency were observed in the groups not exposed to light as compared to the appropriate B(a)P group. These sham irradiated CD-1 pitch groups were not different from B(a)P by the onset rate method. Light exposed CD-1 mice, concurrently receiving condensed pitch fumes, had increased incidences of percent TBA, averaging 19.9 ± 6.0 . Here as before with the non-solar asphalt fume groups, the lower temperature materials induced more dramatic results. Survival was significantly reduced an average of 8.9 ± 2.1 weeks in these groups compared to the B(a)P group and latency time was generally decreased (11.2 ± 2.3 weeks) to a significant degree. Onset rate analysis showed significant differences for all light exposed pitch groups of CD-1 mice. The combination group reacted as anticipated and appeared to be more affected by the pitch component.

Combination Treatment

In comparing the combination group to their respective controls, the C₃H/HeJ strain showed no differences in latency, but the combination group showed a significantly increased response by the onset rate analysis method as compared to the asphalt group (Type III--316° C) without light. The responses in CD-1 mice were similar in that the non-solar exposed combination group had an significantly increased tumor response by the onset rate method than the asphalt group. They also died earlier and developed tumors at a significantly earlier time than the asphalt group. When the combination groups were compared to the responses of the pitch groups, onset rate analysis showed that the sham irradiated pitch group (Type I--316° C) had a significantly greater tumor response than the combination group. No other differences were noted.

To summarize the results, it is obvious from an examination of the data that: the asphalt and coal tar pitch fumes were highly carcinogenic; the condensed coal tar pitch fumes had more carcinogenic activity than the asphalts; the two types of each material had very similar activities; the nonpigmented CD-1 strain was less responsive to the carcinogenic activity of

the materials than the pigmented C₃H/HeJ strain; increased temperature significantly increased the tumorigenic response of C₃H/HeJ mice to the condensed asphalt fumes. (The only pitch group to show a similar increased response was the Type III with concomitant exposure to light); no significant changes in the tumorigenic response of the CD-1 mice to increased temperature of fume generation were noted; simulated sunlight significantly inhibited tumorigenic response in C₃H/HeJ mice to both types of asphalt fumes collected at the lower temperature and no overall significant affects were noted in the CD-1 strain; both strains reacted similarly when exposed to condensed coal tar pitch fumes, B(a)P or the combination of pitch and asphalt in the presence of simulated sunlight, where the general response was a significant inhibition of tumorigenesis; in comparison to the C₃H/HeJ strain treated with 0.01 percent B(a)P, all coal tar pitch groups, the combination groups and those exposed the high temperature asphalt fumes showed an increased tumorigenic response; and, in the CD-1 strain, only non-solar asphalt fume exposed and solar exposed pitch fume groups showed this same significantly increased tumorigenic response when compared to the positive control.

Discussion

Condensed volatiles from heated asphalt roofing materials were strikingly more tumorigenic and carcinogenic to C₃H/HeJ mice than was expected from previous studies^{1,2}; their activity being nearly equal to that of the condensed volatiles from heated coal tar pitch roofing materials diluted to contain approximately 0.01 percent B(a)P. The CD-1 strain was more refractory to the asphalt fumes and the results were more in line with expectations based on historical observations, showing high tumorigenic activity with the coal tar pitch material and lower activity with the asphalts. These observations raise questions about the unique qualities of the two strains of mice used, e.g., levels of inducible microsomal enzymes and metabolic profile differences in handling PAHs. Pelkonen et al.³², have found that additional genes besides the Ah locus may cause a particular mouse strain to be more sensitive or resistant to B(a)P initiated tumors than would be expected on the basis of metabolic induction capabilities. They found the C₃H inbred strain to be 5 to 15 times more responsive to subcutaneous fibrosarcoma initiation than other strains. They did not study the CD-1 strain.

It is interesting to note that Bingham et al.² reported very low or no activity with neat roofing asphalts in toluene (1:1 by weight) using the C₃H mouse strain with essentially an identical protocol to the one used in the current study. Their results with solvent diluted roofing pitch derived from coal tar produced similar tumor incidences but with considerably shorter latent periods than observed in this study. The decreased latency is presumed to be related to the higher dosage of pitch used in the former investigation. This further supports the contention that the thermal history of a material is one of the prime determinants of its carcinogenic activity.

A significantly increased tumorigenic response was observed in the C₃H/HeJ strain using higher temperatures to generate the asphalt fumes. Most of this increased response was attributed to a decrease in time to tumor development rather than an increase in tumor bearing animals. This effect

with asphalt fumes was not observed in the CD-1 non-pigmented strain. The pigmented strain, but not the CD-1 mice, also exhibited a significantly increased tumor response to higher temperature Type III coal tar pitch fumes in the presence of simulated sunlight, in spite of concentration adjustments to standardize the B(a)P content. It is inferred that the higher temperature coal tar pitch fumes may have higher specific activity in both strains when undiluted.

There appeared to be virtually no differences in tumorigenic activity in the same animal strain as a result of exposure to condensates of the two different types of asphalt or coal tar pitch materials generated at the same temperature. Therefore, it can be concluded that there is not a large change in relative carcinogenic risk with the type of asphalt or coal tar pitch material used. The objective to show differences in carcinogenic activity of the different ASTM classifications of materials and hence relative safety of the material could not be demonstrated in this study. However, Lowe et al.³³, noting that many investigators found differences in efficacy using tar to treat dermatoses, demonstrated that pharmacologic action varied significantly among sublots of the same crude coal tar collection. There is no reason to expect that asphalt fume activity would not also vary with different sources of crude oil.

Simulated sunlight, as used in this investigation, clearly caused an inhibition of the tumorigenic response. This is somewhat surprising and quite the opposite effect of that expected. There is some evidence suggesting long wavelength ultraviolet (280-350 nm) may act in a cooperative way with chemicals to induce skin tumors in mice under certain laboratory conditions.^{22,23} Further, Bingham and Nord²⁷ reported that exposure of mice to light at wavelengths above 350 nm coupled with exposure to normal alkanes results in tumor induction, whereas exposure to light alone or alkane alone yields few, if any, tumors. However, inhibition of the carcinogenic effects of UV light and dimethylbenzanthracene (DMBA) on the skin of Swiss mice was observed by Stenback and Shubik.³⁴ This was demonstrated when both UV light and DMBA were administered twice weekly for 4 weeks, with each UV treatment following DMBA treatment by 1 hour. Grossly observed tumors produced by DMBA alone numbered 36, whereas only 21 occurred with the combined treatments. In another schedule, a single UV exposure 1 hour before the first DMBA treatment, followed by 21 weeks of twice-weekly treatment of DMBA alone, resulted in an increased number of tumors as compared to the DMBA controls (52 vs. 30). Although a full comparison of these studies is not possible because of the different experimental conditions, i.e., strains of mice, materials and light source, it is clear that careful consideration must be given to the designs of future experiments involving interaction of light and chemicals. Attention must be given to similarities between the design and actual occupational exposure experience, such as with roofing workers.

Is this inhibition of carcinogenic response due to photooxidation/photodestruction of the carcinogens in these materials or to the modification of the skin, lessening its responsiveness, or to an actual cytotoxic effect on precancerous cells? Zepp and Schlotzhauer³⁵ as well as Mill et al.³⁶, determined half-lives of various photo-irradiated PAH in water to range from less than one hour to several hours. B(a)P and other

carcinogenic PAH were among those most easily photooxidized in less than one hour. Katz et al.³⁷ found the half-lives of many of these same chemicals in simulated sunlight atmospheric conditions to be about the same order of magnitude. The presence of 0.2 ppm ozone markedly decreased the observed half-lives. The relative contribution of this observation to the general inhibition phenomenon observed in the current study remains to be determined. Fisher and Kripke³⁸ using C₃H/HeN(MTV-) mice concluded that suppressor T lymphocytes induced in mice by UV radiation play a decisive role in skin carcinogenesis by reducing the latent period in photocarcinogenesis. This mechanism does not appear to apply to the current study where the general response was an inhibition of the carcinogenic process.

Preliminary chemical characterization according to EPA-RTP Level 1 procedures^{39,40} of a direct extract of coal tar pitch used in these studies and of a pitch fume sample generated from a full-scale roofing kettle (which was not the identical fume material used in these carcinogenicity studies) suggests that heterocyclic sulfur and nitrogen compounds and organic nitriles are present in the pitch samples at levels of 1-10 percent of the PAH content (Table 7). El-Bayoumy and coworkers⁴¹ tested tumor initiating activity of four nitro PNAs and their parent hydrocarbons and found that only nitroperylene induced higher activity than the parent compound; yet, this activity was still much less than that induced by B(a)P. Peiroy and coworkers^{42,43} used microbial mutagenicity methods to assess the activity of sulfur heterocyclics. None of the compounds tested exhibited activity greater than B(a)P or the neutral fractions from which they were isolated. Thus, species of these types may contribute slightly to the carcinogenic effect of the roofing pitch fume materials; however, it remains clear based on the data in Table 8 and from a previous report²⁰ that B(a)P is a reasonable indicator substance of coal tar pitch fume exposure. From Table 8 it can be calculated that B(a)P accounts for approximately 75 percent of the total carcinogenic activity of the coal tar pitch fumes.

Since the PAH content of the asphalt volatiles is very low compared to that of the coal tar pitch preparations, what other chemical components do these asphalt fumes contain which contribute to the observed carcinogenicity? Are these promoters, cocarcinogens, or even other carcinogens not of the PAH chemical class? This point is further emphasized by the analysis presented in Table 8. The amount of total test material applied by the time of 50 percent tumor incidence (C3H mice) is very much larger for the asphalt compared to the coal tar pitch fume. Approximately 10 fold more asphalt fume is required to obtain the same biological effect. The amounts of total PAH analyzed or B(a)P found in asphalt fumes are 30 to 40 fold less than those amounts found to be effective in eliciting the same response with condensed coal tar pitch fumes. This suggests that the asphalt preparations contain other types of chemical components which may augment the activity of the very low concentrations of measured PAH found in this study. It is likely that alkylated PAH derivatives and/or aliphatic hydrocarbons may account for some of this activity. Although complete chemical characterization data of the asphalt and pitch fume samples are not available at this time, the PAH analyses have been supplemented by nuclear magnetic resonance (NMR) analysis³⁹. The latter results imply that the asphalt fume material is <1 percent aromatic, >99 percent aliphatic, with straight-chain,

unbranched materials predominating. In contrast, NMR analysis of the pitch fume indicated >90 percent aromatic content. These analyses are reinforced by earlier work. Puzinauskas and Corbet⁴⁴ characterized the carbon types found in roofing materials. They found roofing pitch was composed of 79, 18 and 3 percent while roofing asphalt was characterized as 37, 23 and 40 percent aromatic, naphthene (cycloparaffins and cyclohexanes) and paraffinic carbon, respectively. The high aliphatic content of the asphalt fume sample is interesting in light of earlier observations^{45,46,47} that some alkyl hydrocarbons exhibit cocarcinogenic activity. Further characterization, fractionation and testing of these materials are now underway which will emphasize the identification of indicator compound(s) for asphalt fume occupational exposure.

In order to more fully judge the comparative risk of exposure to roofing material fumes, Figure 5 was compiled to illustrate relative emission rates, dosage of fumes to elicit an effective 50 percent tumor incidence (ED₅₀) and the number of minutes of generation needed to produce sufficient fumes for an ED₅₀. This was composed for asphalts and coal tar pitches at the suggested kettle operation temperature and at the temperature found during an overheat condition.

Theoretically, an asphalt kettle being maintained at the recommended temperature would emit approximately 37 and 11.5 times less fume than an overheated coal tar pitch or asphalt kettle, respectively. A similar comparison using a high temperature asphalt kettle could theoretically emit as much fume as a properly operated coal tar pitch kettle and approximately one third that of an overheated coal tar pitch kettle. When the time of generation needed to collect sufficient material to produce 50 percent tumor incidence in C3H/HeJ mice is calculated, the ratio of low temperature asphalt fumes to high temperature coal tar pitch fumes represents a greater than 500 fold difference; however, this difference is much less at the lower suggested temperatures of operation for the pitch. This analysis points out the need to maintain kettle temperatures as low as possible in order to reduce the carcinogenic risk.

Penalva and coworkers⁴⁸ studied mutagenic activity of aerosols and vapors emitted by road coating tar at various temperatures and found increasing activity as the temperature of the tar was increased from 250° to 550° C. Similar effects were noted by Mahlum⁴⁹ in his assessment of distillation cuts from coal liquids. This same trend was determined by Tye et al.⁵⁰ in assessing the carcinogenic potency of a catalytically cracked petroleum. He found that tumor initiating activity increased as the boiling point of the fraction increased. These results agree well with the observations from the current study, that higher temperature materials are more hazardous.

Table 9 lists theoretical air concentrations of total condensed fume, PAH and B(a)P from asphalt and coal tar pitch fumes when generated in the laboratory apparatus at 232 and 316° C. Also listed are field concentrations of PAH and B(a)P measured by Malaizandi and coworkers⁵¹ at roofing sites using either asphalt or coal tar pitch materials. The absolute numbers are orders of magnitude higher for the laboratory data as compared to

field data, but this is expected since field concentrations should reflect significant dilution by ambient air. However, there is reasonable agreement among laboratory and field data when the ratios of PAH/B(a)P concentrations are calculated. The ratios for laboratory generation of asphalt fumes is 157 and 122 for low and high temperatures, respectively, and the field collected ratio is 330. For coal tar pitch fumes the laboratory ratio is 88 and 153 for low and high temperature generations, respectively, where the field collected ratio is 159. We conclude from these observations that the relative risk to fumes generated in the field should be similar to those demonstrated in the laboratory setting.

Conclusions

The data from these experiments vindicate the prior concern of NIOSH that there is a greater occupational hazard from exposure to asphalt fumes than was documented in the literature. This investigation clearly demonstrates that exposure to asphalt fumes significantly increases carcinogenic risk, and as expected, higher use temperatures further increase this risk.

The carcinogenic activity of roofing coal tar pitch fume materials may be understandable, to a first order approximation, in terms of their B(a)P content. This ubiquitous carcinogen is therefore a satisfactory indicator substance in estimating relative coal tar pitch fume exposures in roofing operations.

The carcinogenic activity of the asphalt fume materials, on the other hand, cannot even approximately be explained on the basis of their B(a)P content. It can be hypothesized, although it has not been demonstrated, that cocarcinogenic effects of aliphatic hydrocarbons, which may be major components of the asphalt fumes generated in this study, are probably responsible for this enhanced activity. It is evident that further research is necessary before the active components of asphalt fumes are identified. Evidence suggests that emphasis should be placed on the promoting/cocarcinogenic activity of asphalt fumes and that an approach different from that used in studying coal tar pitch must be devised. A protocol using PAH-free asphalt fume condensate administered simultaneously or following subcarcinogenic doses of B(a)P, similar to that used by Bingham and Falk⁴⁶, could provide useful information in the future characterization of this material.

In the interim reasonable measures should be taken to limit exposure as much as feasible to both coal tar pitch and asphalt fumes using available engineering and work practice controls. One major work practice that can be instituted immediately is the maintenance of proper heating conditions in the kettle thereby reducing risk possibly to a substantial degree, i.e., approximately a 7 to 14 fold theoretical decrease for coal tar pitch and asphalt fumes, respectively.

Even though it was not demonstrated in this study, it is quite possible that exposure to both asphalt and coal tar pitch fumes may be more hazardous than exposure to either alone. For if our suspicions prove to be correct, that the activity of asphalt fumes is primarily attributable to its

cocarcinogenic activity, then reduction of carcinogen levels to as low as one thousandth of the original concentration may still lead to carcinogenic expression above the threshold response⁴⁶. This is partially supported in the simulated sunlight experiments where photooxidation probably destroys many carcinogens, but not the cocarcinogenic moieties. One must be very careful in assuming large reductions in risk due to relatively lower exposures to carcinogens and cocarcinogens. As Bingham and Falk⁴⁶ demonstrated, cocarcinogens of the aliphatic hydrocarbon type are most noticeable in their effect when the concentration of the carcinogen is low. They also demonstrated that low levels of cocarcinogens (less than 20 to 30 percent) appear to have the most marked effect on tumor outcome at lower levels of carcinogen exposure. This may be the condition most frequently encountered in the working population and therefore its importance cannot be overemphasized in attempts to determine the hazards to man.

It is interesting that sunlight may offer some protection against the carcinogenic activity of these materials; however, this will be extremely difficult to demonstrate in the working population. The future needs of extensive epidemiological studies, increased medical surveillance and detailed industrial hygiene surveillance in this hazardous work environment are quite evident.

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TABLE 1
Mean Concentration ($\mu\text{g/mL}$) of PAHs and Final Concentration of Condensed Fumes in Skin-Painting Solutions

PAH	Analytical GC/MS	Asphalt						Pitch					
		Type I		Type III		Type I		Type I		Type III		Type I	
		232°C	316°C	232°C	316°C	232°C	316°C	232°C	316°C	232°C	316°C	232°C	316°C
Ion													
naphthalene	128	22	4	17	49	>1800	1770					288	620
Fluorene	166	36	22	39	28	--	740			--	--	--	--
Carbazole	167	20	1	6	--	1980	1450			540	1400		1400
Anthracene/Phenanthrene	178	180	53	300	69	> 950	2960			>2580	>5200		>5200
Fluoranthene	202	86	10	97	7	>2940	2350			> 960	>2800		>2800
Pyrene	202	70	9	63	8	>2070	1790			> 720	>2300		>2300
Benz(a)anthracene	228	11	10	8	6	570	330			330	800		800
Chrysene/Triphenylene	228	25	19	13	14	460	300			290	710		710
Benzo(a)fluoranthene	252	2	4	5	--	230	230			250	250		250
Benzo(e)pyrene	252	6	8	4	1	42	51			45	46		46
Benzo(a)pyrene	252	2	2	3	--	96	85			102	90		90
Indeno(1,2,3-c,d)pyrene	276	3	3	2	--	33	2			11	7		7
Benzo(g,h,i)perylene	276	1	2	1	--	28	2			7	1		1
Dibenzanthracenes	278	2	--	2	--	12	--			4	--		--
Coronene	300	--	--	--	--	--	--			--	--		--
Dibenzopyrenes	302	--	--	--	--	--	--			--	--		--
Final concentration of condensed fumes (mg/mL)		500	500	500	500	78	55			84	30		

Table 2

Effective number of mice per group and week of first Papilloma*

Temperature of Generation	232°				316°			
	Light		No Light		Light		No Light	
Mouse Strain	P	N	P	N	P	N	P	N
Asphalt I	49(33)*	10(56)	49(30)	46(24)	47(18)	10(50)	46(23)	45(24)
Asphalt III	44(34)	48(17)	47(21)	48(31)	49(16)	46(41)	46(24)	45(41)
Coal Tar Pitch I	46(37)	45(31)	47(33)	46(22)	45(37)	45(35)	47(26)	45(31)
Coal Tar Pitch III	48(17)	45(40)	43(34)	49(14)	42(39)	48(23)	46(28)	48(28)
Combination (Type III Asphalt and Type I Pitch)	--	--	--	--	44(21)	41(21)	48(25)	45(30)
<u>No Temperature Variations</u>								
3(a)P	45(40)	49(23)	42(38)	49(28)				
Solvent Controls	33(40)	-(-)	-(-)	-(-)				
Solvent Cage Controls	-(-)	-(-)	-(-)	48(41)				
Untreated Controls	21(30)	-(-)	--	--				

P = pigmented C3H/HeJ

N = nonpigmented C3H/HeJ

(-) = no tumors

-- = not tested

* = week of first grossly observed papilloma

TABLE 3
Percent of Original Mice with Regressed Tumors

Material	Temperature	Light Exposure	C3H/HeJ	CD1
Asphalt I	232	-	2	14
		+	6	28
	316	-	4	16
		+	0	20
Asphalt III	232	-	2	18
		+	8	14
	316	-	4	14
		+	0	12
Pitch I	232	-	10	26
		+	0	26
	316	-	2	28
		+	0	26
Pitch III	232	-	2	28
		+	4	20
	316	-	0	26
		+	4	34
Solvent		-	8	2
		+	12	4
Cage Control		-	2	2
B(a)P		-	6	14
		+	10	22
Combination		-	0	22
		+	0	26
Untreated		+	2	0

TABLE 4
The Effects of Increased Fume Generation Temperature on Net Change in Response

Type (± flight)	C3H/HeJ				C57-1			
	Corrected Percent TBA	Mean Latency (Wks)	Mean Survival Time (Wks)	Onset Rate Analysis	Corrected Percent TBA	Mean Latency (Wks)	Mean Survival Time (Wks)	Onset Rate Analysis
Asphalt	I- - 0.4	-10.9s	-13.1s	s	18.3*	-5.5	-3.5	ns
	I+ 12.0	-11.9s	-11.8s	s	10.0	0.0	-0.8	ns
	III- - 4.6	- 6.1	- 6.5	s	14.0	-1.7	4.7	ns
	III+ 7.1	- 9.1s	- 8.7s	s	- 3.7	3.5	-2.1	ns
Pitch	I- 8.5	- 0.4	1.3	ns	- 3.0	2.6	-1.3	ns
	I+ - 2.2	0.9	0.0	ns	- 8.7	4.8	1.7	ns
	III- 9.4	0.4	4.4	ns	1.3	0.5	3.0	ns
	III+ - 1.2	8.7s	3.5s	s	-11.4	0.9	3.9	ns

* = significant at $p < 0.05$ by Fisher-Irwin Exact Test

s = significant at $p < 0.01$

ns = not significant

TABLE 5
The Effects of Light on Net Change in Response

Type of	C3H/HeJ					CD-1				
	Corrected Percent TBA	Mean Latency (wks)	Mean Survival Time (Wks)	Onset Rate Analysis		Corrected Percent TBA	Mean Latency (Wks)	Mean Survival Time (Wks)	Onset Rate Analysis	
Asphalt	I 232	-12.3	5.7	2.2	s	7.0	0.9	-9.2s	ns	
	I 316	0.1	4.8s	3.5	ns	- 1.1	6.5s	-6.5	ns	
	III 232	-16.7*	6.5s	2.2	s	- 5.2	0.9	0.8	ns	
	III 316	- 5.0	3.5	0.0	ns	-23.9s	6.1	-6.0	ns	
Pitch	I 232	12.7*	2.2	5.2s	s	-17.4	13.4s	2.2	s	
	I 316	- 2.0	3.5s	3.9s	s	-23.1*	15.5s	5.2	s	
	III 232	3.3	0.8	6.5s	s	-12.3	8.7s	-2.5	ns	
	III 316	- 4.9	9.1s	5.6s	s	-25.0s	9.1s	-1.7	s	
3(A)P		-17.0*	24.8s	9.1	s	-44.9s	15.5s	2.2	s	
Combination		1.5	3.4s	1.3s	s	-26.7s	14.3s	0.4	s	

TABLE 6
Group Comparisons to B(a)P Responses

Type (± light)	OC	C3H/HeJ				CD-1			
		Corrected Percent TBA	Mean Latency (wks)	Mean Survival Time (wks)	Onset Rate Analysis	Corrected Percent TBA	Mean Latency (wks)	Mean Survival Time (wks)	Onset Rate Analysis
Asphalt	I- 232	5.8	- 1.3	8.7	ns	-56.4s	17.4s	- 3.0	s
	316	5.4	-12.2s	4.4	s	-38.3s	11.8s	- 6.5	s
	I+ 232	10.5	-10.4s	- 1.8	ns	- 4.5	1.8	-14.4s	ns
	316	22.5s	-22.2s	-10.0	s	5.5	1.8	-15.2s	ns
III-	232	- 0.9	- 4.8	1.7	ns	-48.6s	15.2s	- 4.3	s
	316	- 5.5	-10.9s	- 4.8	s	-34.6s	13.5s	0.4	s
	III+ 232	- 0.6	-13.1s	- 5.2	ns	- 9.9	- 0.4	- 5.7s	ns
	316	6.5	-22.2s	-13.9s	s	-13.6	3.1	- 7.8s	ns
Pitch	I- 232	- 3.0	- 9.6s	- 9.1s	s	- 2.0	- 9.5	- 8.7s	ns
	316	5.5	-10.0s	- 7.8s	s	- 5.0	- 6.9	-10.0s	ns
	I+ 232	26.7s	-22.2s	-13.0s	s	25.5s	-12.6s	- 8.7s	s
	316	24.5s	-21.3s	-13.0s	s	16.8	- 7.8	- 7.0s	s
III-	232	0.3	-11.7s	-13.5s	s	- 8.2	- 4.8	- 6.9s	ns
	316	9.7	-11.3s	- 9.1s	s	- 6.9	- 4.3	- 3.9	ns
	III+ 232	20.6s	-25.7s	-16.1s	s	24.4s	-12.6s	-11.9s	s
	316	21.8s	-17.0s	-12.6s	s	13.0	-11.7s	- 7.8s	s
Combination -		3.6	-11.7s	- 7.4s	s	-16.1	0.5	- 8.2	ns
	+	22.1s	-23.1s	-15.2s	s	2.3	- 1.7	-10.0s	ns

s = significant at p<0.01

ns = not significant

positive number indicates increased response compared to B(a)P

TABLE 7
Results of Level 1 Chemical Characterization of
a Roofing Pitch and a Pitch Fume Sample*

Approximate Concentration (mg/g)

Organic Compound Category	Pitch Extract	Pitch Fume
Aliphatic Hydrocarbons	---	8
Fused Aromatic Hydrocarbons	510	840
Heterocyclic Sulfur Compounds	31	83
Heterocyclic Nitrogen Compounds	15	180
Nitriles	16	10
Amines	8	---

Source: 39

TABLE 8
Total Dose of Test Material Applied up to Time of 50 Percent Tumor Incidence
(C3H Mice--Non Solar)

Test Material	Time to 50 percent T.I.* (weeks)	Amount of Material per application				Total Dose Applied for ED50**			
		Total	PAH	B(a)P	Total	PAH	B(a)P	Total	
		Solids mg	μg	μg					Solids mg
Asphalts									
Type I - 2320	50.4	25	23.2	0.110		2520	2.34	11.1	
Type I - 3160	39.5	25	7.4	0.095		1975	0.58	7.5	
Type III - 2320	46.9	25	28.0	0.145		2345	2.63	13.6	
Type III - 3160	40.8	25	9.1	<0.025		2040	0.74	<2.0	
Coal tar Pitches									
Type I - 2320	42.1	3.90	>560	4.80		328	>47.2	404	
Type I - 3160	41.7	2.75	603	4.25		229	50.3	354	
Type III - 2320	40.0	4.20	>306	5.10		336	>24.5	408	
Type III - 3160	40.4	1.50	>711	4.50		121	>57.4	364	
B(a)P	51.7	-	5.0	5.00		0.5	0.5	517	

* = Time in weeks to produce 50 percent tumor incidence (histologically confirmed) where number of applications = weeks X 2.

** = No. of applications x amount per application = Effective Dose for 50 percent Tumor Incidence

TABLE 9
Asphalt and Coal Tar Pitch
Theoretical Laboratory^a vs. Field Survey^b Air Concentrations

	Total Condensed Fumes (g/m ³)	PAH (μg/m ³)	B(a)P (μg/m ³)	Ratio of PAH/B(a)P
Asphalt				
2320	4.3	3300	21	157
3160	51.3	15000	122	122
IH ^b	--	33.1(± 38.7)	0.119(±0.152)	330
Coal Tar Pitch				
2320	45.8	5.0 X 10 ⁶	55.6 X 10 ³	98
3160	161.7	56.1 X 10 ⁶	366.7 X 10 ³	153
IH ^b	--	476(± 260)	3.1(±4.3)	159

a = from this study based on emission rates at 10 Lpm

b = Malaizandi et al.⁵¹ field study mean (± standard deviation)

FIGURE 1

SCHEMATIC OF FUME COLLECTION SYSTEM

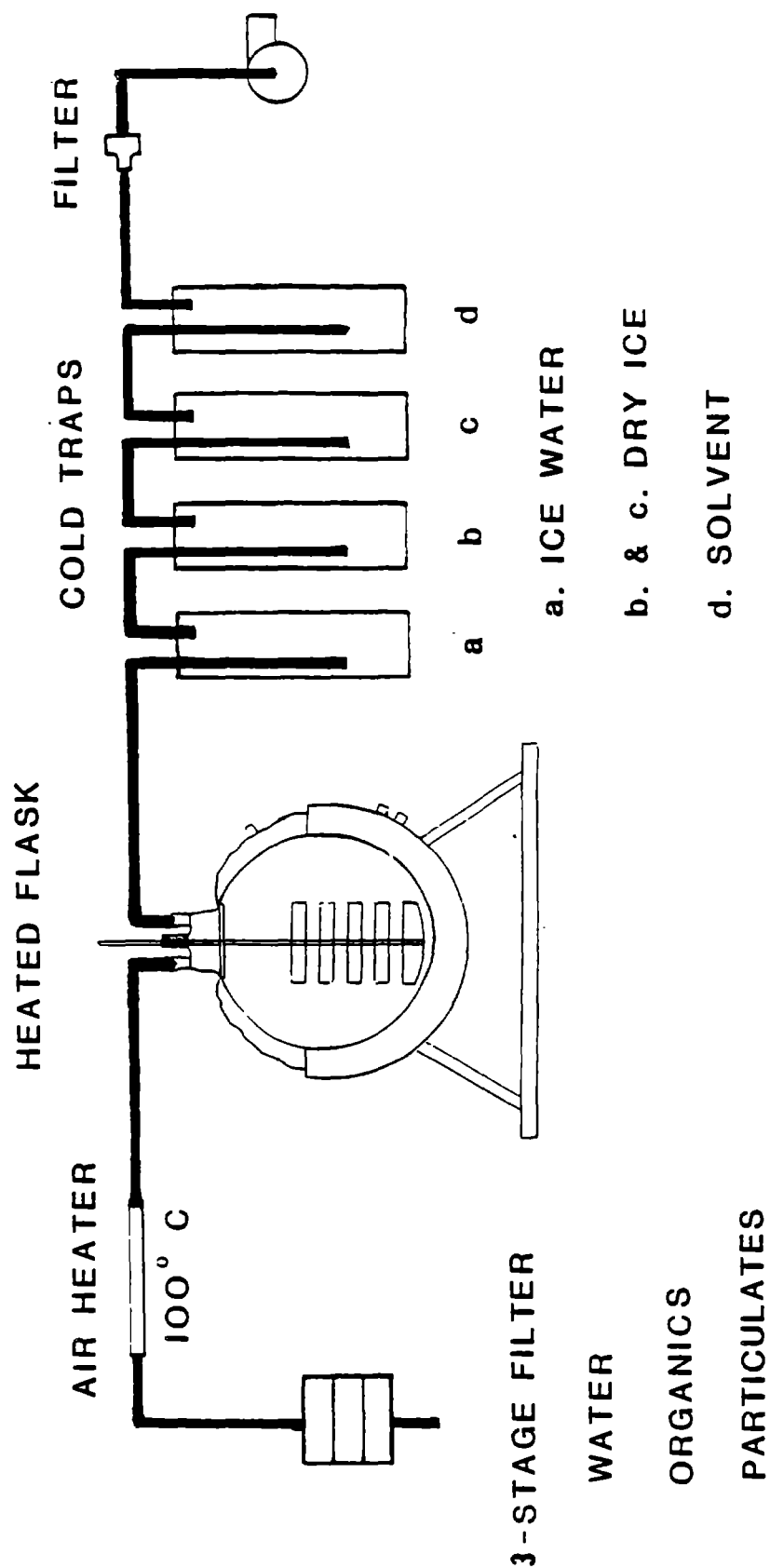
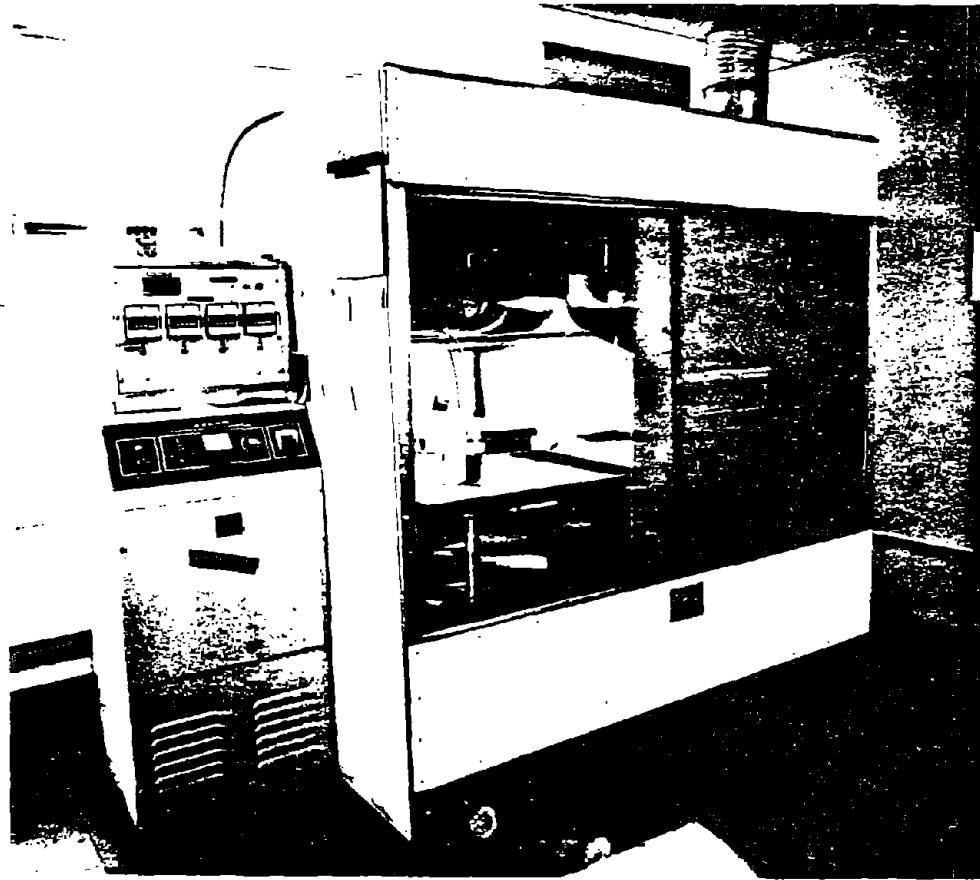


FIGURE 2
SOLAR SIMULATOR



The power source is on the lower left and immediately above it are the exposure counters. The large enclosure houses the light source in the upper center and four turntables, two with light exposure (upper level) and two without (lower level). Only two turntables are shown in this photograph. The exhaust port can be seen in the far upper right portion of the chamber.

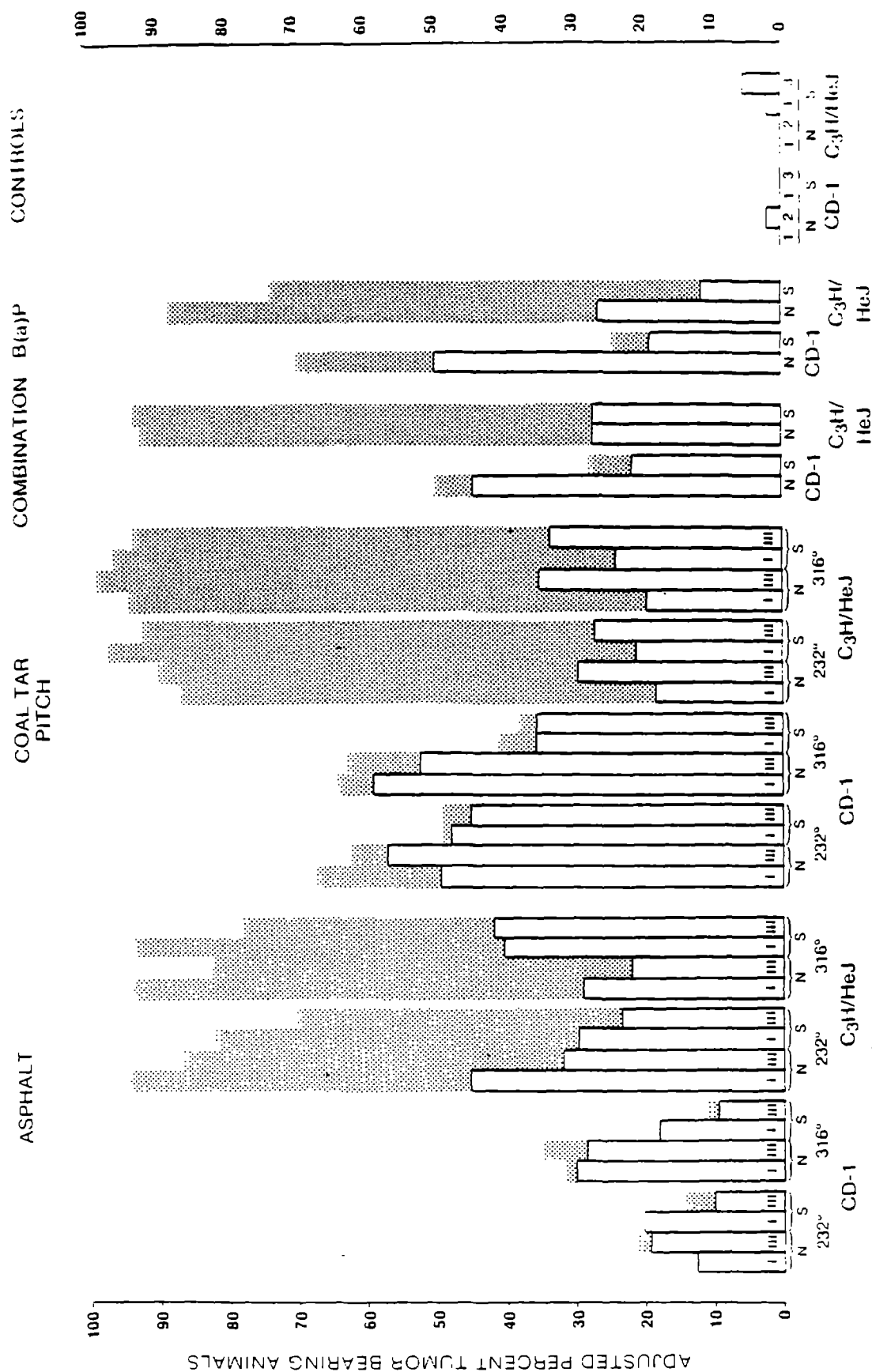


FIGURE 3--SKIN TUMOR INCIDENCE. Corrected for number at risk at the appearance of the first tumor. Open and hatched bars represent benign and malignant tumor proportions, respectively. Controls: 1 = solvent group $\pm$ light, 2 = cage control, and 3 = light exposed only. Types of materials are designated by I or III as noted in the text. N denotes no simulated sunlight exposure; S indicates a solar exposed group.

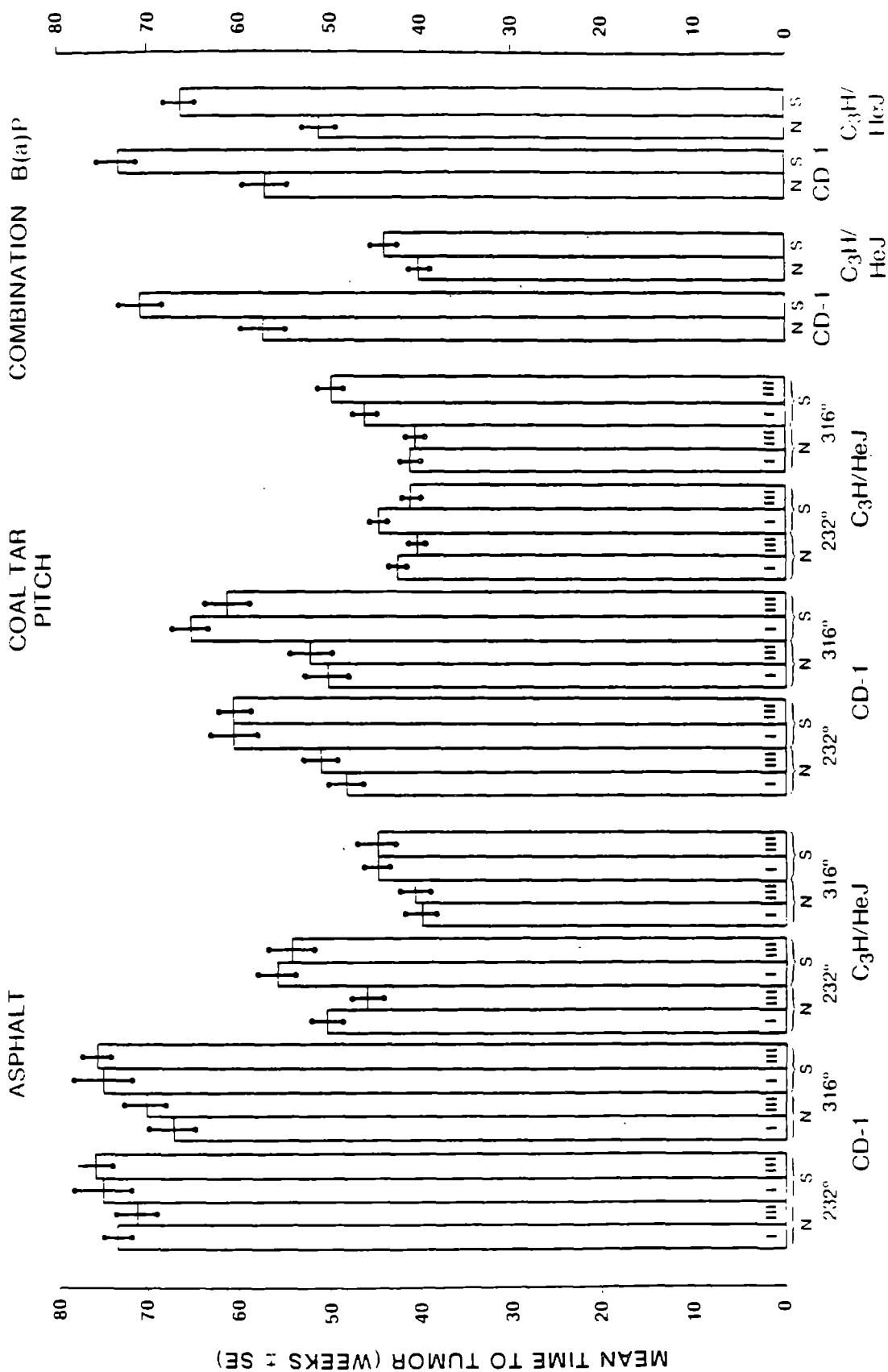
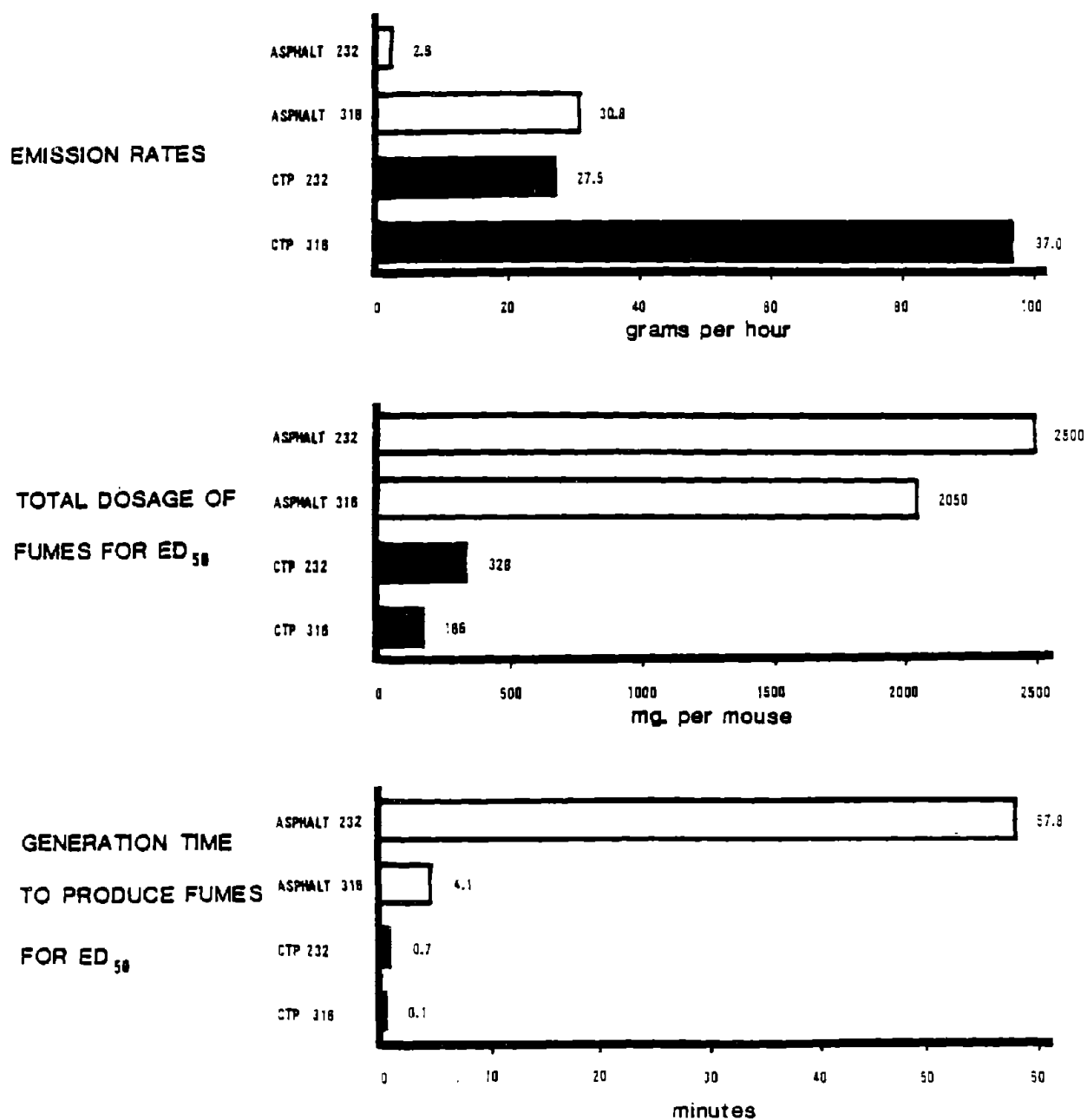


FIGURE 4--MEAN LATENCY TIME. Expressed in weeks $\pm$ standard error based on histopathologically confirmed tumors. Types of materials are designated as 1 or III. N denotes no simulated sunlight exposure; S indicates a solar exposed group.

FIGURE 5

Comparison of Generation Times Necessary to Produce Sufficient
Roofing Material Fumes for an ED₅₀ Tumorigenic Response in
C3H/HeJ Mice



TUMOR INCIDENCE

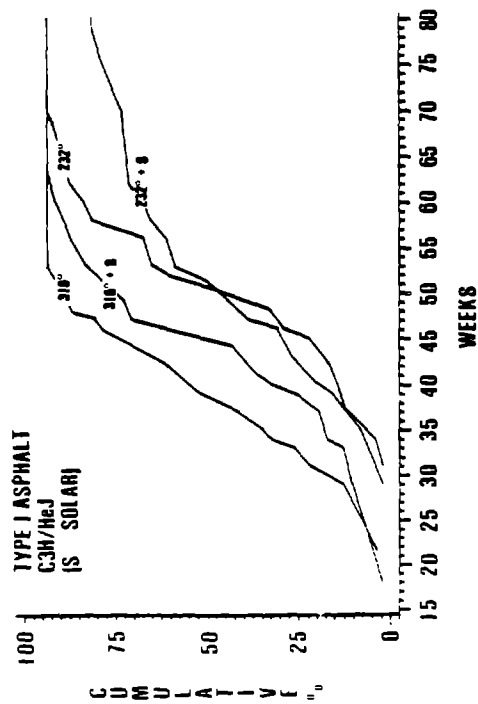


FIGURE A-1

TUMOR INCIDENCE

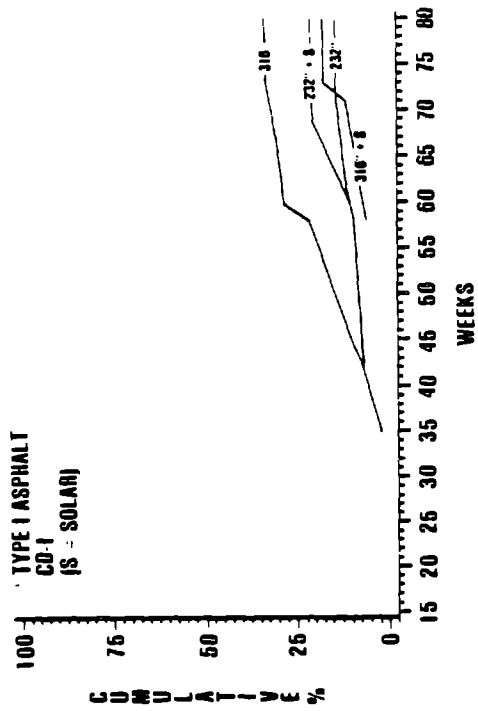


FIGURE A-2

TUMOR INCIDENCE

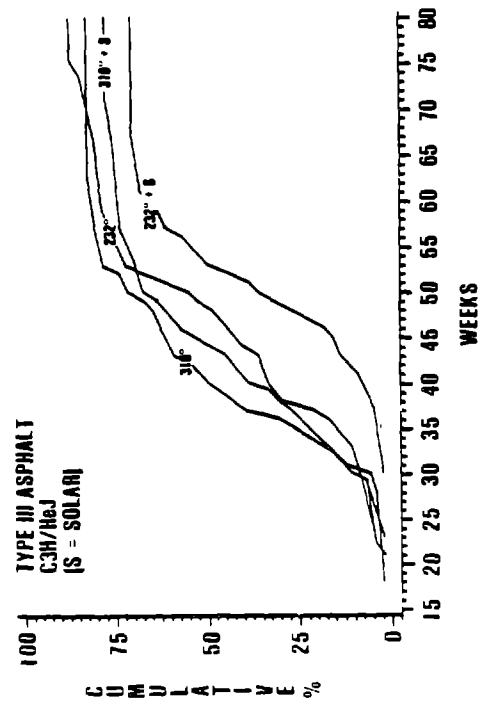


FIGURE A-3

TUMOR INCIDENCE

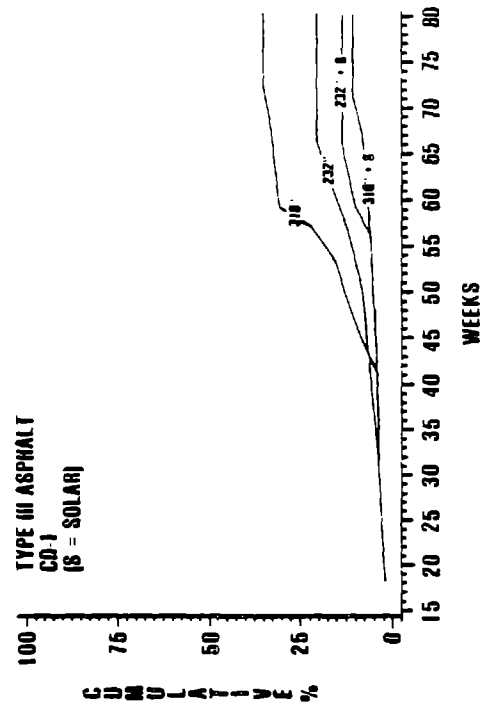


FIGURE A-4

TUMOR INCIDENCE

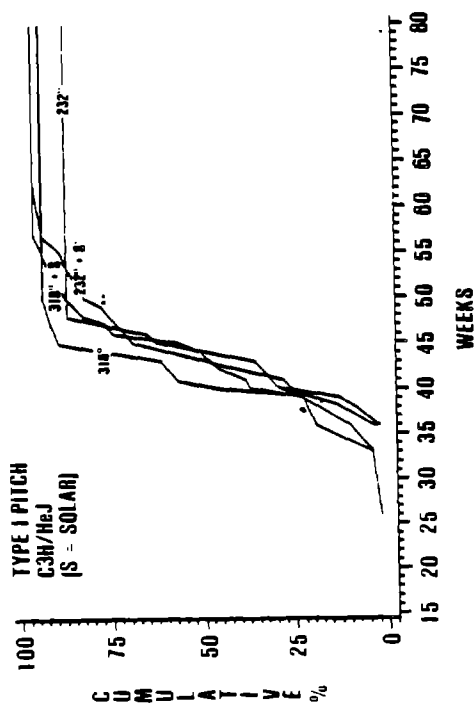


FIGURE A-5

TUMOR INCIDENCE

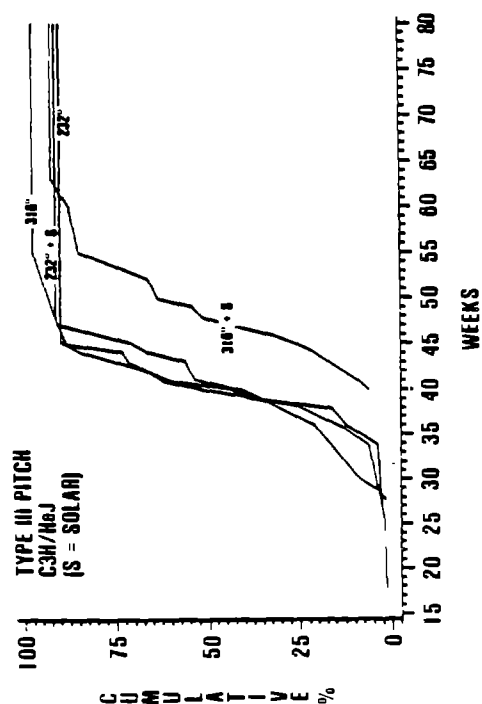


FIGURE A-7

TUMOR INCIDENCE

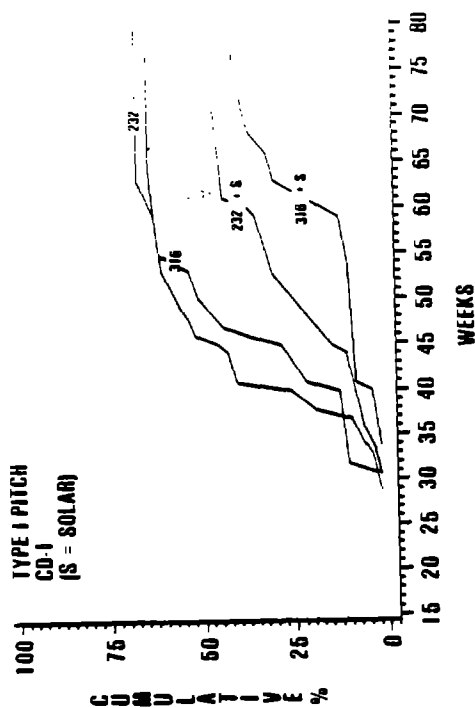


FIGURE A-6

TUMOR INCIDENCE

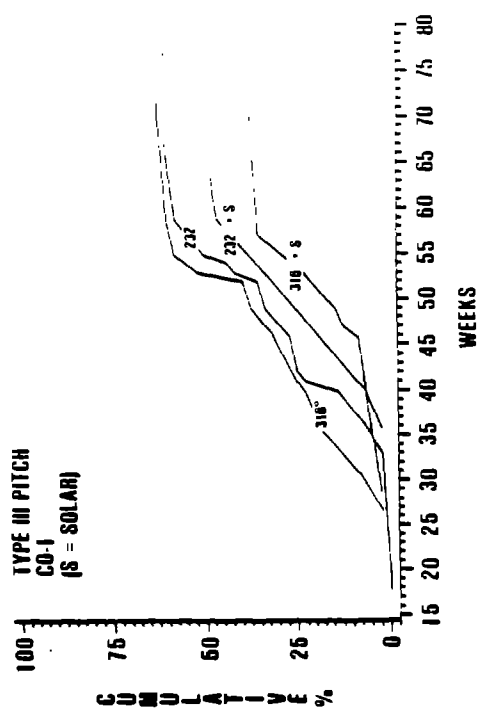


FIGURE A-8

TUMOR INCIDENCE

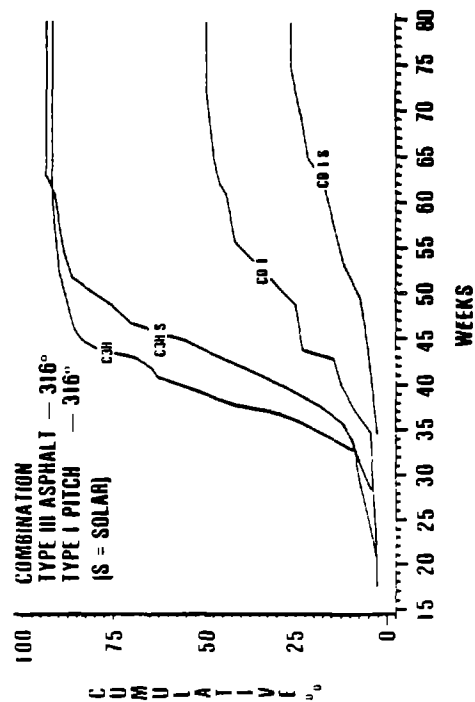


FIGURE A-9

TUMOR INCIDENCE

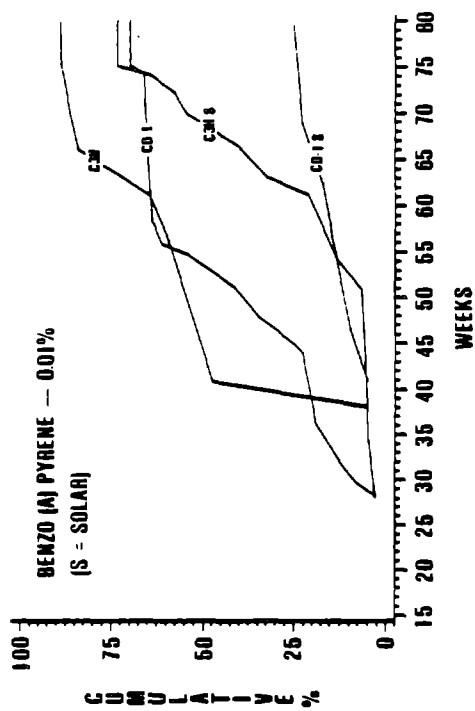


FIGURE A-10

Appendix Figures A-1 through A-10 represent the Cumulative Tumor Incidence expressed as percent of the number of mice at risk at the appearance of the first tumor. All tumors used in these analyses were histopathologically confirmed. -S represents the solar light exposed groups.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION VI - EXPERIMENTAL STUDIES
Chairman: Dr. Edward Bresnick
The Eppley Institute for Research in
Cancer and Allied Diseases

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. BRESNICK: Are there any questions of the four speakers on any of the five presentations?

DR. BEAUMONT: Jay Beaumont of NIOSH, for Dr. Wiser: Regarding these tanning salons that are so popular now, how does your research reflect on their claims of safety?

DR. WISER: In what regard?

DR. BEAUMONT: They say that the rays from their fluorescent bulbs, or whatever they are, are safer somehow. I don't know the details.

DR. WISER: The research we have supported has not looked at that; however, Fred Urbach at Temple University has looked at that. The lamps they used were exactly the lamps we used in the lamps to produce UVB, and we know UVB is harmful. If they try to get rays in low doses, they tan. Some of them have tried to use some other filters, but this investigation is still going on. It is not being done with our program. But yet, those exposures are believed to be extremely harmful.

DR. BRESNICK: Other questions?

DR. VAINIO: Dr. Vainio, IARC, Lyon. This is a question to Dr. Niemeier: Your conclusion was that silica is cocarcinogenic, as well as some of its substitutes. Now, I just wonder if you have any comments on the possibility that silica is a complete carcinogen? There is a recent study from Los Alamos suggesting that you can have lung tumors only with silica alone when the concentrations are very high. Would you like to comment?

DR. NIEMEIER: First of all, I am not familiar with the Los Alamos study, but I know there is other evidence in the literature. Margaret Wagner has published in the Journal of the National Cancer Institute in 1972 and 1976 and described reticuloendothelial tumors found after intrapleural injection of silica (< 5 micrometers) into rats.

However, in the study that I presented today, no lung tumors were found in the group receiving silica sand; however, one hamster out of 35 at risk developed an adenosquamous carcinoma of the bronchi and lung after administration of Minusil control quartz. The total dose received for each type of quartz was 16.5 and 10.5 mg for the silica sand and Minusil, respectively, and was chosen on the basis of its potential to produce a massive fibrotic response in rats. The doses compare favorably to Wagner's 20 mg single injection where tumor incidence ranged from 30 to 40 percent. We hesitated to use very much higher doses, for fear of jeopardizing the entire experiment because of the expected adverse reactions on survival due to the fibrotic response produced by silica.

DR. BRESNICK: Dr. Niemeier, aren't the rat experiments, though, marred a little bit by the fact that it is very difficult to get a clean rat, whereas it is a heck of a lot easier to get a clean hamster? I would trust the hamster studies, I think, a little bit more with silica than I would with the rat.

DR. NIEMEIER: I agree with you. I think our experience now is that we are getting cleaner rats, so the more recent information that is coming out may be closer to the truth. I might add, however, that Wagner used specific pathogen-free rats in at least one of her studies.

DR. BRESNICK: Yes, in terms of lung pathology. Dr. Landrigan?

DR. LANDRIGAN: A question for either Dr. Menter or Dr. Wiser. There has been concern, I have heard in various places in my travels over the last couple of years, that exposure to ordinary fluorescent bulbs in the office environment, not the special bulbs in the tanning salons, might be associated with excessive risk of skin cancer in office workers. And I know that an epidemiologic study to evaluate this question is getting underway up in Canada. I don't think we have any going at the moment.

First of all, could you tell us what is the wavelength in standard fluorescent bulbs?

And then, secondly, have you done or are you planning to do any studies in your test system to perhaps evaluate the effect of standard fluorescent bulbs on tumor incidence?

DR. MENTER: We haven't done any such studies. The wavelengths that are in question are some UVA wavelengths. Ordinary fluorescent lights evidently do have quite a bit of UVA. And these are not carcinogenic so much in themselves, but, as Dr. Willis and I have shown, they can potentiate the effects of UVB. So in that sense, I would say that there is probably some risk there.

We have not considered such studies ourselves. I don't know if Dr. Wiser would like to add to that.

DR. WISER: I believe it is not a continuous spectrum of UVA; there are some lines due to reaction, mercury lines. So there is one sharp line. I forget the wavelength, I guess 366 nanometers or something like that.

VOICE: Is there a problem?

DR. WISER: If they use the bulbs bare, yes, you have that problem. Of course, the intensity is not that high, but after years of sitting under a desk light or in a house, there could be a problem. However, when the fluorescents have a plastic filter over them, you don't have the problem.

DR. BRESNICK: I think that anybody who has been in the business of DNA repair knows that you can't do alkaline elution or any other DNA repair assay in the fluorescent lights that are ordinarily available in laboratories. You have to shut them off or use gold lights.

Are there other questions for the panelists, for the speakers? May I ask a question first? It is for you, Dr. Menter. Has anyone looked at either UVA or UVB as a promoter? I mean, give a dose of benzo(a)pyrene, for example, under an initiating dose on your hairless mice or any mouse, and then use either UVA or UVB chronically in a promotion-type of study?

DR. MENTER: I do not know of any studies of that type. Of course, plenty has been done in the other direction, where UV has been used as an initiator, with chemical promoters. It would be hard to separate the initiator vs. promoter effects of UV if the chemical were used first.

In the studies that we have done ourselves, we really are using UVA and UVB as a combined initiator and promoter.

DR. BRESNICK: You are using it as a complete carcinogen?

DR. MENTER: Yes, but you can also consider it as a promoter.

DR. BRESNICK: Yes. Did you have a comment you wanted to make?

DR. MENTER: I wanted to ask Dr. Niemeier a question. Have you any explanation for the sunlight effect? That the sunlight apparently inhibits your response?

DR. NIEMEIER: There are a number of possibilities. One is the skin-thickening phenomenon, but I don't know how that might relate to the overall tumorigenic response; other possibilities include photodegradation or some type of altered immune response.

There have been some indications in the literature, I believe by Fisher and Kripke (1982) in *Science*, that ultraviolet light can cause an altered immune response; however, they found a reduced latent period in the photocarcinogenic response which was attributed to the role of suppressor T lymphocytes. This is in contrast to our findings of an increased latent period, but similar tumor incidences in the presence of simulated sunlight.

I tend to lean toward the possibility that the altered response that we are seeing with sunlight is due to a photooxidation process.

DR. MENTER: Right.

DR. NIEMEIER: I think that this photooxidation process is especially illustrative with the asphalt materials, where there are very few PNAs present. And if, indeed, the aliphatic hydrocarbons are responsible for the effects observed, invoking the cocarcinogenic response, they are not going to be affected by the sunlight.

DR. BRESNICK: I think there have been a number of studies where you get photodecomposition of, for example, benzo(a)pyrene -- you wind up with all sorts of quinones.

DR. NIEMEIER: That is also my understanding. And, of course, our data demonstrates that using B(a)P alone with sunlight showed a significantly decreased tumorigenic response because of the sunlight exposure.

DR. BRESNICK: Right. Additional comments or questions for our four speakers? (No response.) Well, if not, I think we will call this session to a halt. Thank you to both the audience and the speakers.

(Whereupon, at 11:58 a.m., the workshop was recessed.)

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Friday Afternoon, March 23

SESSION VII - EPIDEMIOLOGY
Chairman: Dr. Carl R. Morris*
Environmental Protection Agency

* Substituting for Dr. William Farland

SESSION VII - EPIDEMIOLOGY
Chairman: Dr. Carl R. Morris, EPA

DR. MORRIS: I think we would like to get started. We have been fairly prompt, I think, in holding to our schedule, and I think that is important.

I obviously am not Bill Farland, nor do I pretend to be. Bill has been off, over the last couple of days due to what we hope will be a brief illness. I am happy to fill in for him.

I would like to start the session this afternoon on epidemiology. It will be sort of a twofold introduction. I will introduce Dr. Robert Spirtas from NCI, and I believe he has someone else that is going to actually make the presentation.

DR. SPIRTAS: Thank you, Carl. The National Cancer Institute has a case control study of mesothelioma underway which involves some 600-700 cases taken from many centers across the country, and we are in the process of gathering that data.

Rather than give you a progress report, I can simply tell you that our measures of exposure are derived from an occupational questionnaire that also has some other aspects to it; but we are hopeful of obtaining another measure of exposure via analysis of the fiber content of lung tissue of some of our cases.

By a fortunate coincidence, visiting with us today is an expert in that type of analysis, Dr. Fred Pooley from the University College, Cardiff, Wales. He is a professor of Minerals Engineering and is also associated with the MRC pneumoconiosis unit in Wales. We have asked him today to discuss the type of analysis that he does on lung tissue, and we hope that we can work this into our ongoing study.

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Techniques for Investigating Mineral Contents
of Diseased Lung Tissue

Dr. Fred D. Pooley
University College
Cardiff, Wales

Techniques for Investigating Mineral Contents of Diseased Lung Tissue

Dr. Fred D. Pooley, University College, Cardiff, Wales

Good afternoon, ladies and gentlemen. It gives me great pleasure to be here to address you. I wasn't dragooned into this, but it does give me great pleasure to demonstrate and illustrate the sort of work that we have been doing in Great Britain with regard to asbestos exposures.

I have been involved with the Medical Research Council in Great Britain for over 15 years, developing techniques to study dust particles in biological airborne and water samples, and we have been accumulating information concerning the mineral dust content of diseased and control tissues.

The area of mineral dust research has grown over this 15-year period with the development of quite sophisticated analytical techniques, and these techniques are now capable of characterizing a single dust particle, a single dust particle which may only be 250 angstroms in diameter and perhaps a half a micron long.

We have very powerful tools which, as I said, can analyze single dust particles and also help us to unravel quite complex mixtures of mineral dusts. We can now split up these complex mixtures into various mineral components; we can analyze them chemically; and we can characterize them very accurately in terms of their physical characteristics: length, diameter, etc. You will hear more about that from another speaker later on.

Using this sort of equipment, we have undertaken and are still involved in numerous studies concerned with the examination of disease and control tissues. What I would like to do is lead you through some of our studies.

(Showing of slides)

First of all, we have some results that we obtained in the years 1977 to 1978. Basically, they are results from mesothelioma cases which occurred in 1976, together with a control series. So it was a prospective study, if you like.

And this is the sort of information that we have gathered. Being able, as I said, to discriminate between fiber types in the tissues, we were able to correlate the number of fibers in the control and the diseased tissues, and here you can see the cumulative percent containing fibers over a certain number.

The interesting thing here, obviously, is that there appears to be very little difference between the control series and the mesothelioma cases. And this is for only one asbestos, chrysotile, which is the largest commercial asbestos mineral; I believe over 90 percent of the world's asbestos usage is chrysotile.

(Change of slides)

But taking amphibole asbestos minerals, and these are crocidolite, amosite, and tremolite, we found in the 1976 study that there were quite marked differences in the levels of fiber between the controls and the diseased cases, an indication obviously that amphibole fibers are perhaps more involved in the production of mesothelioma than chrysotile asbestos.

(Change of slides)

Looking at this, obviously these cases didn't appear to us as one with amphibole and one with chrysotile; they were in fact mixed exposures, and this is the sort of picture we had from the mesothelioma cases of the distribution of fiber types in the lungs of cases of mesothelioma.

Now, the interesting results are the ones dotted on the axis here, where we have high percentages of amphibole, in fact 7 cases here with 100 percent amphibole. We weren't able to detect any other fiber. That doesn't mean that there wasn't any other fiber there, but the amphibole swamped the results.

As you can see, the cases here with chrysotile -- these are the cases with non-asbestos fiber. Note the interesting three specimens up here with fibers in their lungs, with extremely low levels of chrysotile, but large quantities of non-asbestos fiber.

(Change of slides)

We are looking at the controls. We tried to get two types of controls in this study -- cardiovascular controls and also bronchiocarcinoma controls. Unfortunately, we fell short. It was easy to get the mesothelioma samples but not easy to get controls in for this study.

But here is a picture of CVA controls.

(Change of slides)

The interesting thing here is the low levels of amphibole, and yet appreciable levels of chrysotile in the cardiovascular controls.

(Change of slides)

These bronchial cancer controls were very different indeed. Most of the results fell around this non-asbestos fiber position.

(Change of slides)

Following on from that study, in 1977 we collected material from the pneumoconiosis medical panels in Great Britain, and these were diseased cases which were reported to the panel, where the cause of death was related to exposure to asbestos. Also collected were control materials.

When we looked at the asbestos fiber content with respect to diseased cases reported to the panel and the controls, again, this was sort of the picture we have. And these were all asbestos diseases -- asbestosis, mesothelioma, and in some cases lung cancer.

You can see, again, the similar sort of results we had for chrysotile, and the marked difference in the amphibole content between the reported diseased cases and controls, and also there are the non-asbestos fibers down here.

So non-asbestos fibers, chrysotile, apparently were not involved in those cases reported to the pneumoconiosis medical panel where asbestos was supposedly the cause of death.

(Change of slides)

The pathologist at Flandock -- Dr. Wagner -- broke down these cases. We did have results from mesothelioma, but also for asbestosis. And interestingly enough, he graded the cases "not, minimal, asbestosis, 1-2 more severe, 3-4 more severe still" as it were. And these are the results obtained when we looked at the correlation between numbers of fibers in a gram of tissue and the various categories of asbestosis. And the only reasonable correlation was with the amphibole fiber and not with chrysotile or non-asbestos fiber.

What I am doing now is illustrating to you how examination of tissue and quantification of its contents, etc., has contributed to an understanding of what fiber is, perhaps, involved in the production of a particular disease.

(Change of slides)

We are still obviously involved in these studies, and they are of importance, because in the literature there is quite a lot of conflicting information.

This particular plot is by Professor McDonald from Montreal, and it shows lung cancer mortality data for various textile plants in the U.S., textile chrysotile plants, insulating workers, textile plants in the U.K., cement, and chrysotile mining operations. As you can see there is a huge difference in the mortality risk, even though these plants are all supposedly using chrysotile asbestos.

(Change of slides)

And to illustrate possibly why there is such a difference, this is an extract from a lung of a worker in a textile plant in the U.S. If we went back, we would find a very high incidence of lung cancer. And, in fact, the fibers in this lung -- and there were quite a few of them that were quite visible -- are not chrysotile. These fibers are, in fact, tremolite fibers, and, even though there was chrysotile there, when we broke down the mineral content of this tissue, tremolite was present in large quantities and chrysotile in very small quantities. There were other fibers also present.

As I said, supposedly this exposure was to chrysotile in a textile manufacturing situation.

(Change of slides)

Here we are taking another U.S. textile plant, I think in Pennsylvania, where we examined several cases years ago. In fact, they were mesothelioma cases. And again, this is the sort of picture we had from them.

Chrysotile? You can see the chrysotile level in these five cases varying from 54 percent down to as little as 3 percent by number, and large quantities of amphibole fiber including some tremolite.

Crocidolite was in fact used in this particular plant, and amosite, but the tremolite wasn't. And we assumed that, as in the previous case, this tremolite is a contaminant of the chrysotile fiber.

(Change of slides)

Again in the U.K., Rochedale, a much newer textile plant which was supposedly pure chrysotile exposure. In fact, this particular plant was used to establish the chrysotile standard level of fiber exposure.

Looking at the lungs of mesothelioma cases occurring in that plant, we found that, in fact, there were equivalent and in some cases more amphibole fibers, mainly crocidolite in this case, rather than chrysotile. They did, for a small period of time, for a year or so, use crocidolite in the plant. That crocidolite fiber has been retained, and in the cases we looked at, some of them with 40 years exposure in that plant, mainly to chrysotile, they had more crocidolite in their lungs than they had chrysotile.

(Change of slides)

So, this sort of information is very useful, especially when you have got conflicting epidemiologic results to try to sort out.

How does this sort of analysis of tissues help, with regard to the general population? Well, we are at the present time studying some cases from Sweden, a town called Lund, where they have an asbestos cement factory. In that factory they have in the past used crocidolite and amosite, and fortunately for us the University of Lund has kept biological material from all the cases of everybody who has worked in that plant and has died in Lund. And they have also matched it with controls from the town.

This is the sort of result we obtained from the cases there. This is an ongoing study. It is mainly chrysotile used in the plant, as I said, but some crocidolite and amosite is used also. This is the sort of partition of fibers in the cases so far -- mainly chrysotile, as I said, with amosite showing and crocidolite quite predominant, and anthophyllite and tremolite being the contaminants that probably came along with the chrysotile fiber.

This is how the controls and the population are looking -- again, similar quantities of chrysotile, in proportion. These are not actual numbers, by the way; these are just proportions of fibers.

These are the non-asbestos fibers detected in the tissue. We detect chrysotile in the population there, and a little tremolite, and then anthophyllite, but not amosite in any large quantity.

(Change of slides)

Looking now just at the numbers of fibers detected, this is the total number of fibers detected in cases from the plant, and it's a mean value. Here

is the total number of fibers detected in the controls in the town of Lund -- again, as a mean value. These are the levels of chrysotile. As you can see here, this bunch of little peaks, these are the amphiboles detected in the plant, with very much smaller levels in the control case.

What I have done here is try to illustrate to you how looking at tissue specimens, collecting information on fiber numbers, fiber types, etc., can perhaps help to interpret epidemiological studies, interpret biological experiments, and also can help us perhaps with regard to the possible effects of asbestos exposure upon the general population.

Thank you very much.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
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PRESENTATION:

Methods of Control for Smoking in
Occupational Cohort Mortality Studies

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Methods of control for smoking in occupational cohort mortality studies

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STEENLAND K, BEAUMONT J, HALPERIN W. Methods of control for smoking in occupational cohort mortality studies. *Scand J Work Environ Health* 10 (1984) 143–149. Cohort mortality studies, which compare the observed mortality of a historically exposed cohort to an expected mortality based on figures from a national population, are among the most common and useful types of studies done in occupational epidemiology. Such studies are primarily based on records which lack data on smoking. Hence it is often difficult to determine whether an observed excess of a given smoking-related disease is the result of occupational exposures, an excess consumption of cigarettes among the exposed, or an interaction between the two. The purpose of this paper is to outline the different types of control which may be exercised in cohort mortality studies to control for the effects of smoking. Indirect control, which uses only existing data, may be exercised through (i) analysis of other smoking-related causes of death besides the cause-of-death of primary interest, (ii) use of an internal reference group instead of the national population, (iii) adjustment of the excess risk based on hypothetical smoking habits, or (iv) analysis for a dose-response relationship between occupational exposure and disease. Direct control, which requires interviews, may be exercised through (i) a survey of the smoking habits of currently employed cohort members, (ii) a survey of all cohort members, or (iii) a "nested" case-referent study within the cohort. The choice of which method to use depends on the cost and the degree of accuracy required by the investigator.

Key terms: epidemiology, occupation, smoking.

With no data on smoking in a cohort mortality study, it is often difficult to determine whether an observed excess of disease is a result of occupational exposures, an excess consumption of cigarettes in the exposed cohort, or an interaction between the two.

Occupational cohort mortality studies are often conducted using company or union records to assemble the cohort, which is then followed to determine vital status. These studies use a historical cohort design. The exposures have often occurred far in the past, and a large percentage of the cohort is no longer currently employed. The studies are usually done solely from records, which consist primarily of work histories, a list of who is alive and who is dead, and the causes of death for those who have died. Often the whereabouts of those who are still alive is unknown, and no effort is made to contact these individuals. Usually no information about the smoking habits of the cohort is available. An estimation of disease risk is usually made through a comparison of the mortality experience of the exposed cohort with the mortality experience of a large national population (such as

that of the United States), although occasionally other nonexposed groups are used.

Many diseases are associated with smoking, and many of these same diseases are work-related. For example, lung cancer and nonmalignant respiratory disease are among the smoking-related chronic diseases which can be caused by occupational exposures.

If excess mortality in the cohort is observed for a cause of death which is known to be associated with smoking, it is important to attempt to control for the effects of smoking. There are several methods of control, both indirect methods which use existing records and direct methods which require interviews to obtain information on the smoking habits of the cohort. Most of these methods have been discussed at one point or another in the epidemiologic literature (4). Our purpose in this paper is to review all methods which have been used to date.

Some would argue that control for smoking should be built into any study design from the outset, regardless of whether the disease of interest is known to be smoking-related or whether an excess of the disease of interest occurs in the exposed population. This argument is based on the assumption that almost all diseases are smoking-related and that a deficit of disease can also be due to smoking (for example a deficit of heart disease in a nitroglycerine plant). However, obtaining information about smok-

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ing for a retrospective study can be costly, and we would argue that in many cases it may be prudent to determine first if a disease known to be related to smoking is in excess before going to the expense of obtaining smoking information.

Indirect control using available records

Assuming the investigators are unable to obtain any direct information on the smoking habits of the cohort in question, there are still four types of indirect control that can be exercised using available records. They are (i) an analysis of other smoking-related diseases, (ii) use of an internal nonexposed cohort, (iii) an adjustment based on hypothesized differences in smoking habits for the exposed and nonexposed populations, and (iv) an analysis of the data for a dose-response relationship. Except for the use of a nonexposed cohort, all these methods involve little or no expense. However, they all lack the precision of direct control, for which data on smoking habits are obtained.

Other smoking-related causes of death

If a given excess risk for any particular cause is in fact due to excess smoking by the exposed cohort when compared to the national population (the reference group), then other smoking-related causes of death should also be elevated. Coronary heart disease, non-malignant respiratory disease (bronchitis and emphysema), and cancers of the lung, larynx, pancreas, esophagus and bladder are among the diseases associated with smoking. In table 1 we list the standardized mortality ratios for these diseases for smokers in comparison to nonsmokers on the basis of four large cohort studies of smokers, the Dorn study of United States veterans (15), the Hammond study (13), the

Weir & Dunn study (29), and the Doll & Peto study (9). Despite the fact that these data are not quite comparable due to different definitions of smokers (current or ever), different age categories, and different periods of follow-up, for most of these diseases the data are consistent from one study to another. Esophageal cancer is an exception, perhaps due to the inclusion of pipe and cigar smokers in the nonsmoking group in the Weir & Dunn study, since the Dorn study showed cigar smokers to have an excess risk of 5.33, and pipe smokers an excess risk of 1.99, for esophageal cancer. Furthermore, for most of these diseases, a consistent dose response is apparent in relation to the amount smoked, a further confirmation that these diseases are in fact associated with smoking.

If an exposed cohort has smoked substantially more than the national population, then all the smoking-related diseases should be elevated. It is clear from table 1 that the causes of death that are the most associated with smoking are lung cancer, emphysema, and laryngeal cancer. It is these diseases which perhaps can most easily serve as indirect indicators that a given excess mortality is due to smoking rather than to exposure to a toxic agent.

As an example of this technique, in a study of workers exposed to pesticides, an excess of lung cancer was apparent; yet there were no excesses for cardiovascular disease, cancers of the esophagus, kidney, or pancreas, or for emphysema (3). Hence the authors took the lack of elevation of smoking-related causes to be indirect evidence that the excess of lung cancer was unlikely to be related to smoking.

Use of an internal reference group

Rather than using the national population as a comparison group, it is often advantageous to use another reference group, such as a cohort from a

Table 1. Standardized mortality ratios for male cigarette smokers versus nonsmokers from large prospective studies.^a

Cause of death	Study				
	Dorn (15)	Hammond (13)		Weir & Dunn (29)	Doll & Peto (9)
		Age 45-65 years	Age 65-79 years		
Lung cancer	8.84	7.84	11.59	7.61	14.00
Laryngeal cancer	8.88	6.09	8.99	..	..
Esophageal cancer	5.32	4.17	8.99	1.82	4.67
Bladder cancer	1.90	2.00	2.96	2.89	2.11
Pancreatic cancer	1.64	2.69	2.17	2.43	1.57
Kidney cancer	1.34	1.42	1.57	2.46	..
Emphysema	9.09	6.55	11.41	12.33	..
Bronchitis	2.86	..	..	..	..
Emphysema/bronchitis	..	..	..	..	24.67
Coronary heart disease	1.46	2.03	1.36	1.60	1.62

^a Dorn includes men 35-64 years of age at the time of entry with 217,776 person-years between 1954-1962, current smokers versus nonsmokers. Hammond includes men 45-64 and 65-79 years of age at the time of entry with 1,436,573 person-years between 1959-1962, ever smoked regularly versus never smoked regularly. Weir & Dunn includes men 35-64 years of age at the time of entry with 462,658 person-years from 1954-1962, current and exsmokers versus nonsmokers (including pipe and cigar). Doll & Peto include 34,440 men followed for 20 years from 1951-1971, aged 40-85 years in 1971, current smokers versus nonsmokers.

similar socioeconomic class and from the same community. It is well known that smoking habits vary by social class (26). In addition there is evidence for the United States that there is some slight variation according to large geographic regions (5), although there are few published data on this question. Stronger evidence of geographic variation, controlling for social class, is available for England (8). For these reasons, a local nonexposed group, with the same income level as the exposed cohort, may have smoking habits that are more similar to those of the exposed group than those of the national population are.

An example of this approach is a study of welders in a local union where there was a 30 % excess rate of lung cancer mortality compared to that of the national population (2). With the use of nonwelders in the same local union as the reference population, a similar excess risk for the welders was observed. This finding could be taken as evidence that the original observed excess was not due to smoking — assuming the nonwelders from the same local union shared the smoking habits of the welders.

However, there is evidence that smoking rates also vary for different occupations, even within the same socioeconomic categories (7, 21, 25, 28). For example, in one study (25), the range of current smokers within a sample of blue-collar workers varied from 36 % (stationary engineers) to 72 % (roofers). Hence using another occupational group as a reference population may also have its pitfalls. It may be possible to avoid such potential pitfalls if the exposed cohort can be divided into low and high exposure groups. The low exposure group can then be considered as an internal comparison group of workers in the same occupation (see later discussion on dose response).

Adjustment based on hypothesized differences in smoking habits

Axelsson has pointed out that an adjustment can be made under various assumptions about the smoking habits of the cohort, given that the relative risks of smokers for a variety of diseases are well known and the smoking habits of the reference population can be estimated (1). Numerous prospective epidemiologic studies, such as those shown in table 1, have provided relatively stable estimates of the excess risk smokers incur for a number of causes of death. Furthermore several surveys have provided estimates of the proportion of smokers in the United States population. Axelsson chooses an example which considers lung cancer. He assumes that the nonexposed population is composed of 50 % nonsmokers, 40 % moderate smokers, and 10 % heavy smokers, with relative risks of lung cancer of 1, 10, and 20, respectively. He then forms several hypotheses about the smoking habits of the exposed population (see table 2). For example, one such hypothesis is that the exposed cohort is

composed of 30 % nonsmokers, 50 % moderate smokers, and 20 % heavy smokers. Under such a hypothesis, the exposed population would have a relative risk of 1.43 compared to the hypothetical nonexposed population, due to smoking alone. Then, if a study of an exposed population finds an excess risk on the order of 5—10 and if, in fact, the smoking habits of the exposed cohort differ by the hypothesized amount from the nonexposed, then one can conclude safely that smoking cannot account for more than a fraction of the risk found. The point made is that for the degree of risk often found for exposed populations, rather large deviations in the smoking habits of the exposed versus nonexposed populations would have to exist for smoking to explain the excess risk observed.

Clearly, these sorts of adjustments are crude. Lacking any direct information on the smoking habits of the exposed cohort, the investigator can only hypothesize such smoking habits and estimate what degree of relative risk might be explained by smoking.

A recent example of the use of this technique is provided by Suta & Thompson (27), who wished to determine the influence of smoking on a previous finding that automobile workers suffered a 32 % excess of lung cancer. Using data from the annual health interview surveys made by the National Center for Health Statistics in the United States, the authors obtained data on the smoking habits of both automobile workers and the general population in the period 1965—1980. These data indicate that automobile workers smoked more than the general population. Note that these data did not allow Suta & Thompson to know the smoking habits of the automobile workers in the particular study with which they were concerned, but only allowed them to hypothesize a difference between the smoking habits of the exposed group and the national population. The authors then adjusted the observed 32 % excess risk

Table 2. Estimated crude rate ratios in relation to the fraction of smokers in various hypothetical populations.^a

Types of smokers in exposed group (%)			
Nonsmokers (risk of 1)	Moderate smokers (risk of 10)	Heavy smokers (risk of 20)	Rate ratio ^b
100	0	0	0.15
70	30	0	0.43
60	35	5	0.78
50	40	10	1.00
40	45	15	1.22
30	50	20	1.43
10	60	30	1.86
0	0	100	3.08

^a Taken from Axelsson (1). The percentages in the table represent the distribution of smokers among the exposed.

^b Comparing the exposed with the nonexposed group composed of 50 % nonsmokers, 40 % moderate smokers, and 10 % heavy smokers.

downward, and estimated that, after smoking is taken into account, the excess risk declines to somewhere between 6 and 21 %, depending on how the adjustment is made.

The adjustments used by Suta & Thompson contain a measure of imprecision due to several factors, as pointed out by Silverstein et al (23) in an accompanying editorial. One is that the smoking rates for automobile workers obtained from the health interview surveys show an appreciable variation due to sample size limitations, and the differences observed between auto workers and the general population (7—10 % difference in the percentage of current smokers) were not statistically significant. Another is that, while it is possible to adjust for current smokers and those who have never smoked, an adjustment for former smokers is more difficult. The excess risk of former smokers varies according to the age when they quit, to how much they smoked before they quit, and to how long ago they quit. This information may be unavailable in surveys of smoking habits; for the automobile workers in the health interview survey it was not known how much they smoked before they quit. It is this difficulty in adjusting for former smokers which caused Suta & Thompson to give a range of estimates for the excess risk for auto workers after adjustment.

Dose-response analysis

It is customary in occupational cohort mortality studies, if an overall excess risk for a given cause is observed, to estimate different risks according to different dose categories. (Since data on dose is usually unavailable, duration of employment often serves as a surrogate for dose.) If a trend toward increasing risk is seen with increasing duration of employment, it is thought to indicate that in fact the excess risk is associated with exposure.

This concept certainly has some validity. An observed dose-response relationship is frequently taken as one of the criteria for establishing a true association of an agent with a disease, in animal as well as in human studies. Such a dose-response relationship in a human epidemiologic study can be seen as a confirmation of a true association instead of as one spurious due to smoking. However, when duration of employment is used as the measure of dose, there will be a strong correlation with years of consumption of cigarettes. Since increased years of cigarette consumption are associated with increased risk for a number of diseases, an appropriate analysis would attempt to observe an increased risk with duration of employment stratified by age so that workers within the same age categories would be compared according to their duration of employment. Such a stratification for age is customarily done in standard life-table programs used to calculate a standardized mortality ratio. An evaluation of the dose-response relationship through a comparison of standardized mor-

tality ratios may then be a valid way of controlling for the effect of smoking, if one assumes that the smoking habits of the exposed cohort remained fairly constant over time or at least changed uniformly among dose categories over time. In other words, the stratification for age, within the different dose categories of the exposed cohort, must be assumed to stratify broadly on amount smoked also. Furthermore there must be no interactive effect between age (or cumulative years of smoking) and the exposure in question for the disease of interest, or else comparisons of indirectly standardized measures will be invalid.

Direct control using data on smoking obtained by interview

Direct control for the effects of smoking requires contacting members of the cohort themselves, or other individuals such as next-of-kin who can give reliable information about the smoking habits of cohort members. Such an effort usually increases the cost of the study and might be undertaken only if (i) an excess risk is found that is small enough to be in fact accounted for by smoking rather than by exposure and (ii) the much cheaper methods of indirect control are felt to be too imprecise.

It is usually impossible to contact all cohort members or their surrogates directly to determine smoking habits. Smoking data are usually collected through (i) a survey of a sample of currently employed cohort members, (ii) a survey of a sample of all cohort members, or (iii) a case-referent study of a given disease within the cohort and a survey of only cases and referents.

Generally the case-referent approach will be the most precise method of controlling for smoking. Cost considerations may play a role in the decision as to what kind of direct control should be exercised. The cost of obtaining smoking information depends on the number of people who are to be interviewed.

Survey of currently employed cohort members

Conducting a survey of a sample of currently employed cohort members has the advantage of being much easier than a survey of a sample of all cohort members, many of whom are no longer employed. However current workers may not be representative of all workers in the cohort. For example, persons who have smoked more and have had worse health may have left the workforce.

The smoking habits of currently employed workers may be compared with the smoking habits of the current national population of a similar age, which is available from national surveys. However, the national population includes many nonworkers, whose smoking habits may differ from those of the workers. As an alternative, it may be possible to compare the

currently exposed with another group of workers who are current workers but are nonexposed.

A recent example of the use of this technique will serve as illustration. In a recent cohort mortality study of heavy equipment operators (11), the investigators wished to assess whether the cohort's smoking habits differed from those of the national population. A survey of 107 currently working men indicated that 25 % had never smoked. Data from the 1970 health interview survey, conducted by the National Center for Health Statistics in the United States, indicated that 31 % of men in the national population were nonsmokers. The authors conducted a chi-square test and found no significant differences in smoking habits between the cohort and the United States population and hence concluded that smoking was unlikely to have confounded their results. Another example of this approach is provided by Edling et al (10), who conducted a survey of the smoking habits of current workers and then used an Axelson-type adjustment to correct estimates of excess risk of cardiovascular disease mortality.

Survey of past and current workers

A better method to assess the smoking habits of an exposed cohort is to survey a sample of the entire cohort. Such a survey involves tracing employees who are no longer working and who may have died or moved far away from the worksite. When the employees have died, it is necessary to interview a surrogate (such as their next-of-kin or co-workers).

The data which can be derived from interviewing surrogates will presumably be somewhat less reliable than the data obtained from cohort members themselves, but it should be relatively accurate as to whether a cohort member simply smoked or did not smoke. The literature on the validity of surrogate data for smoking is limited. One study (22) indicated that for about 2,000 British and Norwegian immigrants to the United States who died in the 1960s, next-of-kin showed 92 % agreement with the decedent's own information on being a regular smoker versus an occasional smoker or nonsmoker. However, when the comparison was made for more-detailed information about how much an individual smoked (never, occasionally, less than one pack, one pack, and more than one pack per day), agreement between the decedent and the next-of-kin fell to 74 %. In another study of lung cancer among shipyard workers (6), the authors interviewed 24 men with lung cancer and their wives concerning the husbands' smoking habits and found that there was 83 % agreement for smoking category. A similar degree of agreement was found by Flanders et al (12), who interviewed 15 live subjects and their nearest relatives to determine the subjects' smoking habits, and by Pershagen & Axelson (19), who compared information on smoking given for 14 deceased men by the next-of-kin with the information on decedents' medical records. In general,

however, it can be said that more information is needed to determine the validity of smoking data from surrogates.

It is difficult to trace past employees who are still alive but may have moved far away. Fortunately, in many occupational studies, the cohort is composed of individuals who, after retirement, tend to stay in the community where they worked. For example, in a recent study of smelter workers employed at eight different plants (personal communication, Edward Ricci and Phil Enterline, Graduate School of Public Health, University of Pittsburgh, Pennsylvania, United States), the investigators conducted a survey of over 400 cohort members. After cohort members were traced via letters, phone calls, and information provided by friends and relatives, a final response rate of 83 % was attained. These smelter workers came from relatively stable and small communities and were probably easier to trace than workers in large urban centers.

After the survey is conducted, other data must be used to estimate the smoking habits of the reference group. Once data are available on the smoking habits of the exposed and nonexposed, some type of adjustment similar to the one proposed by Axelson must be done to assess the possible confounding effect of smoking on any excess cause-specific risk which was found in the exposed cohort.

An example of this type of adjustment is found in a study of uranium miners (16). In that study a comparison of the smoking habits of the exposed versus those of the national population indicated that an 18 % excess in lung cancer mortality should have been expected for the exposed group due to smoking alone. In fact a 400 % excess of lung cancer mortality occurred.

Nested case-referent study

Once the cohort study is completed and excess risk of a given disease has been found, then an alternative way of controlling for smoking is to conduct a nested case-referent study. In such a study cases are usually all those who died of the disease of interest, and referents are a sample of cohort members who did not die of the disease of interest (or alternatively, had not died of the cause of interest at the time of the case's death). Cases and referents, or their surrogates (usually next-of-kin), must be interviewed to determine smoking habits, and cases and referents must be evaluated for their exposure to the suspected agent. Exposure odds ratios may then be calculated which adjust for smoking. However this approach usually requires that a group of nonexposed individuals is available in the cohort so that there is variability in the cases' and referents' exposure histories.

A good example of this approach is a study of copper smelter workers in Sweden by Pershagen et al (20). From a cohort of some 4,000 workers, 76 lung cancer cases were identified. Two referents per case

were chosen from among those who had died of diseases other than lung cancer. Interviews were conducted with next-of-kin to determine smoking status, and exposure or nonexposure to arsenic was determined from company records. The authors found a threefold risk of lung cancer for nonsmokers exposed to arsenic. An interaction between arsenic and smoking for lung cancer was noted.

In a case-referent study with dead cases, live referents could be used as well, although the question of the comparability of smoking data would arise if some information came from the subjects themselves while other information came from surrogates. Frequently this problem is avoided by interviewing surrogates for the live referents rather than interviewing the referents themselves. On the other hand there is some evidence (17) that dead referents will have smoked more than live referents, a phenomenon which may bias estimates of risk.

The case-referent approach has the value that the investigator cannot only control for confounding due to smoking, but can also determine whether there is an interaction between smoking and the exposure variable. This ability to assess interaction is the strength of the case-referent approach to controlling for smoking. Perhaps the best known interaction between an occupational exposure and smoking is for the carcinogenic effect of asbestos. Other evidence indicates that exposures to arsenic (20), radon daughters, cadmium, and toxins in the rubber industry (18) may also interact with smoking to increase health risk. A recent report has suggested that smoking may also interact with occupational exposures to *decrease* lung cancer (24), under the theory that smoking causes an increase in airway mucus, which protects the lung from carcinogens. However, the epidemiologic data to document this last suggestion are weak or are contradicted by other studies (14). Alternatively, if there is such a protective effect, it may be restricted to very specific situations; for example some authors (14) have noted that British coal miners with smoking-induced bronchitis have had less coal dust in their lungs.

A disadvantage of the case-referent approach may be the necessity of having a group of nonexposed individuals for a reference group. Without such nonexposed individuals, everyone in the case-referent study will be exposed, and no risk for disease conditional on exposure can be estimated.

In some cases it may be possible to conduct a nested case-referent study to control for smoking within a cohort where everyone has been exposed, if there is sufficient variation in exposure levels. In such a case, the risk of low-exposed individuals can be compared to the risk of high-exposed individuals, smoking being controlled. Whittemore & McMillan (30) have recently provided an example of this approach in their case-referent analysis of a cohort study of uranium miners.

Discussion

It is very rare that an epidemiologist possesses information on the smoking habits of a historical occupational cohort; yet this lack of information has not prevented occupational cohort mortality studies from being undertaken. Indeed occupationally exposed cohorts have provided the definitive evidence of carcinogenicity for most known human carcinogens, including benzene, vinyl chloride, asbestos, bis(chloromethyl)ether (BCME), beta-naphthylamine, coke oven emissions, radon daughters, arsenic, nickel, and hexavalent chromium. Sometimes the excess risks observed have been for cancers which are unrelated or only slightly related to smoking (eg, hepatic angiosarcoma and vinyl chloride, leukemia and benzene). In other cases the risks have been of such magnitude that smoking could obviously not account for them (eg, BCME and radon daughters and lung cancer, b-naphthylamine and bladder cancer). Occasionally data on smoking were available for the cohorts (eg, asbestos, arsenic, radon daughters). Finally, in some cases an internal reference group has been used (eg, coke oven emissions).

Occupationally exposed cohorts will continue to provide the most solid evidence of associations between many toxins and chronic human disease. This is so because occupationally exposed cohorts have often been exposed sufficiently long ago to relatively high concentrations of toxins so that an effect can be measured. Furthermore mortality studies, rather than morbidity studies in which smoking information can be more easily collected, will continue to be common because of the relative convenience of using routinely collected records. Therefore the problem of disentangling the effects of exposure and smoking will continue. Many investigators are already currently using some method of control when the disease in question is known to be associated with smoking and when an excess risk is observed for an occupational cohort. Our purpose in this report has been to list these methods in a systematic way and stimulate further discussion.

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PRESENTATION:

Progress Report
Lung Cancer Mortality Among Underground Uranium Miners
With Low Lifetime Exposures to Radon Daughters

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PROGRESS REPORT
LUNG CANCER MORTALITY AMONG UNDERGROUND URANIUM MINERS
WITH LOW LIFETIME EXPOSURES TO RADON DAUGHTERS

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Prompted by a request to the Surgeon General, the U.S. Public Health Service began a cohort mortality study in 1950 to examine the radiation hazards associated with underground uranium mining. The following significantly elevated standardized mortality ratios were previously reported for white miners with follow-up through 1977: lung cancer, 482; tuberculosis, 409; accidents, 331; nonmalignant respiratory disease, 324; and chronic nephritis, 262.

The primary objective in continuing this research is to quantify lung cancer mortality risk through 1982 by radon daughter exposure level, thereby establishing a dose-response curve. Special attention will be given to lung cancer mortality risks for cohort members with lifetime exposures less than 120 working level months (WLM). Analysis of these data will provide important information concerning the present U.S. exposure limit of 4 WLM per year which corresponds to 120 WLM for a working lifetime of 30 years.

To calculate lifetime exposures to radon daughters, a questionnaire survey will be administered to update mining and other occupational exposures and medical and smoking histories for cohort members with incomplete data. These data were last updated in 1969 when 721 white and a smaller number of American Indian miners had not yet accumulated 120 WLM of exposure. Progress will be reported.

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INTRODUCTION

Since excess lung cancer mortality was first noted in the late 1800's among the miners of the Joachimsthal-Schneeberg region of central Europe, there have been a number of studies of the long-term health effects among various types of miners exposed to elevated levels of radon-222 and its radioactive decay daughters (polonium-218, bismuth-214, lead-214, polonium-214). Excess lung cancers have been reported among miners in the Joachimsthal region by Sevc et al. (1976); among lead-zinc miners in Sweden by Axelson and Sundell (1978); among fluorspar miners in Canada by deVilliers et al. (1971); among iron miners in Sweden by Renard (1974); among uranium miners in Canada by Hewitt (1976); among metal miners in the U.S. by Wagoner et al. (1963); and among uranium miners in the U.S. by Wagoner et al. (1964), Lundin et al. (1971), Archer et al. (1976), and Waxweiler et al. (1982).

(Slide 1) The study group of U.S. uranium miners reported on by Wagoner, Lundin, Archer and Waxweiler is the U.S. Public Health Service (PHS) uranium miners cohort. The cohort consists of 3362 whites and 780 American Indians who mined uranium on the Colorado Plateau anytime between 1950 and 1963. To be included in the cohort, miners must have been examined in the PHS surveys that took place between 1950 and 1960 and must have worked at least one month underground in a uranium mine (Lundin et al.: 1971). (Slide 2) Besides having experienced an increased lung cancer risk (standardized mortality ratio (SMR) = 482), white cohort members, when compared to the U.S. mortality rates for white males and with follow-up through 1977, also experienced significantly elevated risks from: tuberculosis, SMR=409; accidents, SMR=331 (the main component of this risk came from a subcategory consisting of accidents other than those due to transportation, poisoning and falls with an SMR=707); nonmalignant respiratory disease, SMR=324 (the main component of this risk came from a subcategory consisting of emphysema, fibrosis, silicosis and chronic obstructive pulmonary disease with an SMR=499); chronic and unspecified nephritis and renal sclerosis, SMR=262. White miners had an all causes SMR of

158, one of the highest risks experienced by any recent occupational cohort (Waxweiler et al.: 1982).

(Slide 3) American Indian cohort members, when compared to the nonwhite mortality rates for Arizona and New Mexico and with follow-up through 1973, also experienced a significantly elevated risk of death from lung cancer (SMR=423) while their risk of death from all causes was in deficit (SMR=86) (Archer et al.: 1976).

(Slide 4) This lung cancer risk is especially interesting because of the smoking habits among the Indians. Sixty-three percent (487) never smoked; of the 33% (256) who did smoke, 46% (117) smoked only 2 cigarettes per day and 90% (230) smoked 10 or less cigarettes per day. Since separation of the effects of radon daughters on lung cancer from the effects of smoking is necessary to adequately characterize the radiation hazard, further analysis of the Indians might be especially fruitful.

(Slide 5) Although members of the PHS uranium miners cohort have been exposed to silica, diesel exhaust and other exposures characteristic of underground, hardrock mining, the primary exposure has been radon daughters. The unit of measurement for radon daughters is the Working Level (WL) where one WL is defined as any combination of radon daughters in one liter of air which results in the ultimate release of 130,000 megavolts of potential alpha energy. The cumulative exposure of radiation from radon daughters to individual miners is measured in Working Level Months (WLM) where one WLM is defined as exposure to one WL for one working month (170 hours). The mean exposure, from uranium mining only, to white members of the cohort as of 1969 was 821 WLM (median=430 WLM).

The current U.S. exposure limit for radon daughters is one WL in any mine at any time miners are present and four WLM per year for any miner (Environmental Protection Agency: 1971). This four WLM per year exposure limit corresponds to a 120 WLM accumulation over a thirty-year working lifetime. NIOSH's primary concern is to quantify the mortality risks among cohort members who received less than 120 WLM so that the Institute can make recommendations on the adequacy of the four WLM/year exposure limit both now, based on current information and in the future, based on this and other new information.

While the 120 WLM level has been greatly exceeded by many members of the PHS cohort, 721 white and a smaller number of Indian miners had accumulated 120 WLM or less from uranium mining as of their last WLM compilations. These last WLM compilations did not include WLM's acquired from other hardrock mining prior or subsequent to uranium mining; these WLM's should be included or accounted for, especially

for the miners who apparently received a lifetime exposure of 120 WLM or less. Most miners had their last WLM compilations in 1969 when work histories and medical records were last updated. About 486 white and Indian miners were still mining uranium as of 1969. These 486 have incomplete uranium mining histories which require updating to ascertain which lifetime exposures remained under 120 WLM. Some misclassification of exposures among miners, especially those receiving less than 120 WLM, is anticipated. This misclassification can, hopefully, be controlled through exposure reevaluations and through analyses in various exposure groupings. Occupational exposures other than uranium mining are also of interest.

(Slide 6) Continuing the follow-up effort on this cohort is necessary because while 84.8% of the white cohort members have had 20 years since their first exposure to uranium mining, only 33.6% have had 25 years and only 13.7% have had 30 years (Waxweiler et al.: 1982). (Slide 7) The epidemic curves for lung cancer and for nonmalignant respiratory disease indicate that the excess risks extend past 25 years since the onset of uranium mining; this is a situation which about two thirds of the cohort has not yet reached.

In the collaborative research described herein, NIOSH and NCI intend to follow up all cohort members to update their smoking, work, and medical histories from 1969 through 1982. NIOSH's primary responsibility will be to quantify lung cancer mortality risk by lifetime level of exposure to radiation from radon daughters. NCI's primary responsibility will be to evaluate sputum cytology as a screening tool for lung cancer by evaluating the medical histories of uranium miners for whom adequate sputum series were recorded.

OBJECTIVES

1. (Slide 8) Quantify the risk of lung cancer mortality for cohort members through 1982 by exposure to all levels of radon daughters thereby establishing a dose-response curve. Special concentration will be focused on risks for cohort members with low (i.e., less than 120 WLM) lifetime exposures.
2. Analyze the effects of smoking, latency, race and age on lung cancer risk within the cohort.
3. Analyze the risks for causes of death other than lung cancer within the cohort through 1982.

MATERIALS AND METHODS

(Slide 9) The basic design of this research remains a prospective cohort mortality study since this research is a continuation of a project begun in 1950. A nested case-control design would not be appropriate because there are no unexposed miners to act as controls. In this proposed analysis, vital status will be updated from 1977 through 1982, and work, smoking and medical histories will be updated from 1969 through 1982.

Employment and some medical data, including smoking histories through 1969, are already coded on computer files. The updating of these data through 1982 will be a major component of this proposed study. The new data will be collected by questionnaires, administered by mail, by phone, and in-person if necessary. The questionnaires will be administered to all live members of the cohort and to the next-of-kin of all deceased cohort members who died after 1965. The questionnaires will be modifications of questionnaires previously developed and cleared by NIOSH and NCI for collection of data similar to the data needed in this proposed study; many of the questions about smoking and occupation are identical to those previously asked of this cohort. The questionnaires will be pretested to assure they provide valid and comparable responses and are likely to avoid interviewer or responder bias. All four questionnaires (alive and next-of-kin for both whites and Indians) will be field tested on up to 9 persons with ethnic and occupational backgrounds similar to the target cohort. After the field testing, modifications will be made as needed. A period of overlap from 1965-1969 in questionnaire coverage has been allotted to evaluate the current responses against previously acquired information. Differences in the information for the overlap period will be reconciled by examination of original raw data and by recontacting interviewees.

Realizing the potential problems in interviewing Indians, Indian interviewers will be trained and other expertise, such as researchers who have previously worked with the Indian population and tribal or reservation authorities, will be sought to help overcome these problems.

The questionnaire is designed to gather data in four areas. 1) smoking histories will be collected because this research is primarily aimed at analyzing lung cancer mortality, which is related to cigarette smoking. Smoking histories will also be considered in the analysis of nonmalignant respiratory disease and other diseases for which smoking is considered a risk factor. 2) occupational histories will be collected because the prime exposure to this

cohort thought to be contributory to the excess lung cancer mortality is radon daughters. In order to assess the individual exposures to radon daughters, mining, in particular uranium mining experience, must be documented. Other occupational exposures, especially those which might increase the risk of lung cancer, are also of interest. 3) medical histories of disease incidence will be collected for lung cancer in order to supply NCI with the data necessary for their sputum cytology analysis. Disease incidence data for other cancers and other diseases are also of interest to NIOSH for future analyses. These data might be especially important in assessing the true risk for diseases with lower case fatality ratios than lung cancer such as certain nonmalignant respiratory diseases and kidney diseases. A medical release authorization will be included so that NIOSH or NCI may pursue further detail on medical conditions discovered from the questionnaire. A special mailing will be required to acquire these signed authorizations for those interviewed by telephone. Also included in the medical histories is a request for a list of the sex and birthdates for each child; this is included to make possible further analyses of possible effects of radiation exposure to fathers on the sex ratio of their offspring. Waxweiler et al. (1981) have published some preliminary results of sex ratio analysis of these uranium miners. 4) A brief food frequency history will be collected in order to investigate the possibility that varying levels of intake of foods high in vitamin A or beta-carotene might affect the lung cancer mortality risk.

Exposures to radon daughters for uranium mining documented after 1969 will be added to the existing exposure records. These additions will be reviewed by an industrial hygienist. The industrial hygienist will collect WL data on individual mines and WLM data on individual miners from the available company, state, and Mining Safety & Health Administration (MSHA) records and reconcile possible differences in these records (e.g., company-MSHA measurement discrepancies). Where necessary, the industrial hygienist will calculate WLM's from the WL's and work histories in accordance with formulas used for previous calculations.

Vital status follow-up from 1977 through 1982 will be accomplished using the routine NIOSH methods such as the Social Security Administration, the National Death Index, and the Internal Revenue Service.

The cohort's mortality experience will be analyzed using the Modified Life Table Analysis System (LTAS) developed by NIOSH (Waxweiler et al.: 1983). Cause-specific SMR's will be calculated using U.S. general population mortality rates (both for whites and

nonwhites) or regional or state mortality rates (e.g., the four-state rates for New Mexico, Utah, Colorado and Arizona or New Mexico rates alone) where appropriate. Adjustments will be made for age, sex, and calendar time period. P-values will be calculated for the SMR's to quantify their significance. One-tailed p-values will be calculated when a priori risks are found. The statistical power (one-sided) to detect a relative risk for lung cancer of 1.5 among those thought to have received a lifetime exposure of 120 WLM or less is .47; for a relative risk of 2.0, the power is .89 (Beaumont and Breslow: 1981). These power calculations are based on person-year at risk projections through 1982 and the actual power will probably be somewhat less when all deaths through 1982 are recorded and when miners are found to have moved above the 120 WLM category due to additional mining exposures.

In this proposed study, the relatively rare situation exists in which individual exposures are known so that actual exposure subgroups rather than duration of employment subgroups can be used to look for dose-response relationships. The dosage feature of NIOSH's LTAS has been specifically designed for use in this situation. However, if the age distribution varies among the exposure subgroups and age is an effect modifier, it would be invalid to compare SMR's across exposure categories. Therefore a direct age standardization will be performed on all exposure categories using appropriate weights and standard population for comparison. The standard incidence density ratios (SIDR) would then be calculated for exposure comparisons across categories. As was described for SMRs, appropriate significance testing of the SIDRs will also be performed.

Another criterion used in evaluating whether an exposure is related to a disease risk is induction-latency. This is defined as the time period from initial exposure (first employment) until the disease manifests itself or, in the case of a mortality study, until death. For live cohort members, the observation period stops at the end of study date. This proposed research will extend the median observation period for members of the PHS cohort to about 27 years. The primary cause of death in this analysis is lung cancer which has been associated with induction-latency periods as little as 7-10 years and as long as 40 years.

General population mortality rates, both national and regional, contain both smoking and nonsmoking segments of the population. In this proposed study, miners will be classified into smoking categories in which mortality rates will not be comparable to general population rates. NIOSH will attempt to integrate the American Cancer Society mortality rates (Hammond and Seidman: 1980)

into the LTAS so that smoker and nonsmoker rates will be available, although their comparability to an occupational cohort such as uranium miners is questionable. NIOSH also intends to use the equations published by Axelson (1978) to correct the U.S. rates for smoking and thus provide an additional comparison. All rate adjustments for smoking categories are questionable and thus it seems advisable to have more than one comparison rate.

PROGRESS

Progress, through March, 1984, on this project includes the following: 1) The protocol has been cleared through NIOSH's Human Subjects Review Board and through formal peer review. 2) The package to obtain Office of Management and Budget clearance has been submitted. 3) The contract to perform the questionnaire follow-up has been awarded. 4) A draft of the questionnaires to be used has been assembled and reviewed by both NIOSH and NCI personnel. 5) A list of persons suitable for pretesting the questionnaires has been assembled. 6) Negotiations have begun to arrange for Navajo authorities to assist in the collection of data among the Navajo members of the cohort. 7) Vital status for cohort members has been pursued through the Social Security Administration, the Internal Revenue Service, the Health Care Financing Administration, and the National Death Index. Through these follow-up procedures we have discovered about 400 deaths among cohort members over and above the 950 deaths analyzed in the last analyses.

ANTICIPATED PROGRESS

It is planned that the questionnaire phase of this project will be completed by September of 1985 and that the final report will be completed by September of 1986.

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THE PHS URANIUM MINERS COHORT

Assembled July, 1950 to
December, 1963

3366 Whites

780 American Indians

Uranium mines (2500) on
the Colorado Plateau

LUNDIN ET AL., 1971

FOLLOW-UP FOR WHITE MINERS 1950-1977

CAUSE OF DEATH	OBS.	EXP.	SMR
RESPIRATORY CANCER	185	38.4	482
TUBERCULOSIS	14	3.4	409
ACCIDENTS	155	46.8	331
NONMALIGNANT RESPIRATORY DIS.	103	31.6	324
CHRONIC NEPHRITIS	8	3.1	262
ALL CAUSES	950	600.3	158

WAXWEILER ET AL., 1982

FOLLOW-UP FOR INDIAN MINERS 1950-1974

Cause of Death	Obs.	Exp.	SMR
Tuberculosis	8	3.7	216
Respiratory Cancer	11	2.6	423
All Accidents	39	31.7	123
All ~~Non~~ malignant Respiratory Dis.	7	5.4	130
All Causes	107	123.9	86

Archer et al., 1976

CIGARET-SMOKING HABITS OF THE AMERICAN INDIAN MINERS

Packs per day	Smoking at Time of Examination	Amount Smoked by Former Smokers	Unknown	Never Smoked
0.0	—	—		487
0.1	117	18		
0.2	50	3		
0.3	23	2		
0.4	3	3		
0.5	37	5		
0.6	1			
0.7	2			
0.8	1			
0.9	1			
1.0	20			
1.5	1			
2.0		1		
Unknown			4	
TOTALS	256	32	4	487

ARCHER ET AL., 1976

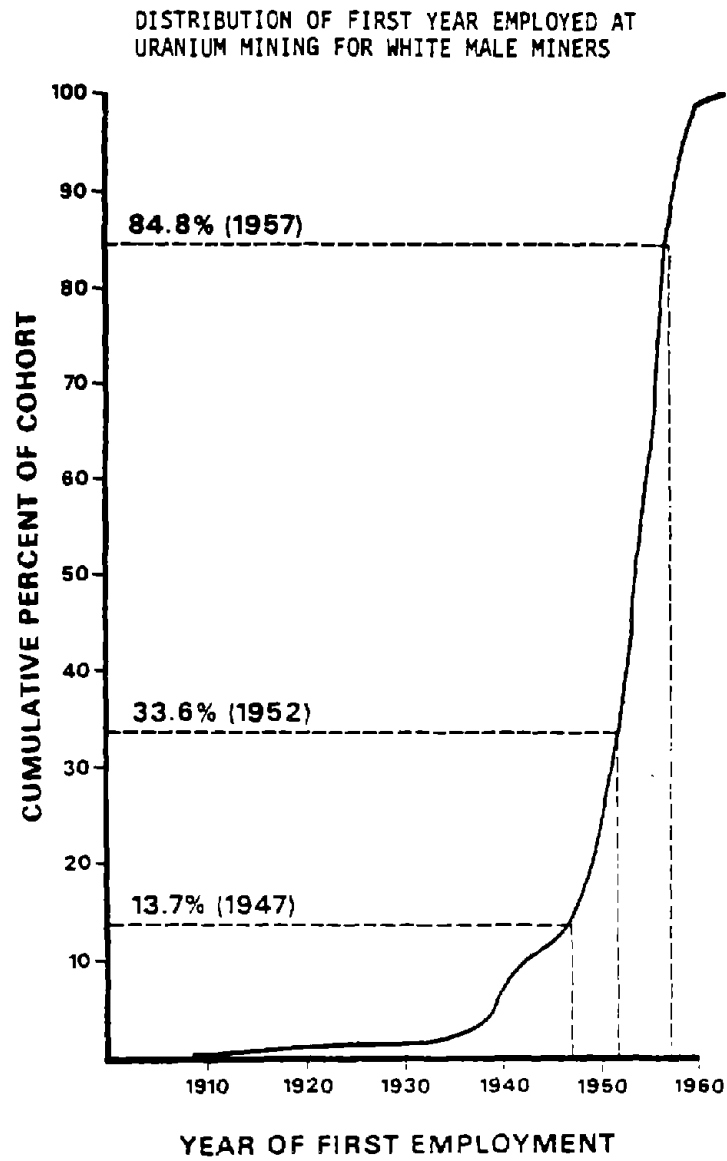
RADIATION EXPOSURES

One working level (WL) is any combination of radon daughters in one liter of air which results in the ultimate release of 1.3×10^5 MeV of potential alpha energy.

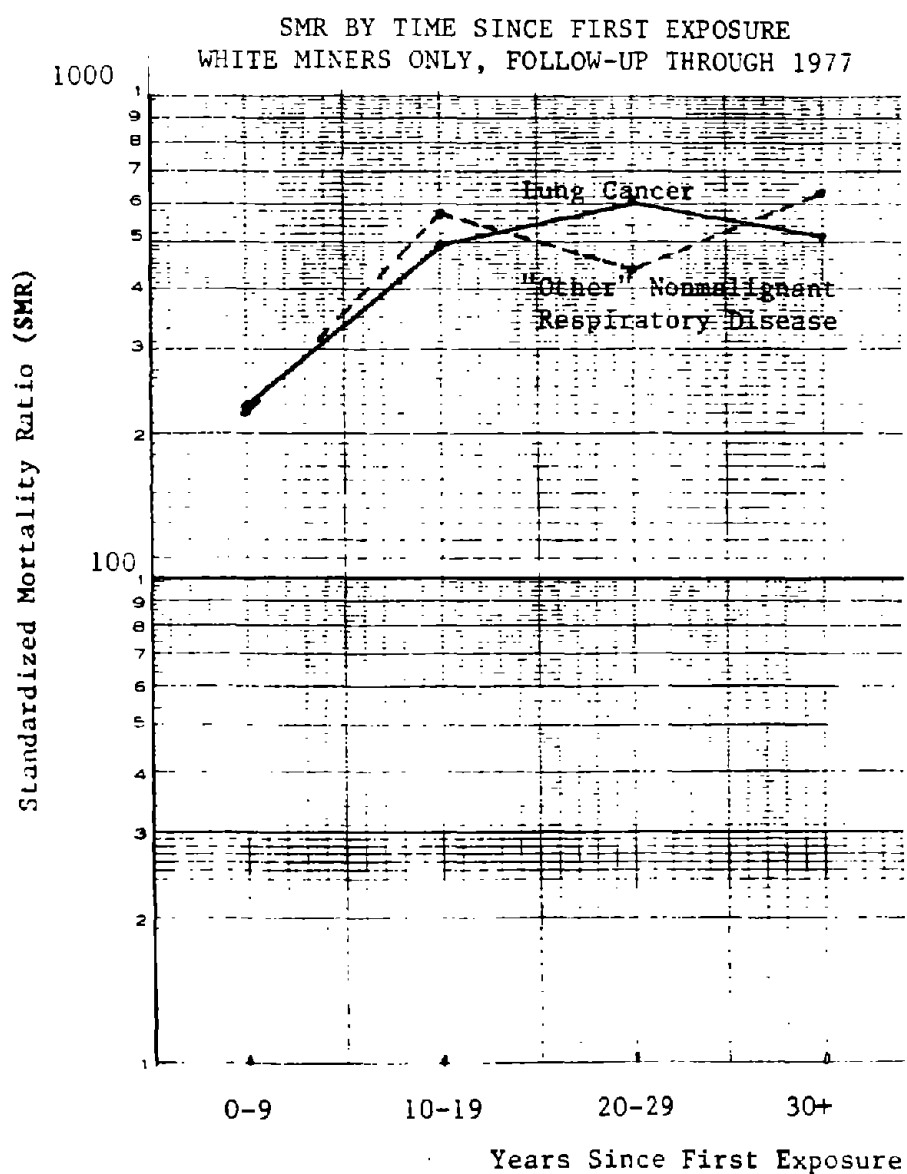
Working levels are annual averages for individual mines arrived at by measurements (43,000), extrapolation, interpolation, or estimation.

Working level months (WLM) are equal to the product of the working level and the duration underground. One working month equals 170 hours.

LUNDIN ET AL., 1971



WAXWEILER ET AL., 1982



DATA FROM WAXWEILER ET AL., 1982

STUDY OBJECTIVES

1. Quantify risk of respiratory cancer from exposure to radon daughters at all levels but concentrate especially on risks associated with lifetime exposures of 120 WLM or less.
($120\text{WLM} = 4\text{WLM} / \text{year} \times 30 \text{ year working lifetime}$)
2. Learn all that this cohort has to tell us about the relationship between exposure to radon daughters and respiratory cancer with respect to latency, dose, dose-rate, tumor cell type, age at exposure, synergism with cigarette smoking, race, etc.
3. Quantify risks and explore possible causes for other causes of death experienced by uranium miners.

METHODS

PROSPECTIVE COHORT MORTALITY STUDY

QUESTIONNAIRE SURVEY TO UPDATE:

SMOKING HISTORIES

OCCUPATIONAL HISTORIES

MEDICAL HISTORIES

FOOD INTAKE HISTORIES

VITAL STATUS FOLLOW-UP THROUGH 1982

MODIFIED LIFE TABLE ANALYSIS

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Development of a Predictive Model for Fiber Carcinogenicity

Dr. Philip Cook
Environmental Protection Agency

Development of a Predictive Model for Fiber Carcinogenicity

Dr. Philip Cook, EPA

I want to thank the National Cancer Institute for supporting this study, and also Bill Farland and Jim Rowe of OTS who have supported the project over the last few years.

This work involves the Duluth Laboratory of EPA, which is primarily an aquatic toxicology lab, but we have some expertise in this rather unique area of asbestos fibers. And working with the cooperative agreement with the University of Minnesota at Duluth, Dr. Jerry Neemy is the principal investigator.

Our work also is in cooperation with Dr. David Coffin of the Health Laboratory of EPA at Research Triangle Park. Dr. Coffin is responsible for the biological work, primarily carcinogenicity studies with rats. Our work involves primarily characterization of asbestos fibers and measurement of fiber doses that are used in fiber carcinogenicity studies. The basic direction of this study is to work towards that day when we can use the properties and characteristics of asbestos fibers to predict their relative carcinogenicity.

EPA has a real need for this type of capability, if it can be achieved. Not only do we want to assess relative health risks for past exposures to the classic asbestos mineral fibers, but there also is a large number of synthetic fibers which are used as asbestos substitutes and a large number of mineral fibers, which bear a great resemblance to asbestos, for which we have very little knowledge on carcinogenicity.

To perform rat tests with each one of these materials obviously would be very expensive and very time-consuming.

(Showing of slides)

A few basic principles involved in our studies in trying to develop our predictive method:

1. We assume that for asbestos fiber exposures a dose-response relationship exists. Dr. Wagner in England and Dr. Merle Stanton of the NCI have done two notable studies which demonstrate that for the common asbestos fibers, a rough dose-response relationship exists. The more fibers the animals receive, by intrapleural injection, the larger the percentage of pleural mesotheliomas observed. In many cases, unfortunately, studies that are reported in the literature involve large mass doses of asbestos, and therefore a plateau on the dose-response curve is reached and we lose the ability to define differences in carcinogenicity.

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2. We also are taking the approach that has in recent years been articulated by Dr. Pott in Germany. We believe that a workable hypothesis is that every fiber in an exposure, regardless of its size and shape, is potentially carcinogenic. Each fiber may have a small fraction of the actual carcinogenic potency of other fibers in the dose, but each fiber can contribute to the overall carcinogenic response. Dr. Pott has provided a model that illustrates this; that is, he has provided for greater carcinogenic potency for long, thin fibers, and lesser for the short and wider fibers.

(Change of slides)

We applied the same principles to all characteristics of fibers as we measured them. This slide provides a very rough outline of the steps we want to accomplish, a pretty ambitious approach. Regardless of whether we achieve all of these things, I think the effort will have been worthwhile.

First, we are attempting to obtain fiber samples with known carcinogenicity. We want to relate the physical and chemical characteristics of the samples to their carcinogenicities.

The second step would be to characterize key fiber properties and develop fiber dose-response relationships. We have decided that not only do we need to know very accurately the actual dose of fibers the animals have received in exposures, in terms of numbers of fibers and even numbers of fibers in different size and shape categories, but we also need to know what is going on inside the animal during the time following exposure and before the appearance of disease. Thus we have endeavored to determine the in vivo fate of fibers in rat lungs following intratracheal instillation.

Because animal exposures are time-consuming and expensive, we are also attempting to model the in vivo dissolution and alteration of fibers that we now observe in these rat lung studies through an in vitro acid-leaching test. We then hope to look for specific associations of fiber properties with observed differences in carcinogenicity through a case-by-case approach. I will attempt to give you some examples of progress in that area.

Ultimately we feel that factors such as respirability, durability, lung clearance, and alteration in the lung need to be modeled and incorporated into the overall approach to predict an effective fiber dose with respect to the time from exposure. Tissue residence time factors may be important in the overall consideration of the relative incidence of disease that is induced.

We are just beginning to use multivariate statistical analyses of our data in a large database that we have accumulated on fiber characteristics, and through this approach we hope to devise quantitative associations of fiber properties with their known carcinogenicity and do this for a large number of fiber samples.

In particular, we had proposed to use a large number of the Stanton set of samples. Dr. Stanton studied 72 different samples of fibers through the pleural introduction route. This is a unique set of samples in that we have a standard uniform biological effect end point for a large number of fiber samples. Unfortunately, we have just received these samples and hope in the very near future to do as much characterization of them as possible.

Finally, we would propose a fiber characterization scheme that would provide data useful for predicting the carcinogenic potential of each sample. Ultimately one could extrapolate from model predictions based on rat carcinogenicity to a human-risk assessment model.

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This is a list of the kinds of properties that we are measuring for each sample. The list starts off with the obvious and very important factors, such as size and shape. Thickness is a measurement which we have only recently started to look at. This may add an interesting new dimension. If the width of a fiber has great significance in terms of its carcinogenicity, there is no reason to exclude thickness, which would be the third dimension that we are considering.

Mass, surface area, number concentration of fibers, durability -- which I will speak a lot more about in a few minutes -- elemental composition, tensile strength, crystal structure, surface properties, and so on, must be considered as potential determinants of fiber carcinogenicity.

(Change of slides)

Some of the first work we did involved looking at three specific minerals: UICC amosite asbestos, compared in its carcinogenic response to a sample of ferroactinolite, which is another amphibole mineral although a non-asbestos fiber, and a sample of non-fibrous grunerite which was not only non-fibrous but occurred in very blocky form as a particulate.

The incidence of tumors for intratracheal exposures are shown here. To our surprise, the amosite asbestos induced lung cancers at a far lower rate than the ferroactinolite. We attempted to explain this on a number of different dose-response bases. For example, we considered the number of fibers for each of the samples which would meet the minimal definition of a 3-to-1 length-to-width ratio, the number of fibers meeting the "Stanton criteria" of length longer than 8 microns and width less than 0.25 microns and the number of fibers with aspect ratios greater than 10. None of these dose expressions seemed to explain the greater carcinogenicity of the ferroactinolite sample.

(Change of slides)

We have devised a procedure for quantitative analysis of fiber concentrations in rat lung samples. The entire rat lung is processed as one sample so that we can, on the electron microscope, do a quantitative determination of the concentration of fibers in the entire lung.

(Change of slides)

For the ferroactinolite sample, which would seem to have a remarkable carcinogenicity compared to amosite, we saw the following things when we looked at its preparations from the rat lungs in the electron microscope:

Six weeks after intratracheal instillation, the fibers appeared much like those at the time of exposure -- fairly blocky, less severe length-to-width ratios as compared to the amosite.

One year following intratracheal instillation, two remarkable things were observed: first, the average shapes of the fibers were longer, and thinner, in general; and the number of fibers at this time was a factor of 5 greater than the number of fibers which were originally introduced into the rat lung. This was strange because normally, one would expect to see about 10 percent of the number of fibers introduced into the lung due to a simple clearance mechanism.

When we looked at these fibers very closely, we could see that a dissolution effect was taking place. There were certain planes parallel to the fiber axis along which preferential dissolution was taking place. The result of that is that the fiber splits in the tissue to produce many more fibers and also fibers which are a great deal thinner than in the original sample.

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If you follow the concentrations in the rat lung over time from the original exposure, the amosite concentration decreases as one would expect from lung clearance. However, the ferroactinolite fibers actually increase in concentration as a result of the splitting reaction, even though lung clearance is going on.

(Change of slides)

If one looks at the concentrations of thin fibers, the difference between minerals is even more dramatic.

(Change of slides)

Thus the factors that we feel are important in determining the sizes, shapes, and numbers of fibers that will be retained in lung tissue include respirability, or those fibers that can get in there in the first place; lung clearance rates; dissolution of the fibers, which we have just observed; fiber splitting, comminution, which would include other kinds of fiber breakage; and translocation of fibers to other tissues.

(Change of slides)

If one looks at human lung, you can also find similar fibers which have been altered in vivo. This is an anthophyllite fiber in human lung tissue. The same type of dissolution appears to take place. One can see how individual smaller fibers could split off during this process.

(Change of slides)

To model this effect, we devised a leaching test. We are not attempting to model the exact reactions that take place during lung dissolution, but we want to model the effects that are observed in the fibers themselves. And we want to do this in a rapid way. So we use a strong acid. After stopping the acid at some desired reaction time with the introduction of base, we filter the samples so that we can prepare electron microscope grids.

(Change of slides)

This is a comparison of what we see in the rat lung situation for the ferroactinolite sample, where in the rat lung the number of fibers actually increased over time, to the same effect observed in the leaching test.

We have attempted to correlate the time scales. For this sample we let one year of a rat's life equal about one minute of acid leaching. We then observe these maxima in fiber concentrations in a rough agreement for the two sets of samples.

The reason that the acid-leaching test produces far greater maxima is that the fiber splitting which takes place is not being counteracted by lung clearance.

(Change of slides)

Some important observations come from this technique. This is an example of acid leaching of chrysotile fibers, an important human carcinogen. If one compares the concentrations in the test of fibers longer than 5 microns to short, thin fibers, less than 5 microns long and less than a tenth of a micron in diameter, there is an obvious difference in the way they behave in this acid environment. The long fibers, although they are in low concentration, are relatively durable and maintain essentially a constant concentration. The smaller fibers are rapidly diminished in number during leaching. We think we are seeing the same type of thing happening in lung exposure situations.

(Change of slides)

A cross section view in a very high resolution electron micrograph shows the fiber bundle structure of crocidolite. Essentially, it is made up of microfibrils which appear to come apart during the splitting process. This was a sample of a crocidolite fiber which was isolated from blood serum, so the dissolution process had actually already begun between the individual microfibril boundaries.

(Change of slides)

A comparison of fiber durability can be made using this acid-leaching test. Crocidolite asbestos is very durable and essentially retains the same concentration of fibers over the test. Chrysotile asbestos is far less durable, and toward the end of the acid-leaching time, essentially reaches a zero level. Fibrous glass has even lower durability. This may be an important factor in determining the relative risk of exposure to fibrous glass.

(Change of slides)

This is an attempt to summarize the rat lung fiber residence studies that I have described for a number of different minerals. The area of each symbol is proportional to the fourth root of the fiber concentration. Where you can notice a change in the area of a symbol, there is an actual change in concentration that would be 10,000 times greater than that, so we are looking at a large change. Fibrous glass goes from a concentration which would approximate 10,000 fibers per nanogram to a concentration which would be only slightly greater than 100 fibers per nanogram. And this is within one year's residence time in the rat lung.

(Change of slides)

For the same type of demonstration of results for the acid-leached samples, we have quite parallel effects. One interesting mineral, erionite, which we have studied, has been reported to induce a very high level of mesothelioma in villages in Turkey where the population is apparently exposed to that kind of fiber. Erionite, however, appears to have very low durability. At essentially one minute of acid-leaching time, it has disappeared.

(Change of slides)

We can express the sizes and shapes of the fibers that we are looking at on these samples this way. These are mean shapes and sizes. We now have introduced the thickness measurements, so we get a look at the third dimension. The number that you see on the slide represents the number of fibers per nanogram of the mineral or synthetic fiber sample. Obviously, where you have a very blocky type of structure, the number of fibers that you can get from a particular unit mass is far less.

(Change of slides)

The same type of representation for chrysotile and glass, which are more cylindrical in shape, shows that the glass fibers in one year of rat lung residence dropped from a concentration of 5,660 fibers per nanogram introduced to only 436 fibers per nanogram of originally introduced material.

(Change of slides)

Using a multivariate analysis technique, we are attempting to look for the most important properties so that we can simplify our attempts at developing fiber property-biological activity relationships. To do that we are using Principal Component analysis.

This is an example of our approach. In this case, PC-1, which would be Principal Component 1, accounts for 77 percent of the variability among the different properties which were considered in this analysis. PC-1 essentially equates to increasing size.

The other Principal Component which is plotted accounts for another 23 percent of the variability and is related primarily to increasing aspect ratio or shape.

As one would expect, the concentration of fibers per nanogram of material becomes larger as one goes to decreasing sizes, such as for thin chrysotile asbestos and fibrous glass fibers.

(Change of slides)

This is the same type of Principal Component analysis, but we have added the thickness factor that I just mentioned.

(Change of slides)

One of the problems that you have, in trying to really understand the characteristics of these fibers, involves limitations of the application of such measurements as the mean length and width. One wants to look at the whole distribution of particles in a very thorough way. I think we have discovered a new way to do that.

Essentially, here we have a three-dimensional plot with color coding of points adding a fourth dimension. We can plot any three variables in this way and look at the distribution of individual particles. In this case, we have plotted a ferroactinolite sample as purple, a fiberglass sample as light blue, crocidolite as red, chrysotile as yellow, and amosite asbestos as green.

I am not going to go into any particular profound findings from this, I just want to demonstrate the utility of the technique for finding new relationships. For example, fibrous glass appears to cluster in this region.

(Change of slides)

If one describes a different space -- in this case we are looking at the smaller range of particles with fibers having a length range of 0 to 2.5 microns, aspect ratios of 3 to 20, and thickness, on the Z-axis, of 0 to 0.15 micron. In this case, we are seeing the distribution of particles in space, and we are also seeing projections on two sides of the fiber space.

(Change of slides)

If one looks at this in space only, you can see the fibrous glass here.

(Change of slides)

Finally, if one changes the orientation with which you are looking at this distribution, you begin to see some very remarkable clusters of fibers. The ferroactinolite sample seems to be in this band. The amosite, crocidolite, fibrous glass, and chrysotile are in this area. I was just amazed that we could see this much differentiation between fiber samples.

Not only can we plot in this way different samples with different carcinogenicities, but we can look at samples such as the ferroactinolite over time of residence in the rat lung and observe the changes that take place.

(Change of slides)

This is the same type of plot, looking at it from the rear of the fiber space. I am very anxious to get back and do some more work with that particular procedure.

Thank you.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION VII - EPIDEMIOLOGY*
Chairman: Dr. Carl R. Morris*
Environmental Protection Agency

Questions and Discussion

*Substituting for Dr. William Farland

QUESTIONS AND DISCUSSION

DR. MORRIS: I wonder if our speakers could take the stand, so to speak, and we will have an opportunity, for perhaps another 10 or 15 minutes, to talk to them and raise questions and hopefully get them clarified.

Any questions from any of the panelists?

DR. KRAYBILL: Kraybill, NCL. I would like to ask Dr. Cook a question: so much of this work with the asbestos fibers or asbestiform particles has been recorded, as you well know, on inhalation studies directed towards the observation of mesotheliomas, etc.

In the past there have been many symposiums, including one I remember that the New York Academy of Sciences had, probably 8-10 years ago. But at that time the investigators from America, South Africa, and probably Australia and New Zealand, were not quite sure what the insult of asbestiform particles would mean in terms of gastrointestinal absorption, because indeed these particles pass across that gastrointestinal mucosa.

So, correct me if I am wrong -- as I recall, I think there was a study done, and I believe it was rodents and the hamster, which the so-called "million dollar experiment" referred to. And I believe that the study was more or less negative, which probably answered the question that indeed these particles do not migrate across the villi, microvilli, in the gastrointestinal mucosa to get into the blood stream, and, ergo, go to the liver, lung, etc. Would you clarify this situation, please?

DR. MORRIS: Well, he only has two minutes, Herman.

DR. COOK: And I thought that I was all through answering questions on the GI cancer problem associated with ingestion of asbestos.

My understanding is that the NIEHS feeding study that you refer to was essentially negative. But I don't think I would agree that it indicates that fibers don't pass through the GI mucosa.

I just wrote a review about a year ago of that particular problem, and there are some negative studies, but there are more positive studies. I personally feel that the positive studies were done in a more thorough, sensitive, analytical way, and the available evidence indicates that some fibers do pass through the GI mucosa. But we are talking about a very small fraction, perhaps 1 in 10,000 or 1 in 100,000.

Several years ago we made measurements of human urine from the Duluth area, where people were ingesting about 100 million amphibole fibers per liter of drinking water at that time. And we could see these rather unique fibers in the human urine as the result of that exposure.

DR. MORRIS: Thank you. Any other questions?

DR. BLAIR: Aaron Blair, the National Cancer Institute. I have a comment on Dr. Steenland's very fine summary of techniques that are used by occupational epidemiologists that deal with the problem that has plagued us and continues to plague us.

One of our thoughts about having Kyle present here today was the idea that it might be timely to have a conference dealing with a review, essentially, of methods for getting information on smoking and maybe other lifestyle confounders that typically are not available for occupational cohorts from record systems that are used to develop them.

All of the techniques described by Dr. Steenland are used, but what is really not known is what is the most efficient method? How can it be done most cheaply? And what gets you the information that is necessary to make the appropriate adjustments?

We may not need smoking information to the detail that you might need in other sorts of studies; we are essentially going to adjust for smoking and not try to investigate the relationship between smoking and a particular cancer or cause of death.

We wanted to generate some discussion here of the usefulness of such a conference and possibly expand it a bit to deal with practical issues of estimating exposures in retrospective cohort studies, also. There are many ways you can go about it, and to my knowledge there have been few studies that have compared the different approaches. I would be interested in hearing any thoughts or comments that other people may have.

DR. MORRIS: Would anyone else from the audience like to comment on that particular position? Yes?

DR. SHY: Carl Shy from the University of North Carolina. I would like to support Aaron's feelings about the need for methodological studies to evaluate different ways of assessing smoking habits.

I think we have some situations where smoking habits were determined on cohorts prospectively, and the possibility then of following up those cohorts to compare, say, smoking information obtained from the relatives, especially of those who have died, and of co-workers would be valuable, since many case control studies will obviously not let us get direct access to persons who had lung cancer and to assess their smoking habits directly.

But I think this kind of methodological study is really important, and perhaps there are data sets now available which, if followed up on, would allow us to make those comparisons.

DR. MORRIS: All right. Thank you. Anyone else like to comment on that issue?

DR. STEENLAND: I mentioned the uranium miners and Bob's work, and some of the few cohorts where there is smoking data available. It might be useful to try to compare next-of-kin response about smoking habits. As I mentioned, there is very little information about how good that data is, although it is routinely used by epidemiologists.

There are other studies besides the Rogot and Reid, but they are usually looking at 10 people or 15 people and comparing next of kin. So I would agree with you that more work needs to be done.

I think, also, when you have good data on the relationship between exposure and smoking, work needs to be done on what you do with it in terms of multivariant comparisons and how you can simultaneously adjust it.

The work by Whittemore on the radon daughters is maybe exemplary and shows how you can use multivariant modeling to learn about the interactions.

DR. MORRIS: Well, can we gather from the comments at least of the three individuals -- and anyone else is welcome to comment -- that that would be a reasonable recommendation that we might put forward certainly to the NCI for consideration and perhaps in another forum at some future time? Is that sort of the consensus that I'm hearing?

Perhaps for the record we might indicate that, and we certainly ought to consider it further in our planning, I believe.

Are there any other questions for the speakers during this session?

DR. MORRIS: Hearing none, I want to thank them all for their participation today and for each of you listening, and remind you that we have to return to the last session, which is to begin hopefully promptly at 3:15.

Thank you.

(Whereupon, at 2:55, the workshop was recessed.)

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

Friday Afternoon, March 23

SESSION VIII - EPIDEMIOLOGY
Chairman: Dr. Barry L. Johnson
National Institute for Occupational Safety and Health

SESSION VIII - EPIDEMIOLOGY
Chairman: Dr. Barry L. Johnson, NIOSH

DR. JOHNSON: Good afternoon. I would like to welcome you to the eighth and final session of this symposium. This is a session that is concerned with epidemiology. There are two papers on the program at this time.

I am going to change slightly the order of events, in the sense that we will receive questions after each paper, as opposed to waiting until the end when both papers have been presented. This is in the interest of perhaps acknowledging some tight travel schedules on the part of some members of the audience.

Our first paper is to be presented by Dr. Elizabeth Fontham. The title of it is "Cancer of the Lung, Stomach, and Pancreas in Southern Louisiana." Our presenter is from Louisiana State University.

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PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Cancer in Louisiana: Case-Control Studies of
Environmental Determinants in High Risk Parishes

Pelayo Correa, M.D.
Elizabeth T.H. Fontham, Dr.P.H.

Louisiana State University
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Final Report

**Cancer in Louisiana: Case-Control Studies of
Environmental Determinants in High Risk Parishes**

Pelayo Correa, M.D.
Linda W. Pickle, Ph.D.
Elizabeth T. H. Fontham, Dr.P.H.

March 22-23, 1984

Introduction

A recently completed case-control epidemiologic investigation of cancer of the lung, cancer of the stomach and cancer of the pancreas was undertaken in southern Louisiana in mid-1979. This area of Louisiana was targeted for study because significantly elevated mortality rates for these three cancers have been observed in one or more sex/race groups for several decades (1-2).

Death rates for lung cancer for 1968-78 were higher in Louisiana than in the U.S. for all sex-race categories except black females as shown in Table 1. These rates are higher in southern Louisiana than in the state as a whole. Stomach cancer death rates are higher in Louisiana compared to the U.S. average for blacks and slightly lower for whites. There is a clustering of high death rates, however, for all sex-race groups in south Louisiana. Stomach cancer rates for south Louisiana blacks are well above those of whites and well above the U.S. average. While the U.S. death rates for pancreatic cancer are highest for black males, in Louisiana we have an excess for whites compared to the national average and a deficit for blacks (3).

The Gulf Coast area of south Louisiana is distinctive as compared to the northern part of the state and other southern states in a number of ways which may have potential impact on the incidence and mortality rates for these cancers. The economy of the southern region of the state is dependent on diverse natural resources, agri-business, manufacturing, and seaport activities. The primary manufacturing industry in the south is petrochemical. The economy of northern Louisiana is heavily dependent on small farms. South Louisiana also differs from north Louisiana and other southern states in that a large proportion of the population comprises a cultural group known as Cajuns. Cajuns originally were the Acadians, French Catholic refugees exiled from Nova Scotia, who settled along the bayous of south Louisiana. Today the lifestyle of the descendents of these settlers is pervasive throughout the region and can be aptly described as "laissez bon temps roulé" or "let the good times roll."

It was the hypothesis of this study that diet, tobacco use, alcohol and coffee consumption, and occupation in certain high risk industries were responsible for the excess mortality in this region for cancers of the lung, stomach, and pancreas. The purpose of the study was to evaluate the environmental determinants of each of these cancers in south Louisiana.

Materials and Methods

Current primary lung cancer cases, stomach cancer cases and pancreas cancer cases were identified from admission and pathology records of all participating hospitals in twenty-six south Louisiana parishes. All major hospitals in the study area participated except some in the city of New Orleans where, for logistic reasons, interviewing was deliberately limited to two

large hospitals serving the medically uninsured population and two large private hospitals. For each subject a control was randomly selected from patients attending the same hospital and matched by race, sex, and age within 5 years. Patients whose main diagnosis was emphysema, chronic bronchitis, chronic obstructive pulmonary disease, or cancer of the larynx, oral cavity, esophagus or bladder were excluded from the control selection procedures for lung cancer cases. Similarly, patients whose primary diagnosis was ulcer of the stomach or duodenum were excluded from the stomach cancer control selection and patients with cirrhosis of the liver or cancer of the abdominal cavity, not otherwise specified, were excluded from the pancreas cancer control selection. The admission diagnoses of controls for each cancer type are shown in Table 2.

Personal interviews of the cases and controls, or their next-of-kin in the event they had died, were conducted by locally hired interviewers. Each interviewer was trained in interviewing techniques and in the use of the questionnaire. Sample questions from the interview were re-asked of 5% of the respondents by phone and a sample of questionnaires were blindly recoded.

Interviews for 1253 lung cancer cases or 76% of those ascertained for the study were completed and 1274 or 85% of the controls were completed. 391 stomach cancer cases (81%) and 390 stomach cancer controls (93%) were successfully interviewed. Interviews for 346 (81%) pancreas cancer cases and 354 (94%) of the pancreas controls ascertained for this study were completed. The proportion of next-of-kin respondents by cancer site is as follows: lung cancer cases, 23%, and controls, 9.5%; stomach cancer cases, 37%, and controls, 16%; and pancreas cancer cases, 51%, and controls, 19%. Respondents were questioned about occupation, residency, diet, smoking and drinking habits, health, water supply, and other related items including information on the smoking habits of spouse and parents.

Histologic confirmation was obtained for 97.8% of the lung cancer cases, 97.2% of the stomach cancer cases, and 84% of the pancreas cancer cases as shown in Table 3.

Results

Smoking

An increased risk of lung cancer with increasing amounts of cigarette use can be seen for all sex-race groups as shown in the next table (Table 4).

There were few non-smokers among the lung cancer case series: 97.7% of white male lung cancer cases were smokers; 97.6% of black males; 85% of white females; and 94% of black females.

Crude odds ratios for lung cancer shown in Table 5 indicate increasing risk of lung cancer for non-filter only versus filter only relative to non-smokers. Similarly, increasing risks are seen the younger the starting age; for quitters compared to non-quitters; and light versus deep inhalation.

In addition to the large impact of active smoking on lung cancer risk in Louisiana, an increased risk of lung cancer was found for non-smokers married to smokers (Table 6). Among non-smoking, ever-married men and women the relative risk of lung cancer rises with pack-year consumption of cigarettes by their spouses. No such increased risk of lung cancer associated with smoking of spouses was seen for active smokers except for light smoking males (<20 pack-years) married to heavy smokers (41+ pack-years). Likewise, no significantly increased risk of either stomach cancer or pancreas cancer was found among non-smokers married to smoking spouses.

An examination of the effect of parents' smoking on offspring was also made (Table 7). As expected, the smoking habits of the parents were found to have a strong influence on the smoking habits of their offspring. Heavy smokers were more likely to have had smoking parents and non-smokers were more than twice as likely to have had non-smoking parents. Further, the smoking histories of parents were associated with each other. When the smoking status of each parent is classified separately the relative risk of lung cancer for persons whose father smoked was not significantly increased, while for those with a positive maternal smoking history the relative risk was 1.66 ($p < 0.01$). Adjustment for active smoking, including age started, tar content of usual brand, degree of inhalation, use of hand-rolled cigarettes, years of smoking and maximum amount smoked, reduced the estimated relative risk associated with maternal smoking to 1.36 ($p < 0.05$). No increased risk for either stomach cancer or pancreas cancer was found associated with a positive maternal smoking history.

An approximate two-fold increased risk of pancreas cancer was found associated with ever having smoked cigarettes among white males and females ($p < .05$). No increased risk associated with active cigarette smoking was seen among black females and for black males the odds ratio of 2.6 did not differ significantly from unity (Table 8).

Coffee Consumption

Coffee was examined as a risk factor for pancreas cancer. Table 9 shows the age adjusted risk estimates for the four sex-race groups. No significantly elevated risks of pancreas cancer were found associated with coffee consumption. For white males and females and black males the odds ratios for 5 or more cups of coffee per day range from 1.32 to 1.68, but do not significantly differ from one. An estimated relative risk of 2.2 for current smokers is observed for both whites and blacks after adjustment for coffee consumption, sex and age; however, no significantly increased risk associated with coffee intake is seen after adjustment for smoking, age and sex (Table 10).

Table 11 displays the relative risk associated with coffee consumption for each of these cancer sites. Only for lung cancer

was a significantly increased risk seen, and the relative risk increases with increasing consumption. Adjustment was made for age (<50, 50-59, 60-69, 70+), income (<\$10,000, \$10,000+/year), sex, race, alcohol consumption (below median, median +), fruit and vegetable intake (below median, median +), cigarette use (never, 1-40 pk-yrs, 41+ pk-yrs), and Cajun ethnicity. The lung cancer risk related to coffee drinking persisted and no significantly increased risk was seen for either stomach or pancreas cancer. Because coffee consumption and cigarette smoking are strongly related in the data set, coffee consumption was examined separately for smokers and non-smokers (Table 12). For non-smokers the highest risk estimates are for the lowest level of intake (1-2 cups) and decrease with increasing dose. For cigarette smokers the increasing risk with higher consumption is seen.

Diet

A total of 57 food items were grouped by type and relative risk estimates were computed for each of the three data sets. A significantly decreased risk of lung cancer was found for above median consumption of total fruits and vegetables, dietary vitamin C and dietary beta-carotene after adjustment for age (<50, 50-59, 60-69, 70+), sex, race, income (<\$10,000/yr, \$10,000+), cigarette smoking (0, < 40 pk-yrs, 41+), Cajun ethnicity, respondent status and alcohol consumption (ever, never). See Table 13. Adjusting for these same variables, a significantly elevated risk of stomach cancer with above median consumption of pork meats and nitrite preserved meats was seen, 1.80 and 1.66 respectively. Significantly reduced risk of stomach cancer was found associated with above median consumption of vegetables, fruits, total fruits and vegetables combined and dietary vitamin C (Table 14). No protective dietary items were noted for pancreas cancer as seen in Table 15. After adjustment, significantly elevated risks of 1.64 and 1.56 were found for above median consumption of pork meats and all meats respectively.

Proximity to Industrial Plants

Respondents were asked whether the subjects had ever lived near an industrial plant. If so, they were asked the location, name and type of plant(s), the distance of the plant(s) from the home, and the number of years in which the subject lived near the plant(s). When type of plant was examined and "near" restricted to residence within two miles for five or more years, a significantly elevated relative risk of 3.58 (1.40-9.15) for lung cancer was found associated with lumber-related industries. The estimated of relative risk was little changed after adjustment for income, cigarette smoking, race, and sex, 3.48 (1.10-10.98). See Table 16. No association between residential proximity to industry was observed for stomach cancer. An elevated risk of pancreas cancer was found associated with living within two miles of an industrial plant or five or more years. When type of plants were examined, an elevated risk was noted only for residency near a petroleum refinery, 1.99 (1.01-3.94). After adjustment for income, cigarette smoking, race and sex the odds ratio was reduced to 1.77 with 1.0 included in the 95% confidence interval.

Discussion

The results presented have been broad by design to cover a range of variables for each of the three cancer types. On-going analysis in Louisiana and NCI includes an examination of the contribution of occupational exposures to these cancers, in particular lung cancer. Detailed dietary analyses, including beverage consumption, have also been undertaken for each of the cancer sites. Undoubtedly additional information will be available to enhance our understanding of these cancers and the risks relevant especially to the Louisiana population.

Cigarette smoking as a risk factor for lung cancer is overwhelming in the magnitude of risk and the proportion of the population exposed. Our data further suggest that passive exposure to cigarette smoke may have a detrimental effect on non-smokers married to heavy smokers and to smokers who were exposed many years earlier to maternal smoking (4).

The finding of increased risk of lung cancer associated with increased daily intake of coffee was unexpected. It seems unlikely that this relationship is causal. Because coffee and smoking are strongly associated in our data set, it may be that we failed to control adequately for smoking in our analysis. The finding of a positive dose response for smokers, but not non-smokers, suggests that this is the case.

Results from this study are, in general, consistent with other investigations of diet and lung cancer (5-7). A protective effect was found for above median consumption of fruits and vegetables, dietary vitamin C and dietary β -carotene.

Only close proximity to lumber mills and wood products industries carried an increased risk of lung cancer. Currently residential proximity to high risk industries is being analyzed using addresses given in the residential section of the questionnaire and a comparison of these results will be made.

The dietary findings related to stomach cancer are generally consistent with findings of other studies and the etiologic hypothesis postulating intragastric N-nitroso compound formation (8). Diets high in intake of pork and nitrite-containing meats and low in fruits, vegetables and dietary vitamin C carried high risk of stomach cancer.

The results of our examination of the association of coffee and cigarettes in pancreatic cancer are similar to those of Wynder et al (9) - that is, a positive association with cigarette smoking and no increased risk from coffee consumption. Above median consumption of pork and total meats increased risk of pancreas cancer approximately 60%. These two food groups may be indicators of a diet high in fat consumption which has been implicated as a risk factor for pancreas cancer (10-11). Occupational and industrial exposures and their association with pancreas cancer will be examined and may enhance our understanding of this disease.

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TABLE 1
Average annual age-adjusted mortality rates
per 100,000 population for selected sites
by sex and race in United States and Louisiana

		White Males	White Females	Black Males	Black Females
<u>LUNG CANCER</u>					
1930-32*					
	U.S.	3.2	1.8	1.4	0.8
	LA.	4.0	1.6	1.9	0.6
1968-78**					
	U.S.	61.2	13.4	68.7	13.4
	LA.	78.6	15.5	73.0	13.9
<u>STOMACH CANCER</u>					
1930-32					
	U.S.	24.9	17.9	17.6	15.4
	LA.	21.7	16.0	21.2	17.1
1968-78					
	U.S.	9.6	4.7	17.7	7.8
	LA.	8.1	4.0	20.6	9.7
<u>PANCREAS CANCER</u>					
1930-32					
	U.S.	2.9	2.5	1.6	1.0
	LA.	3.7	1.5	2.5	0.3
1968-78					
	U.S.	11.0	6.7	12.7	8.0
	LA.	12.6	7.5	12.3	6.7

* Adjusted to 1930 U.S. population

** Adjusted to 1970 U.S. population

TABLE 2

Admission Diagnoses of Controls
by Cancer Type

Diagnosis	Lung Cancer Controls %	Stomach Cancer Controls %	Pancreas Cancer Controls %
Circulatory System	21.7	26.4	21.8
Gastrointestinal	12.5	9.5	14.7
Musculoskeletal	6.0	6.1	2.8
Genitourinary	7.7	7.7	5.1
Nervous System & Sense Organs	2.6	2.5	1.7
Other Tumors	4.9	9.2	9.0
Endocrine Disorder	5.7	5.4	5.4
Accidents, Injuries	4.9	4.1	4.8
Pulmonary	2.1	8.5	7.1
Infections	19.1	10.3	15.8
Other	12.8	10.2	11.8

COMPLETION RATE :	Lung Cancer			Stomach Cancer		Pancreas Cancer	
	<u>Cases</u>	76.2%		81.4%		81.2%	
	<u>Controls</u>	84.9%		92.6%		93.6%	

TABLE 3

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Table 4
Odds ratios for lung cancer in Louisiana
by pack-years of cigarettes smoked

Pack-years	ODDS RATIO			
	WM	WF	BM	BF
0	1.0	1.0	1.0	1.0
1-20	3.6	1.5	4.0	13.3
21-40	9.5	7.7	14.6	19.5
41+	24.5	26.2	18.2	52.8
Total	17.2	8.6	13.1	22.0

Table 5
Odds ratios for lung cancer in Louisiana
by smoking characteristics

	O.R.
Non-Smokers	1.0
Filter*	8.4
Non-Filter*	15.2
Age Started*	
<16	24.2
16-20	17.4
21+	8.3
Current smoker	12.6
Quit	
3-5 years	7.7
6-20	7.0
20+	3.9
Inhale	
None	4.6
Mouth	6.3
Chest	11.3

* Adjusted for sex and age

TABLE 6

Non-smoking, ever-married lung cancer cases and controls according to lifetime consumption of cigarettes by their spouses.

Cigarettes smoked by spouse (pack-years)			
	None	1-40	≥ 41
<u>Males</u>			
Cases	6	2	0
Controls	154	20	6
Odds Ratio	1.0	2.0	
<u>Females</u>			
Cases	8	5	9
Controls	72	38	23
Odds Ratio	1.0	1.18	3.52*
		2.07	
<u>Both sexes</u>			
Odds Ratio	1.0	1.48	3.11*
(adjusted for sex)			

*p<0.05

TABLE 7

Lung cancer cases and controls (both sexes combined) according to paternal and maternal smoking history.

	Father smokes		Mother smokes	
	Yes	No	Yes	No
Lung Cancer	579	590	182	1054
Control	615	652	126	1214
Odds Ratio - Crude	1.04		1.66**	
Adjusted for active smoking (logistic regression-see text)	0.83		1.36*	

* p<0.05

** p<0.01

TABLE 8

Distribution of Cases and Controls According to
Cigarette Smoking Habits and Estimates of Risk Ratios
for Pancreas Cancer

TOBACCO USE	ADJUSTED RELATIVE RISK*			
	Male		Female	
	White	Black	White	Black
Never Smoked	1.0	1.0	1.0	1.0
Pipe/cigar	2.77 (0.86-8.92)	1.07 (0.14-8.20)	-	-
Ex-smoker	1.27 (0.62-2.6)	2.34 (0.72-7.42)	1.68 (0.65-4.3)	0.44 (0.10-1.99)
Current Cigarette				
< 1 pkg/day	1.66 (0.38-7.2)	5.17** (1.01-26.4)	1.37 (0.49-3.79)	0.78 (0.26-2.41)
≥ 1 pkg/day	2.71** (1.31-5.62)	2.62 (0.70-9.76)	2.66** (1.34-5.29)	2.74 (0.76-9.88)
Ever Smoked Cigarettes	1.94** (1.03-3.67)	2.60 (0.89-7.61)	2.17** (1.23-3.85)	1.03 (0.45-2.39)

*Adjusted over age in decades

**Significant at 0.05 level

TABLE 9
Distribution of Cases and Controls
by Coffee Drinking Habits and Estimates of Risk Ratios

COFFEE (CUPS/DAY)	ADJUSTED RELATIVE RISK*			
	Male		Female	
	White	Black	White	Black
0	1.0	1.0	1.0	1.0
1-2	0.86 (0.41-1.84)	0.73 (0.26-2.12)	0.87 (0.39-1.95)	0.62 (0.22-1.80)
3-4	0.79 (0.35-1.82)	2.35 (0.69-7.99)	1.24 (0.47-3.24)	0.54 (0.13-2.30)
5+	1.68 (0.74-3.78)	1.57 (0.50-4.88)	1.32 (0.53-3.29)	0.87 (0.15-5.11)
Total	1.02 (0.51-2.05)	1.13 (0.47-2.74)	1.04 (0.49-2.19)	0.59 (0.22-1.58)

*Adjusted over age in decades

TABLE 10
Estimates of Relative Risk of Cancer of the Pancreas
Associated with Use of Coffee and Cigarettes**

Use of Cigarettes	Relative Risk						Total [@]	
	WHITE			BLACK				
	Coffee (cups/day) 0	1-2	3+ Total [@]	Coffee (cups/day) 0	1-2	3+ Total [@]		
Never	1.0	0.71	1.72	1.0	0.19*	0.41	1.0	
Ex-smokers	0.81	1.25	1.74	1.38 (.76-2.51)	0.63	0.50	4.31*	1.01 (0.41-2.51)
Current smokers	3.27	2.21	2.27	2.22* (1.38-3.58)	-	0.98	1.21	2.28 (0.97-5.35)
Total [@]	1.0	0.74 (0.39-1.41)	1.21 (0.64-2.27)		1.0	0.36* (0.15-0.87)	0.56 (0.19-1.65)	

*Significant at 0.05 level

**The referent category is the group that uses neither coffee nor cigarettes. Estimates are adjusted for sex and age in decades

^aValues are adjusted for the other variable in addition to age and sex and are expressed in relation to the lowest category of each variable

TABLE 11

Estimated Relative Risk Associated with Coffee
Consumption for Cancer of the Lung, Stomach and Pancreas

DAILY COFFEE CONSUMPTION	LUNG CANCER		STOMACH CANCER		PANCREAS CANCER	
	Crude	Logistic*	Crude	Logistic*	Crude	Logistic*
Cups						
0	1.0	1.0	1.0	1.0	1.0	1.0
1-2	1.49 (1.14 - 1.94)	1.80 (1.25 - 2.59)	1.47 (0.99 - 2.17)	1.39 (0.86 - 2.24)	0.80 (0.52 - 1.24)	0.73 (0.44 - 1.19)
3-4	1.97 (1.49 - 2.63)	2.32 (1.58 - 3.41)	1.36 (0.86 - 2.17)	1.13 (0.64 - 2.01)	0.94 (0.58 - 1.54)	0.89 (0.51 - 1.56)
5+	3.01 (2.27 - 4.00)	2.48 (1.69 - 3.65)	1.67 (1.05 - 2.66)	1.51 (0.86 - 2.67)	1.36 (0.84 - 2.20)	1.17 (0.67 - 2.02)
Total	1.96 (1.53 - 2.51)	2.10 (1.49 - 2.96)	1.48 (1.03 - 2.14)	1.34 (0.86 - 2.06)	0.96 (0.65 - 1.43)	0.85 (0.55 - 1.32)

Test for trend $\chi^2_1 = 74.01$

*Logistic model includes confounders: age (<50, 50-59, 60-69, 70+), income (<\$10,000/yr, \$10,000+/yr), race (black, white), sex, alcohol (below median, median +), fruit and vegetable intake (below median, median +), cigarettes (never, 1-40 pkys, 41+ pkys).

TABLE 12
Estimated Relative Risk of Lung Cancer
Associated with Coffee Consumption by Smoking Status

DAILY CONSUMPTION	NONSMOKERS		CIGARETTE SMOKERS	
	Crude	Logistic*	Crude	Logistic**
Cups				
0	1.0	1.0	1.0	1.0
1-2	2.78 (0.94 - 8.22)	4.87 (1.09 - 21.70)	1.38 (1.03 - 1.86)	1.63 (1.11 - 2.39)
3-4	2.24 (0.69 - 7.32)	4.10 (0.85 - 19.84)	1.86 (1.36 - 2.56)	2.22 (1.48 - 3.34)
5+	2.02 (0.48 - 8.56)	2.74 (0.43 - 17.67)	2.32 (1.70 - 3.16)	2.41 (1.61 - 3.60)
Total	2.53 (1.34 - 7.28)	4.34 (1.00 - 18.77)	1.83 (1.36 - 2.47)	1.98 (1.38 - 2.83)

* Logistic model includes: age (<50, 50-59, 60-69, 70+), sex, race, SES (<\$10,000/yr, \$10,000+), alcohol (below median, median +), fruit & vegetables (below median, median +), Cajun (yes, no).

** Logistic model includes: age, sex, race, SES, Cajun, alcohol, fruit & vegetables, packyears (≤ 40, 40+).

TABLE 13
Estimated Relative Risk of Lung Cancer
Associated with Above Median Consumption of Dietary Items

	Crude	Adjusted [†]
Dairy Products	0.90	0.91
Pork Group	1.20*	1.10
Nitrite meats	1.16	1.08
All meats	1.09	0.93
Seafoods	1.01	0.93
Breads, etc.	0.99	1.09
Vegetables	0.80*	0.84
Brassica	0.84*	0.90
Fruits	0.75*	0.82
Fruits & Vegetables	0.67*	0.75*
Vit. C index	0.70*	0.76*
Vit. A index	0.79	0.84
β-carotene index	0.74*	0.81*

* p < .05

† Logistic model includes: age (<50, 50-59, 60-69, 70+), sex, race (B,W), SES (<\$10,000/yr, \$10,000+), cigarettes (0, ≤ 40 pkyr, 41+), Cajun, respondent status, alcohol (ever, never).

TABLE 14
 Estimated Relative Risk of Stomach Cancer
 Associated with Above Median Consumption of Dietary Items

	Crude	Adjusted [†]
Dairy Products	0.95	0.82
Pork Group	1.47*	1.80*
Nitrite meats	1.31	1.66*
All meats	1.09	1.27
Seafoods	1.08	1.20
Breads, etc.	1.29	1.11
Vegetables	0.75	0.72*
Brassica	0.99	1.00
Fruits	0.58*	0.58*
Fruits & Vegetables	0.52*	0.41*
Vit. C index	0.53*	0.54*
Vit. A index	0.80	0.78
β-carotene index	0.79	0.76

* p < .05

† Logistic model includes: age (<50, 50-59, 60-69, 70+), sex, race (B,W), SES (<\$10,000/yr, \$10,000+), cigarettes (0, ≤ 40 pkyr, 41+), Cajun, respondent status, alcohol (ever, never).

TABLE 15

Estimated Relative Risk of Pancreas Cancer
Associated with Above Median Consumption of Dietary Items

	Crude	Adjusted [†]
Dairy Products	0.87	1.00
Pork Group	1.48*	1.64*
Nitrite meats	1.35*	1.41
All meats	1.43*	1.56*
Seafoods	1.16	1.46
Breads, etc.	1.53*	1.18
Vegetables	1.24	1.25
Brassica	0.88	0.88
Fruits	0.87	0.83
Fruits & Vegetables	1.06	1.06
Vit. C index	0.87	0.86
Vit. A index	1.04	0.96
β-carotene index	0.89	0.87

* $p < .05$

† Logistic model includes: age (<50, 50-59, 60-69, 70+), sex, race (B,W), SES (<\$10,000/yr, \$10,000+), cigarettes (0, ≤ 40 pkyr, 41+), Cajun, respondent status, alcohol (ever, never).

TABLE 16

Relative risk associated with proximity
to industrial plants - residence within 2 miles
for 5 or more years (South La)

	<u>Crude</u> O.R.	<u>Adjusted</u> O.R. ⁴
Lung cancer		
Any plant	1.12 (0.92 - 1.36)	1.21 (0.97 - 1.52)
Lumber ¹	3.58*(1.40 - 9.15)	3.48*(1.10 - 10.98)
Petroleum refining ²	1.06 (0.75 - 1.49)	1.21 (0.96 - 1.53)
Chemical ³	1.09 (0.76 - 1.55)	1.01 (0.68 - 1.50)
Stomach cancer		
Any plant	0.98 (0.68 - 1.41)	0.97 (0.66 - 1.43)
Lumber	1.00 ----	-- ----
Petroleum refining	0.95 (0.47 - 1.90)	0.98 (0.48 - 2.01)
Chemical	1.25 (0.60 - 2.64)	1.43 (0.63 - 3.26)
Pancreas cancer		
Any plant	1.69*(1.14 - 2.51)	1.51 (1.00 - 2.30)
Lumber	0.62 (0.15 - 2.58)	-- ----
Petroleum refining	1.99*(1.01 - 3.94)	1.77 (0.85 - 3.71)
Chemical	1.70 (0.84 - 3.44)	1.62 (0.74 - 3.55)

¹ SIC: 2400-2499

² SIC: 2900-2999

³ SIC: 2800-2899

⁴ Logistic model includes SES-Low <\$10,000, high \$10,000+/year;
cigarette smoking - ever, never; race - white, non-white; and sex

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

PRESENTATION:

Etiologic Study of Respiratory Cancer
in Coastal Texas

Dr. Patricia Buffler
University of Texas
Houston, TX

Etiologic Study of Respiratory Cancer in Coastal Texas

Dr. Patricia Buffler, University of Texas

We have been involved in three studies in Texas on respiratory cancer, three of the broad areas of respiratory cancer: a study of lung cancer in white females, a study of lung cancer in white males, and a study of laryngeal cancer in white males.

These three investigations were embodied in one overall study, and that is what I am going to be describing today. I will give you an overview of the methods, but I thought what might be of interest to this group is to describe our results to date for the three separate investigations. The analyses are underway, and only those analyses which we feel fairly comfortable with will be discussed here.

Our study was similar in design to the study described by Dr. Fontham. Our study was a population-based study, a case-comparison interview type, of respiratory cancer conducted in the three years 1979 to 1982 in six Texas coastal counties.

(Showing of slides)

This is a very familiar slide. Our interest in lung cancer in the Texas Gulf Coast was initiated by looking at the availability of the cancer atlas provided by Tom Mason and McKay. As you see, it's the coastal area here, and the darkened areas are those counties that have significantly high mortality rates. And this is the lung cancer map for white males.

The map for white females, interestingly, is similar, in that the same Texas coastal counties, that were dark red for the white males -- that is, the top group, which are significantly high and in the highest decile -- were also noted for white females. So that the question with regard to an occupational component was particularly interesting because one would not expect to see that as strongly for the white females.

Let's move on.

(Change of slides)

This is a map of Texas which allows you to see six coastal counties -- Orange, Jefferson, Chambers, Harris, Galveston, and Brazoria. It is one of the areas in Texas where our population is concentrated. It is also an area where our industries are concentrated. We have the largest gasoline refinery in Galveston County, some of the largest chemical production facilities, and a significant concentration of metals industries.

We were particularly interested at the time we initiated the case-control study of lung cancer in looking at what the rates for the periods subsequent to when the map was produced looked like for the coastal counties.

We examined 10 cancer sites for the 9-year interval, 1970 to 1978, and with the age adjusted over to the estimated total Texas population, for that interval, 1974, and separated the Texas counties into the six coastal counties of the study area and the 248 remaining Texas counties.

Here what is shown, the top solid line, is the lung cancer mortality rate for white males in the six-county coastal area. The dotted line is for white males in the 248 counties remaining in Texas. This line is for white females in the six coastal counties and the bottom line is white females in the remaining 248 Texas counties.

The overall age-, race-, and sex-adjusted mortality rates for lung cancer in the six-county coastal area were 48 per 100,000 compared to 37 for the rest of the remaining counties in Texas. So for lung cancer we found a very significant difference. Lung cancer accounted for 40 percent of the overall excess in cancer when we compared the coastal counties with the rest of Texas.

For the study itself, cases were ascertained from among the 67 hospitals located in the six-county study area. Fifty-six of those hospitals participated in the study, and I am going to give you the numbers of case and controls in the three separate studies.

For white males, we were able to ascertain 96 percent of the cases estimated to occur during the 4-year interval in the five counties surrounding Harris County. We did not include Harris County in the study for white males.

The incident cases were estimated on the basis of age, race, and sex mortality rates, the population increase from 1970, and an incidence-to-mortality ratio of 1.35-to-1. So this provided us with an estimate of all incident cases in this 5-county area in white male residents, and the number of ascertained cases was 96 percent of that number.

Of that total, we interviewed 476 cases who were subsequently eligible for the study, eligible by virtue of histologic confirmation of their disease. In our study, 7 percent of the ascertained cases did not have histologic confirmation. Subsequently, of the interviewed cases, some were excluded from the analysis because of poor data quality.

We also interviewed 466 comparison subjects. Our comparison subjects were selected from the general population. For the living cases, we used the records of the Department of Motor Vehicles for persons under 65, and the HCFA files for persons over 65.

For decedent cases -- that is, cases who were deceased at the time of ascertainment -- we used the state mortality files, selecting individuals who had not died of any number of excludable conditions. These were individuals who had not died of cancer or chronic respiratory diseases.

For white females, we ascertained 88.7 percent of the estimated number of incident cases for a 4-year period in the five counties surrounding Harris County, and for a three-year period from mid-1977 to mid-1980 for Harris County.

For the male laryngeal cancer study, let me give you the number for females. The ascertained cases were screened for eligibility. We then attempted to locate them. After all exclusions, we analyzed 460 cases and 482 comparison subjects. We interviewed a larger number, but excluded some cases based on poor data quality.

For white males, we were able to ascertain approximately 92 percent of the estimated incident cases for a longer time interval, a 5-year time interval, mid-1975 up through mid-1980.

The resulting study population, after all exclusions, was 209 cases, and 250 comparison subjects, making the laryngeal cancer study one of the largest case comparison studies for laryngeal cancer, and making the study of lung cancer in white females one of the largest case-comparison studies.

Now, let me run through some of the significant findings for the laryngeal cancer study.

In terms of data analysis, a number of activities are in progress. One analysis has been completed, by a graduate student working with me at the University of Texas School of Public Health. Dr. Mason served on her committee.

Her particular interest in analyzing these data was to look at the potential contribution of exposure to asbestos to laryngeal cancer. To proceed with this, she systematically reviewed all of the DOT and SIC codes. The detailed occupational histories were obtained as part of the study, because the question regarding the contribution of occupational factors to risk was a prominent one.

These detailed occupational histories were coded to the occupation and to the industry; we used the SIC codes for industry and the Dictionary of Occupational Titles for the occupations.

The detailed listings were reviewed a priori, and all occupational titles and industrial codes or industrial categories where there was potential for asbestos exposure were identified. This became the high-risk group, or the high exposure group, in contrast to the others, which were considered not exposed.

In the analyses, smoking by itself was found to be a significant risk factor for laryngeal cancer, with an odds ratio of 4.6. Alcohol by itself was found to be a significant risk factor, with an odds ratio of 2.2.

When the potential for asbestos exposure, based on having been employed in one of the high-risk groups, was looked at and adjusted for smoking and alcohol, the odds ratio was 1.2 -- elevated, but not significant. The elevated odds ratio was more marked before the smoking and alcohol adjustments were made.

In the detailed review of the occupation and industry codes that were part of the exposed groups, two groupings were noted as being significant. They were people who worked in processing occupations within the chemical

industry and people who worked in construction within the construction industry. The odds ratio for the first group was 9.5, and the odds ratio for the second group was 4.7.

Additional analyses, looking at all of the occupational information, are underway. We have two major efforts in progress, one headed by Dr. Mason and Dr. Morris at the NCI, and the continuing work that we are doing at the University of Texas.

With regard to the white females, as I mentioned, they were white female all-incident cases for the designated time intervals in the six coastal counties. These were Harris County and the five surrounding, somewhat less urban counties, where one or two industries -- the chemical, petroleum and petrochemical industries -- are dominant.

Among the female cases, 91 percent were smokers, compared to 59 percent of the controls. This was the percentage who "ever-smoked." Current smokers comprised 68 percent of the cases, compared to 38 percent of the controls.

Let me move in sequence now.

(Change of slides)

This shows the estimated number of cases, and those ascertained and interviewed for the white males and females in the lung cancer studies.

(Change of slides)

This is the smoking information. I am not going to go through all of the detailed smoking information in terms of odds ratios for current smokers, ex-smokers, those who use filters, etc.

Our experience for men and women was similar in terms of the effect of smoking. Here, what we have done is examine the odds ratios associated with smoking for men and women by type of cancer, splitting the lung cancers into the adenocarcinomas and the non-adenocarcinomas, a grouping of the squamous small-cell and other lung carcinomas.

Here you see this first broken line, which is non-adenocarcinoma in males, and this line here, which is the odds ratio. This is 1. This is elevated. These are all significantly elevated above 1.

This is the odds ratio for the low-smoking category, based on pack-year: 6.6, 11.3, and going up to 15.4. Similarly, for non-adenocarcinomas in women, the ratios are 4.2, 11, and on up to 19. For the adenocarcinomas, the solid line is the male group, with ratios of 3.8, 4.5, and 5.4. The dotted line is females, where similar patterns exist when seen by cell type.

The cell-type information is particularly interesting. Adenocarcinoma was the predominant cell type in both young men and young women. And a strength of the analysis with regard to cell type is the study design itself. This was a study based on all newly-diagnosed, histologically confirmed cases within a defined population group. So we were impressed that, contrary to some popular

notions but not contrary to published case theories, adenocarcinoma does occur almost as frequently among women, in terms of the relative distribution, as in men. It is the predominant cell type in young men as well as young women.

Among older women, the epidermoid squamous was the predominant type among women over 60. There is a slight shift there. There was a clear association with age with cell type, and a clear association with smoking status with cell type. Among nonsmokers, adenocarcinoma was the predominant cell type.

(Change of slides)

The analyses? We are in a holding pattern, because the analyses for white females to date, in terms of any preliminary findings, have been limited to the white females in Harris County. This work has been done by a doctoral student at the University of Texas School of Public Health.

The Harris County series consists of 259 cases and 278 controls. For this group it was important, in terms of pursuing the occupational question, to identify occupations and industrial settings which may present a potential for exposure to respiratory carcinogens. All job titles and all SIC categories were reviewed. Based on the literature, which is predominantly based on studies of males exposed to respiratory carcinogens, a series of DOT and SIC codes were identified as possibly high risk, and the analysis was conducted using those exposed categories.

"Exposed" means being employed in either a high-risk occupation or a high-risk industry, as designated a priori. This was important, because slightly in excess of 60 percent of the female cases and comparison subjects indicated that their usual occupation was being a housewife. The occupational information, even though the case series was quite large, was somewhat limited. So it was not possible to productively examine the specific occupational groupings or industrial groupings.

For the high-risk group thus designated, those who were employed once vs. more than once and adjusted for smoking in terms of pack-years, the odds ratio was 2.4, a significant elevation.

When high-risk industry was looked at -- that is, having been employed once vs. more than once in a high-risk industry -- the odds ratio was again elevated, and this was adjusted for smoking in the same way.

Additional findings of particular interest are still being refined. There may be a possible confounding if the male spouses in the female cases and comparison subjects worked in selected occupations. Our initial analyses show that there is a bystander effect for occupations that may present a potential for exposure to dust and asbestos, but this does need to be controlled for the employment status of the cases and the comparison subjects.

Now, moving along to the males. We have the white males for lung cancer. We have 476 cases and 466 comparison subjects, 86 of whom were decedent cases. The comparison subjects, which were frequency-matched to the cases, were matched also in vital status.

(Change of slides)

Here is the information with regard to the usual occupation or employment. Using the clerical sales occupation as the referent group, there were five categories that showed a significant excess. Those are noted.

When we looked at these same occupational data for "ever employed," the only group that was significantly different was the clerical sales group, the referent group.

I am going to move quickly on to the industry information, because I think most of the important information is there.

Before that, I want to point out that the slide I passed up earlier spoke specifically about the model that we used for our analysis. It was a categorical multiple regression analysis that controls simultaneously for smoking, age, ethnicity, education, and vital status. Smoking was defined in terms of duration of time, which we found to be the most explanatory variable, in preference to a smoking index where intensity, age, and start are incorporated. The duration-of-time worked the best.

The other factors were age, ethnicity (or Hispanic vs. non-Hispanic), number of years of education, and vital status.

(Change of slides)

This is when we looked at the data with regard to the usual industry of employment. Here we noted four industrial categories that were associated with an increased odds ratio: construction, chemical, metals, and transportation. The most marked is that for metals, with an odds ratio of 6.57. However, if you will note the numbers in parentheses, there are only 24 cases and controls in the metals group. This is based on "usual industry."

This is the industrial setting, a review of the cases, their detailed work histories, where they spent most of their time. We then looked at these data with regard to "ever employed" in a particular industry. Here it was an "ever-never" comparison. There is the reference group who are the "never employed," in contrast to the previous analysis. Here, the increased odds ratio for chemicals and metals are seen again, but not those that were observed previously for construction and transportation.

In addition, you note the significant decreased odds ratio for sales-clerical. That was the referent group in the previous table.

(Change of slides)

We then looked at these results with regard to a time effect, duration of time in the specific industries, duration of time employed. And what is plotted here is the estimated increase in the odds ratio per 10 work-years. There are 10-year time intervals, but it is actually plotted from the baseline.

This is the increase that would be estimated based on the analytic model that I have outlined. The top line for metals is significant; it represents a 56 percent increase per 10 work-years plotted upwards. The information with regard to work-years becomes fairly scant after 30 to 40 years. So I really want to caution you with regard to interpretation outward there. The second

line is that for construction, with 15.5 percent per 10 work-years, also a significant increase. The line immediately under that is for employment in the chemicals industry, which is associated with a 15 percent increase.

(Change of slides)

We then looked at these data for the different cell types. Here is the information for the adenocarcinoma series, in which 94 cases remain of the original 104 adenocarcinoma cases in our total case series.

The top line is that for employment in the metals industry, and it represents an increase in the risk or odds ratio of 85 percent per 10 work-years. The next line is that for construction, and it represents an increase of 26 percent per 10 work-years, followed by transportation and chemicals.

The increase for metals was not as striking when the association was examined in the non-adenocarcinoma series.

Here, the series is larger, with 283 cases. The top line, for metals, represents a 35 percent increase, followed by chemicals with 18.6, and then construction and transportation.

None of these increases were statistically significant. In the previous table, the increase is noted for those two categories, metals and construction, where it is statistically significant.

Thank you. I would welcome your comments.

PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

SESSION VIII - EPIDEMIOLOGY
Chairman: Dr. Barry L. Johnson
National Institute for Occupational Safety and Health

Questions and Discussion

QUESTIONS AND DISCUSSION

DR. BLAIR: Aaron Blair, the National Cancer Institute. The interesting finding that shows up in a lot of places is the very strong inverse relationship with year first started smoking. Have you been able to look at that? The question arises because they start earlier or they have a greater number of pack-years. You could look at that if you have enough ex-smokers. Have you done so?

DR. FONTHAM: We did adjust for age and pack-year, and the relationship does not persist.

DR. SPIRTAS: Bob Spirtas, NCI. I missed part of it, I think. Is there an occupational history component to this?

DR. FONTHAM: There is, but the analysis isn't here.

DR. SPIRTAS: All right; because I think it would be particularly interesting with the petrochemical industry and the sugar cane, too.

DR. FONTHAM: That's right. I can tell you that a crude analysis does not indicate any striking effects, but we are looking into it in greater detail.

DR. SPIRTAS: Would you care to tell us a little bit about any unusual characteristics of the coffee, the tobaccos, or the foodstuffs consumed in the area?

DR. FONTHAM: Well, the foodstuffs are probably legendary, in terms of spices and lots of seafood, although we didn't find case control differences in seafood for any of these three cancer sites.

The coffee is also different -- the way it is prepared and the roast. Of those who drink pure coffee, almost everyone drinks what is known as "dark roast." It has been roasted a lot longer. We did have "roast" on a similar questionnaire in a study of stomach cancer precursor lesions, and almost all coffee drinkers reported drinking dark roast.

Lots of people don't drink pure coffee; they drink coffee with chicory as an additive. We don't see a chicory effect. We didn't look at it for lung cancer because it wasn't particularly of interest, but we did not see an association for either stomach or pancreas cancer.

There was a relatively large group of people who used hand-rolled cigarettes. Eighty-seven of the study subjects hand-rolled cigarettes only. Much of this was commercially-available tobacco. Some of that group used home-grown tobacco.

At one time in Louisiana there was a small area where a very strong type of tobacco was grown, but it was not very prevalent and not very many people were exposed to it in our case-control study.

DR. BUFFLER: Pat Buffler from the University of Texas. With regard to the significant associations that you noted, lung cancer residing in close proximity to lumber yards, and for pancreas, residing in close proximity to petroleum refineries, did you control in those analyses for the occupations of the cases and controls, in that we do observe that people certainly want to live as close as possible to where they work?

DR. FONTHAM: Right. Obviously, that is something that needs to be done. This analysis we presented today was based on the question: Have you ever lived near an industrial plant? I don't know if that was on your questionnaire or not, but . . .

DR. BUFFLER: We had a similar question.

DR. FONTHAM: We also had the residential history. And what we are doing now is looking at residential proximity to industries, using the exact addresses on the residential history, and occupation is being controlled for in that analysis.

DR. CANTOR: Ken Cantor from NCI. A question for both speakers. These studies were originally targeted in this region because the maps were read, and I was wondering if, from the results of the study, you can now comment on why there are high levels of lung cancer and laryngeal cancer in your case, and the three sites in the Louisiana case?

DR. BUFFLER: I think any comments would be rather preliminary. As you can see, we tried to give you sort of an overview, both of us.

DR. CANTOR: Well, maybe some specific comments. Are there unusually high levels of cigarette smoking, for example?

DR. BUFFLER: The proportion of females, cases and comparison subjects, who report ever-smoked, or current-smokers, are higher than the information that is published.

The proportion of our male controls looks slightly higher. But, if you have worked with smoking histories, you know that there is so much more to a smoking history than "did you ever smoke?" or "are you a current smoker?" in terms of the intensity and the age they started, with regard to a risk in an individual, and then the subsequent expression of disease.

We are concerned, as you have noted, in terms of both reports -- more so in the analyses presented by the Louisiana investigators -- with paying very careful attention to the contribution of these confounding factors, because the differences that we are seeing with regard to occupational or industrial effects in most instances are fairly low or moderate relative to the overwhelming contribution of smoking to laryngeal and lung.

What I presented with regard to the white males in lung cancer does take into account most of the confounders that we are able to address. We are not able to address nutrition in our series, since 86 percent of our cases were decedent cases. We have information on dietary history for only a very small proportion of those cases.

I haven't answered your question directly, but maybe Terry would like to, or Linda.

DR. CANTOR: Do you want to comment on the cigarette smoking question in Louisiana?

DR. FONTHAM: We have one measure. We did a much smaller study using the same questionnaire in North Louisiana, which has lower rates of lung cancer, at the same time we did it in South Louisiana. And the complete analysis of the comparison of those two areas is not finished, so I can't give you the bottom line. But prior to doing either one of these studies we did a survey of lifestyle -- not a case control study but selecting a sample of individuals who live in high-risk parishes in the South, and a sample who live in low-risk areas in North Louisiana. And we did find lots of differences -- we found dietary differences, occupational differences, and we certainly found smoking differences as well, including differences in the proportion of these individuals who reported their parents having smoked. In other words, smoking has been going on in South Louisiana in a larger proportion of the population than in North Louisiana, and it is not just this generation, but goes back at least one.

I don't know if that answers your question, but there are smoking differences.

DR. BUFFLER: In terms of -- if I could comment further -- occupational differences, I know I went through some of the occupation and industry slides pretty fast, but if you looked at the numbers in parentheses next to the occupational, particularly the industrial categories, one of the things that is very striking is one that we did anticipate, which is why the study was done in those particular counties. This is the fact that a very high proportion of our cases and controls were employed at one point in shipbuilding and repair; a very high proportion of our cases and controls were employed in the petroleum industry, the chemical industry, and a much smaller proportion in metals. But we knew that we had a fairly large complex of small metals industries in the Houston area.

What I didn't point out was that we did not find an association in terms of the increased risk in lung cancer with having been employed in shipbuilding and repair, even though we have a very large number of study subjects that report that they were employed at one point. The risk ratio initially was 1.5, and that was when we only controlled for ever-never smoking. When we controlled for duration of time smoked, which in the view of our data we considered to be the tightest smoking control we could employ, age, ethnicity and the other factors listed, the odds ratio dropped down to 1.08. That was somewhat of a surprise, but we were also surprised to see very high odds ratios for metals.

This is being pursued in the laryngeal series, and it will certainly be pursued in our series much more carefully, so we have a better understanding of what exposures may be involved.

DR. JOHNSON: This has been a very informative session, I believe. Among other things, we have learned a great deal about the geography of Texas.

It has been an excellent session, and I thank the two speakers who presented.

I believe Dr. Cameron from NCI is going to have summary wrap-up comments. Tom?

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PROCEEDINGS OF THE
THIRD NCI/EPA/NIOSH COLLABORATIVE WORKSHOP:
PROGRESS ON JOINT ENVIRONMENTAL AND
OCCUPATIONAL CANCER STUDIES

WORKSHOP SUMMARY

Dr. Thomas P. Cameron
National Cancer Institute

WORKSHOP SUMMARY

Dr. Thomas P. Cameron, NCI

I think it's apparent that we really don't need a wrap-up. As a comment on Dr. Buffler's Friday afternoon comment, it can be worse than that. About 13 years ago in Las Vegas at a large meeting, I walked into the 8:30 a.m. meeting, and the speaker was addressing an audience of two -- one was the session chairman.

He later told me that he was there because he hadn't gotten to bed at all.

I think we are talked out, and we are sat-out. I really appreciate it that you have stayed. I think it has been a very enjoyable two days.

Two comments: We will be working on that confounding-factor (tobacco) workshop. I think we will create something between NCI and NIOSH and take it to our Board of Scientific counselors and suggest that we hold a workshop.

Other than that, if anyone here has any suggestions on projects that have not yet been covered by the interagency agreements, and you think they would fit into their mandate, please let us know.

Good night, and thank you very much.

(Whereupon, at 4:20 p.m., the workshop was concluded.)

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POSTER/DISPLAY PROJECTS

SURVEY OF COMPOUNDS WHICH HAVE BEEN TESTED
FOR CARCINOGENIC ACTIVITY

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The Survey of Compounds Which Have Been Tested for Carcinogenic Activity (USPHS Publication No. 149) is a unique data publication on the tests of chemical substances in experimental animals for carcinogenic activity. The data are compiled from scientific literature published since 1939; it is the largest survey of chemicals that have been tested for carcinogenicity. It is not a critical review and assessment of the carcinogenicity of the tested substances.

When the 1979-80 volume is complete, the database will consist of 11 volumes of material. It will contain data on approximately 6700 chemical substances for which long-term bioassay tests have been conducted. Over 8500 literature references are included, selected from the screening of over 440,000 articles included in over 47,000 journal issues.

The data are extracted in units of data lines reflecting the test protocol for each bioassay experiment. Each data unit incorporates: literature reference; animal species and strain information; methods of preparation and dose level of the chemical substance(s) administered; the route and site of administration; level of pathology examination; total number of animals with tumors for each experimental group; the number and histopathological description of the tumors; survival data; duration of experiment; and information on control groups.

To facilitate users' access to the database, a number of indexes are provided; literature reference; author cross-reference; chemical name; Chemical Abstracts Services (CAS) registry number; route of administration; site of application; animal species/strain; and tumor site and vehicle. A machine-readable version of the index material is also being prepared.

The database has continued to evolve in response to the needs of the scientific community and regulatory authorities. The data format has been slightly modified, and additional details on each experiment have been added in the latest three volumes. Scientists in carcinogenesis research throughout the world have continued to express their support to NCI for the PHS-149 publication because it provides ready access to the world's literature on the subject for its structured and detailed presentation of data as well as providing an overview of research activities in the field of chemical carcinogenesis. Regulatory authorities have also found it an important information resource in the development of regulations and in responding to hazard assessment issues associated with chemical waste and spills problems.

This database has also been employed as a source of information input for other efforts related to chemical-biological structure activity studies. Portions of the data have been included as a part of other databases that are utilized in such studies.

This research is supported by the National Cancer Institute.

DEVELOPMENT OF A NATIONAL DATA BASE ON BODY BURDEN
OF TOXIC CHEMICALS INCLUDING CARCINOGENS

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Background

In 1975, it was suggested by the ICGEC and staff at the EPA that a more relevant exposure assessment to toxic agents and environmental carcinogens could be achieved by the acquisition of data on human body burden (organ, tissue, and body fluid levels) which reflect exposures to food, air, and water contaminants, as well as pharmaceuticals. In addition, chemical residues found in feral populations and food animals were felt to be important as indicators of potential human exposures. Such data are needed by the EPA to assist in the identification of industrial chemicals of concern and in the setting of appropriate regulatory limitations as well as by the NCI in their nomination and selection process for carcinogenesis bioassay.

In 1976, the Task Group on Chemicals in Human Tissues was established under the aegis of the ICGEC to identify and plan activities in the area of human body burden. One of their first objectives was the establishment of a human body-burden data base. After two pilot efforts to determine appropriate procedures, the establishment of the data base was identified for funding through the NCI-EPA Collaborative Program. Work began at Oak Ridge National Laboratory in September 1978.

The primary objective of this program is to provide a central resource or guide to data on chemical compounds that have been identified in human tissues and body fluids and in food and feral animals. The data base does not attempt to replace the original literature or data source. As appropriate data sources are identified, primarily in the open literature, pertinent information is extracted and entered into the data base. The wide variety of information collected included chemical, analytic, toxicologic, pathologic, demographic, and bibliographic data. The major data fields are listed below

- Type of data source (e.g., report, journal, letter)
- Language (other than English)
- Chemical name, as used in the data source
- CAS preferred name and registry number
- Chemical formula, Hill configuration
- Major use(s)
- Immediate source or possible source of chemical levels measures in biological media, mean and range
- Analytical technique employed
- Route of exposure
- Half-life
- Number of cases
- Pertinent information, i.e., sex, demographic

Toxicity, health effects
Pathology, morphology information
Pertinent explanatory comments or caveats
Keywords (important descriptors)

The data base is published annually in tabular format, arranged alphabetically by CAS preferred chemical name. Generally, the CAS names are from the 9th Collective Indices.

Retrospective searching to 1974 of a core list of journals is complete and has yielded over 5,000 documents containing human body-burden data. The total human file is comprised of over 8,000 records containing information from approximately 3,000 documents. These documents contain data on over 1,000 chemicals that have been identified in human biological media.

The First Annual Report of the human body-burden file was released in March 1980 and the Fifth will be available in early 1984. Since this is the only comprehensive body-burden data collection of its kind, and due to the keen interest that has been expressed in human body burden, the report has been given wide distribution.

A total of over 2,600 records from approximately 600 documents have been entered into the animal residue file. The third annual publication will be available in early 1984.

The data base is available on-line on the DOE RECON system as well as Lockheed's DIALOG. The National Library of Medicine has expressed interest in making the data base available as part of its extensive and widely-used on-line network.

A number of computerized searches of the human data file have been successfully accomplished upon requests from Federal agencies, state governments, and Congress. A special search for data on cadmium and lead in human blood and kidney was published as a report (EPA 560/13-80-020) for the World Health Organization's Biological Monitoring Project. A similar report on chemicals in human breast milk was published (EPA 560/5-83-009) for the EPA National Human Monitoring Program. It is anticipated that a limited number of special searches of the data base will continue to be required although the on-line and published forms of the database are intended to meet the needs of the majority of users.

The primary plan for the future is to continue to build the human and animal data files. The highly favorable response to the human data base thus far points to its great value and to the need for a centralized source of data on chemicals found in human biological media. The demand for body-burden data is increasing and as the data base has grown and been widely disseminated, it has gone a long way towards filling a previously unmet need of Federal and local government agencies and researchers involved in all aspects of the protection of human health and the environment. The value of the data base is apparent from the overwhelmingly positive response to user survey.

BODY BURDEN OF TOXIC SUBSTANCES IN SUPPORT OF THE TOTAL
EXPOSURE ASSESSMENT METHODOLOGY (TEAM) STUDY

Lance Wallace
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The TEAM Study is designed to measure exposures to about 20 toxic, carcinogenic, or mutagenic substances in the personal air and drinking water of a probability-based sample of several hundred persons in several urban-industrial areas. This project seeks to add to the value of this study by taking breath samples of all subjects and analyzing them for the same compounds. About 250 samples will be collected between January and June 1984 from subjects in Los Angeles, California and Contra Costa, California. The data will be analyzed to determine (1) the frequency distributions of breath concentrations for the two areas, (2) correlations between exposures in air and water and concentrations in breath, and (3) effects of age, sex, occupation, and activities on exposures and breath concentrations.

A two-parameter time-dependent model predicting breath concentrations following 24-hour exposures in air will be tested to estimate effective breath half-lives of some of the target chemicals.

Finally, estimates of exposure and body burden will be made for all residents (about 600,000) of the sampled areas.

Acknowledgement:

This research is supported by the NCI/EPA Collaborative Program.

SYMPOSIUM ON THE APPLICATION OF BIOLOGICAL MARKERS
TO CARCINOGEN TESTING

Harry A. Milman (EPA) and Elizabeth K. Weisburger (NCI)

The Symposium was held November 15 to 19, 1982 at the Holiday Inn, Bethesda, Maryland. Participants in the seven scientific sessions (In Vivo Tests, In Vitro Tests, Enzymes, Oncofetal Antigens and Hormones, Monoclonal Antibodies, Carcinogen-Induced Modification in DNA, Viruses) were requested to submit their manuscripts before the meeting to facilitate processing. The round table discussion was taped and included with the material for publication, as well as abstracts from the poster session.

Associated Universities assumed responsibility for publication and the resulting volume "Application of Biological Markers to Carcinogen Testing" appeared in late 1983.

Acknowledgement:

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