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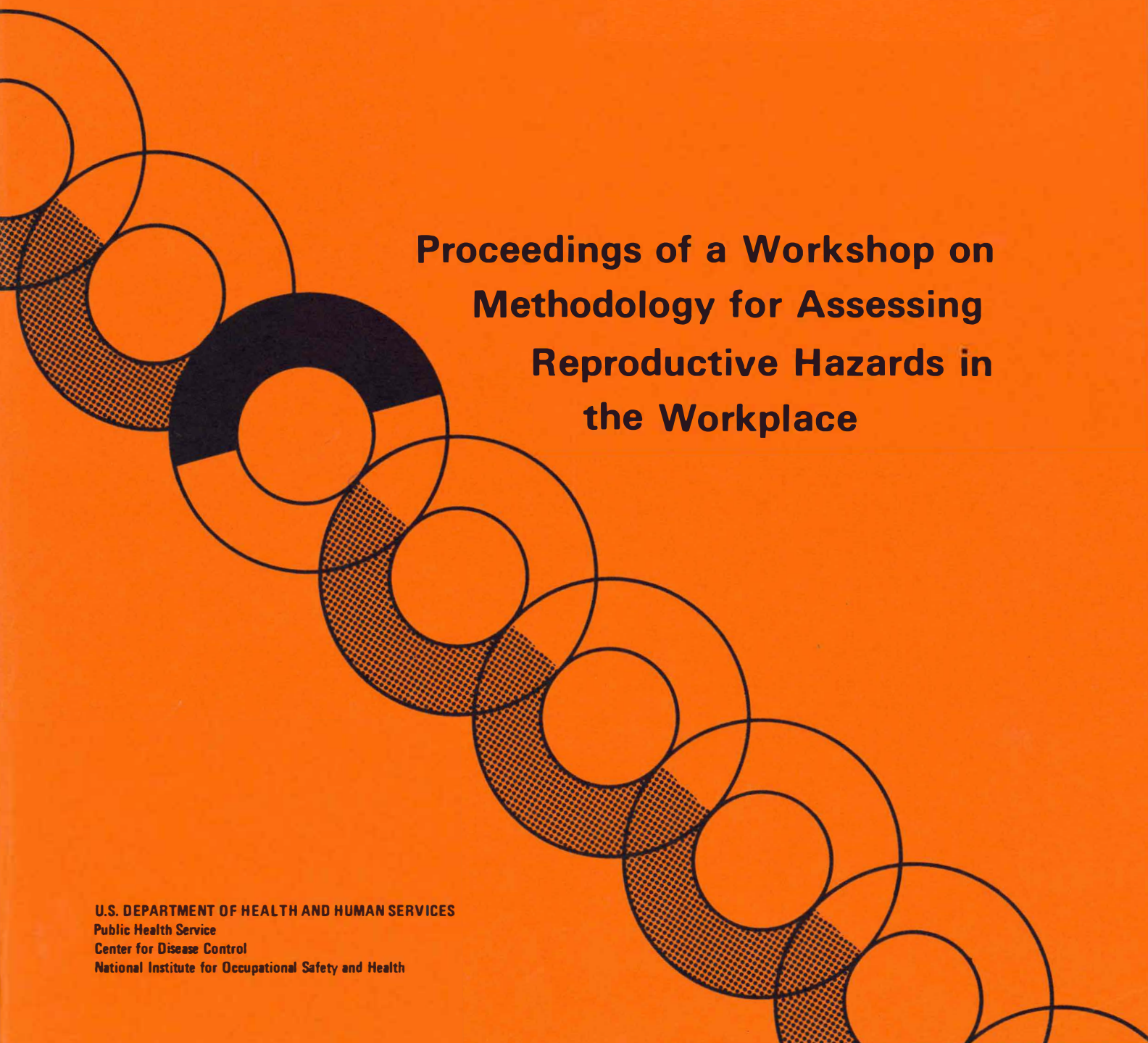
Proceedings of a Workshop on Methodology for Assessing Reproductive Hazards in the Workplace



U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Center for Disease Control
National Institute for Occupational Safety and Health

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PROCEEDINGS OF

A WORKSHOP ON METHODOLOGY

FOR ASSESSING

REPRODUCTIVE HAZARDS IN THE WORKPLACE

April 19-22, 1978

Edited by

Peter F. Infante, DDS, DrPH

and

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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ABSTRACT

A Workshop on Methodology for Assessing Reproductive Hazards in the Workplace was held at the National Institutes of Health in April 1978. Scientists from several countries presented 29 papers on topics which included: (1) case studies of agents associated with adverse effects on reproduction; (2) standard *in vitro* and *in vivo* tests for the identification of mutagens and teratogens; (3) short term methods for human surveillance of mutagens, including cytogenetic studies and sperm assays; and (4) epidemiologic methods for detecting teratogens. Workshop members also made recommendations for future research.

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ACKNOWLEDGMENTS

The Society for Occupational and Environmental Health would like to express its thanks to the speakers who presented papers at the workshop for their willingness to participate and for their contributions to the published proceedings.

The efforts and hard work of the Planning Committee and especially the Workshop Co-Chairpersons, Dr. Peter Infante and Dr. Marvin Legator, deserve special recognition.

And to Sandra Zimmerman, Executive Director of SOEH when this workshop was held, the Society would like to extend its appreciation and thanks for a job well done.

GREETINGS FROM THE SOCIETY AND OPENING COMMENTS FOR THE WORKSHOP

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GREETINGS FROM THE SOCIETY AND OPENING COMMENTS

I would like to welcome all of you this morning on behalf of the Society for Occupational and Environmental Health and its cosponsor of the Workshop, the National Institute for Occupational Safety and Health (NIOSH). We extend particular appreciation to our colleagues, who have traveled to our nation's capital from abroad. Two years ago in Washington, the Society held a meeting on Women and the Workplace. As a result of that meeting, the point was made quite clearly that the study of women in the occupational setting should deal with more than an assessment of reproductive hazards and that reproductive hazards themselves should deal with more than just women. In the interval since those recommendations, two studies addressing carcinogenesis in women have been reported: the first, demonstrating an excess of lung cancer and asbestosis in female asbestos workers and a second, suggesting an excess of breast cancer among women in the PVC fabricating industry.

During this same time interval, the adverse effects of dibromochloropropane (DBCP) to male workers have demonstrated that in the workplace reproductive hazards can no longer be considered an exclusive problem of women. Likewise, agents such as diethylstilbesterol (DES) that are known to cause transplacental effects resulting from female exposure through medicinal use, have now also been shown to cause adverse health effects on males resulting from occupational exposure. I am, of course, speaking of lactation and gynecomastia among male workers exposed to DES.

In view of these recent events, it has become clear that the effects of chemicals in the environment on reproduction need to be assessed from the standpoint of both female and male exposure. Thus, the Society is holding its first Workshop on Methodology for Assessing Reproductive Hazards in the Workplace.

Just as there is a need for researchers from various disciplines to work together, there is also the need for scientists from research and regulatory agencies to work together to eliminate the parochial interests of each agency. For example, in terms of epidemiologic assessment of reproductive hazards, there must be a recognition by epidemiologists, that characteristics of certain populations, due to their defined nature and intensity of exposure, serve as the most advantageous populations for study regardless of where they may be found. The missions of EPA in some situations might best be served by its supporting studies of groups occupationally exposed to pesticides suspected of causing reproductive hazards. DBCP serves as an example where this approach may have identified the problems years ago.

Likewise, the missions of NIOSH and OSHA in some situations might best be served by supporting studies of populations receiving high levels of exposure through medicinal treatment. DES serves as an example. As toxic materials do not recognize national boundaries, these same cooperative efforts must apply worldwide. If clearly defined populations for assessing specific suspect reproductive hazards exist only outside the United States, the United States should encourage and support the conduct of these studies in other countries.

No doubt, in the next few days, we will raise many questions we cannot answer. This will not be surprising as we have only recently begun to focus on some of these issues. This recent focus is the result of women entering blue collar jobs in relatively increasing numbers.

It is not common for scientists from various disciplines to work together to further the difficult task of trying to assess data, to design and modify methodologic approaches, to identify specific populations for study and to develop recommendations for further research--endeavors all leading hopefully to a safer and more healthy working environment for all men and all women. Let's hope we can do it!

SOME SCIENTIFIC AND SOCIAL ISSUES OF IDENTIFYING REPRODUCTIVE HAZARDS IN THE WORKPLACE

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I am pleased to be here today, and I am particularly pleased to be a part of this continuing discussion of a problem that is still a dilemma to us all--reproductive hazards--how to assess them--and how to protect against them.

Historically--and too often, still today--reproductive hazards have been seen as a "woman's problem"--as if there were no male contribution to the continuation of the species. Recently, many of the lead industries have acted to exclude women of childbearing age, and similar exclusionary practices are apparently becoming more common in the petrochemical industry as well. This discriminatory trend is alarming to me. It also worries many in the unions and other groups. Ironically, such practices could ultimately result in discrimination against the male worker, since many substances, such as lead, which affect female reproduction and the fetus, are also harmful to men.

One company excludes fertile women from its lead operations expressing concern for the fetus. But that same company does not test male workers for effects on the sperm, even though recent studies show that lead can affect male fertility.

Just in the last year with the nematocide DBCP we saw an instance where, clearly, the male reproductive capacity was affected--in some cases resulting in sterility. Nobody seriously suggested that we remove all males from that operation. Would that have been the social response if the workers in that instance had been women?

The more we learn about toxic effects on reproduction, in fact, the more we are aware of the importance of male vulnerability. Research on vinyl chloride and anesthetic gases, for example, has shown higher rates of birth defects, spontaneous abortions and other reproductive abnormalities not only among female workers, but among the wives of exposed male workers.

At OSHA we are dedicated--and it is our legal responsibility--to assure, as far as possible, safe and healthful workplaces for all workers--men and women. And that includes protection of all functional capacities, including the reproductive capacity. We have a responsibility to insure the continuation of a healthy human population--as well as an obligation to protect every working person from the avoidable tragedy of a spontaneous abortion or stillbirth, or the procreation of children with birth defects.

Those of us who are scientists and those of us who are involved in Government regulations have a lot of catching up to do. For too long we have been concerned with protecting only the healthy, white, male worker. And yet, paradoxically, one important reason for the discriminatory practices we are

now seeing is that we know more about reproductive effects on females, especially during pregnancy, than we know about the effects on males.

For some inexplicable reason, little research has been done on the effects of workplace toxins on male reproduction. I think it is time we recognize the discriminatory effect of this emphasis, and make sure that in the future we do not foster a continuing bias by picturing reproductive hazards as primarily a female phenomenon.

We must be sure that attention is also addressed--where indicated--to seeking out effects on the male reproductive capacity. This does not mean ignoring gender-specific effects where they do occur. We need to be aware of these, and to use that information--not to exclude workers, but to protect them.

It is particularly important to have meetings such as this, because as a regulatory agency, OSHA needs to be guided by the information the medical community can generate. And in this area of reproductive hazards, it is easy to lose focus, since the scientific responsibility is so diffuse among many diverse agencies, including not only our own sister agency, NIOSH, but also the Environmental Protection Agency, the National Cancer Institute, Environmental Health Sciences, the Food and Drug Administration, the new Department of Energy, and others.

This is still the frontier in learning about toxic effects on sexual capacity. We have vast gaps in knowledge left to fill. And our job at OSHA is that much harder because we have to develop standards for worker protection now. We have to address the social and legal dilemmas and we are, but we are limited to some degree by how little we know, scientifically, about the risks involved.

From a regulatory standpoint, we need answers from scientists in many areas:

- What more do we need to know about mutagens? We know that certain substances are mutagenic, but we have not yet been able to trace a specific birth defect in a human population to a specific mutagenic substance.

- When are epidemiological studies useful in evaluating reproductive effects? And when are they not?

- Is there any need for a tier--or hierarchical approach--in evaluating mutagenic and other reproductive hazards?

- What other information do we need about the effects of toxic substances on male and female physiology under stress?

- Can we relate data from animal tests to human populations in determining safe exposures?

- Can we detect toxic substances that exert a weak effect? Can methodologies used in testing safety in pharmaceutical drugs be used in assessing workplace and other environmental exposures? Or do we need new testing protocols?

- Should teratogenic tests consider toxic exposures prior to conception?
- What do we need to know about the synergistic and additive effects of multiple toxic exposures?
- In terms of social responsibility, at what point should industry, in its testing programs, inform employees and regulatory agencies of abnormal results?
- Given the number of chemicals which still need to be evaluated for reproductive toxicity, what should be the method for assessing priorities?

These are only a few of the questions that scientists can help us to answer. None of them have simple solutions. But in talking about methodology in this field, I want to emphasize to you how important it is, because we are dealing here with substances that have a vital impact on people's lives. And the methodologies used in testing are going to be scrutinized more closely than they ever have been before.

At OSHA, we are beginning the long overdue task of formulating a fair, uniform policy aimed at protecting all workers, male and female. And by protection, let me emphasize again that our commitment is to protection, not exclusion. Employers who look at the exclusion of any group of workers as an answer should be warned that it could have legal implications both under the Occupational Safety and Health (OSH) Act, which explicitly seeks to assure a healthful working place for every man and woman, and also under the Equal Employment Opportunity Act, which protects against job discrimination.

We really don't know the scope of the problem we are facing--but we know that, socially, we have a problem. To illustrate: 7% of all children born in the United States suffer from serious birth defects--a total of some 200,000 a year--accounting for a vast medical problem. One out of three beds in children's hospitals are taken by children with congenital defects. Some of these are hereditary. About 10% we know are environmentally induced, and the vast majority--two-thirds of all defects--are of unknown origin. And we have similarly disturbing and inexplicable statistics on spontaneous abortions and stillbirths. Yet the more we learn about the effects of environmental toxic exposures on reproduction, the more we suspect that it is a substantial burden. The list of chemicals and other toxic substances such as radiofrequency/microwaves grows almost daily as our research efforts expand. NIOSH now lists 56 substances which are mutagenic in animal tests and 471 teratogens.

We have created a new internal task force to consider a whole series of issues related to reproductive function. The social and legal implications in this area are complex, and at the present time we don't have many answers. Women employees are worried about their jobs in work situations that have been traditionally male-dominated. Now that women have managed to get a foot in the door, they fear this reproductive issue is being used to exclude them. Other workers, male and female, are planning families, and they are concerned about whether their workplace exposure is safe. Too often we don't have the answer. To be candid, employees are also afraid some companies are acting from simple fear of liability. But in many instances

they, too, are at a loss to know what policies to adopt. Our internal committee will be looking at these problems and such issues as rate retention, maternity and paternity leave or temporary transfers. We will also be looking at the feasibility of generic standards for certain classes of reproductive toxics.

Discussions have been underway for some time between OSHA and other involved agencies, principally the Equal Employment Opportunity Commission, which has responsibility for protecting workers' rights to fair employment. These discussions are still in the early stages, and will continue.

We are also concerned about increasing public understanding in this area. We are committed to educating our constituencies--both workers and employers--about this area, which is still so poorly understood by the public.

Above all, I think it is important that we keep separate which issues are medical ones, and which are social and legal ones, so that the actions we take as scientists don't simply open the door for more discriminatory practices. The goal at OSHA is to insure that no man or woman has to choose between a job and the right to procreate healthy children.

REPRODUCTIVE HAZARDS FROM PRENATAL ALCOHOL USE: THE FETAL ALCOHOL SYNDROME

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Since the advent of modern industry, ethyl alcohol has been one of the most important and ubiquitous chemical agents associated with the workplace. However, it occupies a unique position of importance as an environmental hazard as a result of its additional social uses and abuses which transcend the confines of the workplace itself. Thus, in any consideration of environmental hazards, alcohol deserves early consideration. Recent studies of the effect of prenatal maternal alcohol consumption on fetal well-being emphasize that this generalization applies to reproductive hazards as well.

THE FETAL ALCOHOL SYNDROME

Although concern regarding the effects of ethanol on the fetus dates back at least to the early Greeks and other nearby cultures, delineation of a recognizable pattern of fetal abnormalities associated with prenatal maternal drinking awaited the studies of Jones, Smith, and co-workers in this country[1,2] and Lemoine in France.[3] This pattern of abnormalities of form and function has been called "the fetal alcohol syndrome." It is now recognized that children displaying this recognizable phenotype represent the more severe end of a spectrum of abnormalities attributable to maternal prenatal alcohol use. Such children are characterized by:

1. a characteristic facial appearance
2. prenatal onset growth deficiency
3. alterations of central nervous system performance
4. an increased frequency of malformations

The facial appearance of children with this condition is often sufficiently striking to suggest this diagnosis even in the absence of a maternal history (FIGURE 1). The midportion of the face is flattened with a short, upturned nose with a broad, low bridge. The eyes appear small because of narrow palpebral fissures and epicanthic folds. The upper lip is broad and somewhat convex, with a narrow mermillion border.

Growth deficiency is a striking feature in more severely affected children. Most are small for gestational age at birth, and continue to grow poorly during subsequent postnatal life. Weight gain is often proportionately worse than length gain. Thus many children are seen by physicians with the complaint of "failure to thrive."[4]

Growth in head circumference is also below normal and frank microcephaly is not uncommon. As microcephaly reflects poor brain growth, it is not surprising that mental deficiency is a common finding among these children.[5] However, poor coordination, poor fine motor function, "hyperactive" behavior, increased distractibility, and tremors are also commonly reported.

The last of these features in the neonate may suggest the "jitteriness" of drug withdrawal, hypoglycemia, or hypocalcemia. However, this sign may persist, despite adequate metabolic management, for months or even years. Likewise, the mental deficiency is a permanent feature, and may range from borderline abnormalities to severe mental retardation.

A wide variety of major and minor malformations are also seen with increased frequency in children with the fetal alcohol syndrome.[6] Defects reported in these children to date are summarized in TABLE I. Particularly frequent are the ocular anomalies,[7] cardiac defects,[8] and cleft palate.[4] CNS malformations are of particular importance for survival and function.[9] Overall, 40% or more of infants born to chronic alcoholics may have significant structural malformations.[10,11]

PATHOGENESIS

The recognition of this pattern of alterations of fetal growth and morphogenesis, and its association with maternal alcohol consumption, has stimulated widespread concern and interest in this country and abroad. Of particular concern has been a delineation of the specific factors related to maternal alcoholism which result in the observed abnormalities. Concern has been expressed that the effects on the fetus could be mediated through some non-specific factor associated with maternal drinking such as maternal malnutrition, a specific nutrient deficiency, or through concomitant exposure to some other agent either contained in alcoholic beverages, or used in association with them. A variety of lines of direct and indirect evidence at the present time implicate ethanol, or one of its major metabolites, as the most likely causal factor. However, the exact pathogenetic mechanism remains obscure.

Although infants of poorly nourished mothers may be smaller than infants of other mothers, they do not display a characteristic pattern of altered facial development or a consistent pattern of birth defects. Furthermore, experience in Denmark during World War II, when maternal malnutrition during pregnancy was widespread and severe, resulted in no recognized increase in the frequency of birth defect problems. Furthermore, in carefully controlled pair feeding animal experiments in several different rodent species, the effects associated with alcohol appeared to be independent of maternal nutritional status.[12,13]

Specific nutrient deficiencies, with the possible exception of iodine, are not known to produce an excess of birth defects in man. Furthermore, among several mothers of children with the fetal alcohol syndrome, no specific serious nutritional deficiency could be identified. Although not conclusive, these studies would suggest that maternal nutritional status is not responsible for the abnormalities seen in children with the fetal alcohol syndrome.

Children with the fetal alcohol syndrome have been born to mothers consuming a wide variety of different alcoholic beverages. No correlation between the type of alcoholic beverage and the abnormalities observed has been possible to date. Thus, it would appear that some toxic factor such as one of the congeners in various alcoholic beverages is also not likely to be important from a pathogenetic standpoint.

Chernoff[12] and Randall[13] have been able to recreate animal models of the fetal alcohol syndrome in rodents with striking similarities to the fetal alcohol syndrome in man. These investigators have demonstrated the importance of ethanol in the pathogenesis of the fetal alcohol syndrome, and have produced evidence that the frequency and severity of anomalies produced are dose-related phenomena. Furthermore, in Chernoff's studies, strains of mice with genetically different capacities to metabolize ethanol have shown differential susceptibility to the adverse fetal consequences of maternal intrapartum ethanol exposures. The frequency and severity of defects seemed to correlate more closely with the blood alcohol level than with actual alcohol intake, suggesting that unmetabolized ethanol rather than one of its metabolites may be the responsible factor. Further information on this aspect is badly needed in order to improve understanding of the sources of variability in the effect of alcohol on the fetus.

VARIABILITY IN THE FETAL ALCOHOL SYNDROME

As mentioned above, it has not become apparent that the fetal alcohol syndrome represents a point near the more extreme end of the spectrum among children damaged prenatally by maternal ethanol abuse. Even among infants born to chronic alcoholics, the studies of Jones, et al. and of Ouellette, et al. have demonstrated that not all of the offspring display serious abnormalities.[10,11] Furthermore, even among the offspring of twin pregnancies, dissimilarly affected individuals have been reported.[14] Ouellette, et al.,[11] and Manzke and Grosse have reported numerous instances of children with only partial manifestations of the fetal alcohol syndrome.[15] Indeed, in the author's experience children with partial manifestations of this condition are frequent both among offspring of chronic alcoholics, and among children of mothers who drink at lesser levels during pregnancy.

The sources of the variability observed among children damaged prenatally by maternal ethanol consumption remain far from completely understood. Experience with other teratogens would suggest several possible sources for this variability, including differences in timing, dosage, host metabolism, and interaction with other factors. As the studies of Chernoff, Randall, and others suggested that in man dosage might be an important consideration, studies attempting to relate abnormalities in the offspring to the level of maternal alcohol consumption during pregnancy in humans have been carried out. Little[16] presented information which suggested that birth weight might be related to level of alcohol consumption, a result subsequently supported by Kaminski, et al.[17] In the latter study, the results were shown to remain significant even when classic maternal risk factors such as tobacco use were taken into account. These authors also demonstrated an increase in perinatal mortality which could be correlated with level of alcohol intake during pregnancy. More recently, we have examined this question with regard to the pattern of abnormalities observed in children with the full fetal alcohol syndrome.[18] In this last study, a pattern of abnormalities similar to, although somewhat milder than, those seen in children with the full fetal alcohol syndrome was seen in 10% of children whose mothers consumed an average of two to four drinks per day during early pregnancy. Among mothers drinking over four drinks per day on the average, 19% showed such abnormalities of growth and development. These results in conjunction with studies of the offspring of chronic alcoholics who consumed very high levels of

alcohol throughout pregnancy suggest a dose-response curve relating level of alcohol intake to the frequency and severity of abnormalities produced. That this is true for both abnormalities of morphogenesis and of central nervous system function is further supported by the recent studies of Streissguth, et al. which show a correlation in severity between abnormalities of morphogenesis and level of mental retardation in children with various degrees of the fetal alcohol syndrome.[19]

Taken in aggregate, these results have led the U.S. Food and Drug Administration to conclude that a level of six drinks per day is sufficient to establish a major risk to the developing fetus, and that even at a level of two drinks per day, there may be an increased risk to the fetus for abnormalities of growth and performance.[20] Indeed, no safe level of maternal alcohol intake during pregnancy has been established, and the placement of warning labels on bottles of alcoholic beverages has been recently advocated.[6]

Information relating the timing of maternal alcohol intake to the nature or severity of defects produced in man remains difficult to obtain. Nevertheless, in our study of the effect of moderate levels of alcohol consumption during pregnancy on fetal growth and development, the strongest correlation was noted between outcome and maternal drinking behavior in the earliest weeks of pregnancy.[18] This result is not unexpected as a knowledge of embryology would predict that in order to produce major abnormalities of morphogenesis prenatal insults to development must occur relatively early (that is, generally within the first trimester of pregnancy). However, this should not be construed to indicate that alcohol consumption during later stages of pregnancy is safe. Indeed, brain growth and organization proceed throughout pregnancy as does growth in other organ systems. Damage accruing from drinking during later stages of pregnancy might also be expected to be more subtle or difficult to detect than would be damage which occurred from early pregnancy alcohol abuse. Indeed, observations in experimental animals would suggest that cerebellar damage could be a problem in infants exposed to alcohol during late stages of pregnancy.[21] This is a question of theoretical importance as ethanol infusions are occasionally used during human pregnancies to arrest premature labor and delivery. Unfortunately, detailed investigations of central nervous system performance in children exposed prenatally under these circumstances have not been reported.

Information on possible host susceptibility factors in man are currently mostly unavailable, although, as pointed out above, in rodents genetic differences in the control of alcohol metabolism seemed to play a role. Likewise, relatively little information is available on possible interactions between alcohol and other environmental factors on fetal growth and morphogenesis. Although it would appear that ethanol exposure is necessary to the occurrence of the pattern of abnormalities referred to as the fetal alcohol syndrome, it may well be that the addition of other prenatal risk factors could have additive effects on fetal growth and morphogenesis resulting either in more serious abnormalities, or additional problems. Indeed, Martin, et al. have presented evidence that alcohol and tobacco may have interactive effects on prenatal development resulting in abnormalities manifest in the neonatal period.[22] Further studies of these relationships are badly needed. It would not be surprising in the future to find that such interactive effects may significantly alter expectations regarding the level and type of prenatal risks in individual patients.

One may conclude from these diverse lines of evidence that a wide variety of damage to the fetus may occur from prenatal ethanol exposures. This damage may cover a wide spectrum with regard both to severity and type of abnormalities. Nevertheless, it is clear that the types of abnormalities observed conform to a relatively specific pattern of alterations which is characteristic of a prenatal effect of alcohol on growth and morphogenesis. At the most severe end of the spectrum, this pattern is sufficiently distinctive to be recognizable as the fetal alcohol syndrome.

EFFECTS OF PATERNAL ALCOHOL CONSUMPTION

The possibility that heavy paternal alcohol consumption might affect the fetus has also been the subject of speculation. A limited amount of animal data raises the possibility that alcohol might be a mutagen,[23] and one study has suggested that the chromosomes of alcoholics show a significant increase in aberrations.[24] However, even if these findings were confirmed they would not account for the high frequency of abnormalities observed in the offspring of maternal alcoholics. Nor would we expect to find any consistent pattern of abnormalities in such children under these circumstances. Thus it seems clear that the most serious and consistent abnormalities among infants born to alcoholics are associated with maternal drinking, thereby implying a direct effect of ethanol on the fetus.[25,26]

EPIDEMIOLOGY OF PRENATAL ETHANOL-INDUCED DAMAGE

If the fetal alcohol syndrome were a rare pattern of abnormalities causally related to some unusual genetic or environmental factor, there would be substantially little cause for public concern. Unfortunately, the above studies strongly suggest a relationship between this pattern of abnormal growth and development, and one of the most ubiquitous drugs in our society: ethyl alcohol. An evaluation of recent reviews would suggest that the full pattern of abnormalities recognizable as the fetal alcohol syndrome may in fact occur with the frequency of between one and two cases per 1,000 live births in the United States and in Western Europe.[18,27,28] Indeed, at least 250 cases have been reported in the world's medical literature in the 5 years since this problem was first publicized in North America.[6] This astonishing accumulation of cases would suggest that the fetal alcohol syndrome may be one of the most frequent recognizable causes of mental deficiency in this country at the present time, ranking with Down's syndrome and neural tube defects.

In fact, this may well represent a substantial underestimate of the impact of this teratogen in that other recent studies would suggest that partial expression of this pattern due to somewhat lower levels of maternal ethanol intake during pregnancy may result in significant alterations of fetal growth, morphogenesis and function in an additional three to five cases per 1,000 live births.[18,29]

If one recalls that in past years the great majority of such children were born to mothers who had become chronic alcoholics toward the end of the reproductive careers, this syndrome takes on added significance. Recent demographic and sociologic studies of alcohol use among younger individuals would suggest that a substantial percentage of children of both sexes leave

high school with an established "problem drinking pattern."[28] As this phenomenon would appear to be generalized throughout the United States, it raises the specter of substantial numbers of young women who are just beginning their reproductive careers consuming excessive amounts of alcohol during pregnancy. Should this dire prediction come true, we can expect a substantial increase in the number of individuals damaged prenatally by ethanol in the immediate future.

The health and educational care as well as other aspects of the burdens imposed on individuals, families, and society by this problem can hardly be underestimated in either financial or humanitarian terms. Thus, a substantial commitment of our efforts and resources to combat this problem would appear to be in order. Fortunately, this is similar to the conclusion reached by the Institute of Medicine of the National Academy of Sciences in advising the U.S. Public Health Service with regard to this problem.[29]

IMPLICATIONS FOR CLINICIANS

The preceding data have a number of important implications for clinicians in health care specialties. Perhaps most important, it is imperative that physicians who care for pregnant or potentially pregnant women recognize that alcohol is a dangerous drug. A careful history of maternal drug use, specifically including questions regarding the amount and pattern of maternal drinking during pregnancy, should be a part of every new prenatal evaluation. Mothers should be cautioned against excessive alcohol consumption at any time during pregnancy, and women who are found to have dangerous drinking patterns should be warned of the risks to their infants. Indeed, in some cases the risk may be sufficiently high to warrant consideration for termination of pregnancy.

Furthermore, patient education regarding the hazards of alcohol consumption during pregnancy should be a part of the routine care of women, including adolescent girls. This becomes particularly important as studies cited above suggest that the period of pregnancy of most concern may be the first few weeks, a time during which the mother may not be aware that she has become pregnant, and may not have sought medical consultation. For this reason, it has become our practice to advise women to avoid alcohol consumption if they are of a reproductive age and are doing nothing specifically to avoid a pregnancy. This educational process should be extended to high school age groups, and perhaps to preteens as well, as those youngsters most at risk for alcohol-related problems are often found to have begun drinking by fifth grade levels. It may well be that such early education as a part of our health curriculum may be the only effective mechanism for prevention of this needless tragedy.

Thirdly, it is important for pediatricians, family physicians, and other specialists who care for children, to recognize the pattern of abnormalities associated with prenatal maternal ethanol use. The early identification of affected children may allow diagnosis and treatment of specific health problems in these individuals. It may allow the more appropriate use of medical facilities for the evaluation of problems of growth, development, and nervous system performance. It may also lead to more appropriate educational planning for affected children. Finally, recognition of an affected infant may

lead to recognition of a mother who is at a substantial risk for problems in previous or future offspring, thereby leading to improved care for other members of a high-risk family and prevention of this problem in additional children.

Several important questions have yet to be resolved with regard to the effects of alcohol on the fetus. As pointed out above, we need an improved understanding of the factors which may modify the expression of this condition such as the relationship between amount and timing of drinking and fetal outcome, interaction with other environmental or genetic factors and the possible effects of drinking late in pregnancy. Of particular importance will be an investigation of fetal hazards related to low levels of maternal alcohol intake during pregnancy. Such exposures include those occurring in and about the workplace. At the present time, there is no specific data to suggest that an occasional drink or low level industrial exposures during pregnancy do harm to the fetus. However, it should be stressed that a safe lower threshold of alcohol consumption during pregnancy below which fetal damage does not occur has not been defined to date. One recent paper has raised the question of a possible association between a rare type of childhood malignancy and prenatal ethanol use. This hypothesis remains to be critically evaluated in man.[30]

Further investigations of the efficacy of alternative modes of medical, nutritional, or educational therapy of these children need to be carried out, and new methods need development.

Finally, new methods of prevention of this disorder are badly needed. This is a particularly tragic condition as it is potentially 100% avoidable. It remains to be seen whether or not expanded educational programs or other forms of intervention designed to prevent this condition will be effective in reducing the impact of this problem on our society.

SUMMARY

Current data clearly point to an association between chronic maternal alcoholism and serious morphological and developmental abnormalities in the fetus. When these abnormalities are severe the condition is recognizable as the fetal alcohol syndrome. However, this represents only the most severe end of a spectrum of abnormalities, some of which may be found in a proportion of infants exposed to lesser amounts of alcohol during pregnancy. Alcohol is an avoidable hazard for the fetus, but only through increased awareness by the medical community and improved education of the lay public may we hope to control this problem.

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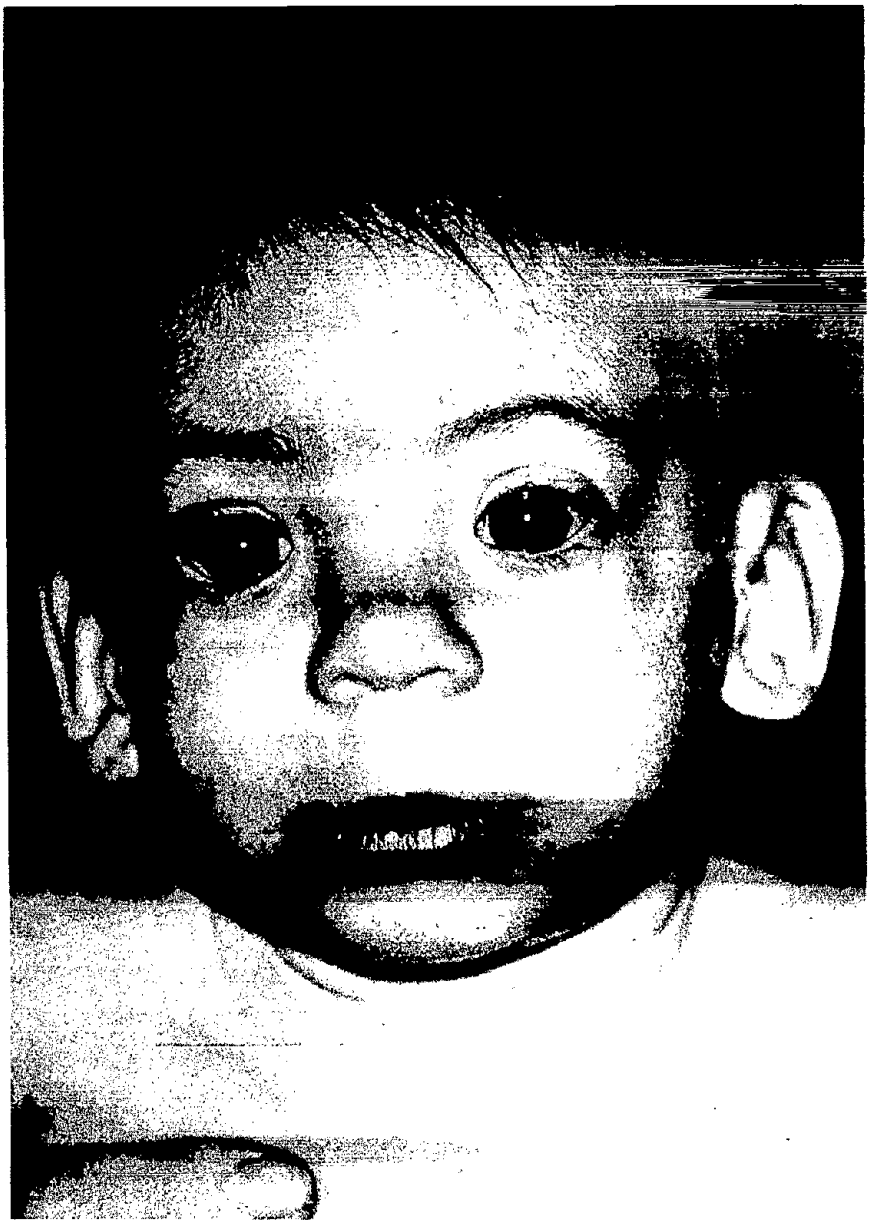

FIGURE 1

Facial features of an infant with fetal alcohol syndrome. Note especially the narrow palpebral fissures, low nasal bridge and short nose, epicanthic folds, flat midface and broad upper lip with narrow vermillion border.

TABLE I

BIRTH DEFECTS ENCOUNTERED IN THE FETAL ALCOHOL SYNDROME

A. Craniofacial Region
   1. auricular anomalies
   2. ocular defects
      a. anophthalmia/microphthalmia
      b. colobomas of iris and/or retina
      c. optic nerve hypoplasia
      d. ptosis
      e. strabismus
   3. cleft palate

B. Cardiovascular System
   1. septal defects (especially VSD)
   2. cyanotic heart defects

C. Skeletal System
   1. vertebral segmentation anomalies
   2. limited joint mobility

D. Central Nervous System
   1. neuroglial heterotopias and neuromigrational anomalies
   2. cerebellar hypoplasia
   3. absence of corpus callosum
   4. hydrocephalus
   5. ? neural tube defects

E. Genito-Urinary System
   1. renal malformations
   2. external genital anomalies

F. Integumentary System
   1. capillary hemangiomata
   2. hirsuitism

EFFECTS OF IN UTERO DES-EXPOSURE ON HUMAN ADULTS: TESTICULAR HYPOPLASIA AND ABNORMAL SEMEN IN MALES; VAGINAL ADENOSIS AND RIDGES IN FEMALES

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The transplacental hazards of DES on the developing human female fetus were originally described by Herbst and coworkers in their report of clear-cell adenocarcinoma of the vagina in the adolescent woman.[1] The transplacental effects of DES on the human male fetus were originally reported by our group.[2,3] Both anatomical (epididymal cysts, hypoplastic testes, ...) and functional (abnormal semen) abnormalities of the genital tract were significantly greater in the DES-exposed males as compared to placebo-exposed control males whose mothers were all participants in a prospective, randomized, double-blind study of the effects of DES on pregnancy at the Chicago Lying-In Hospital during the early 1950's. This is an updated report of 308 DES-exposed males and 307 placebo-exposed control males and 346 DES-exposed females and 319 placebo-exposed control females.

MATERIALS AND METHODS

The effects of DES on pregnancy were evaluated two decades ago at the University of Chicago in a prospective, double-blind randomized study of 2,162 consecutively registered pregnancies which resulted in 840 women receiving DES and 806 women receiving placebos.[4] DES was administered orally at the rate of 5 mg per day starting with the seventh week of gestation, and increased by 5 mg per day every second week up to a maximum daily dose of 150 mg by the 34th week. Over 250 males in each of the two groups (DES-exposed and placebo-exposed) have been traced and evaluated to date. Neither the patients, nor the examining physician and laboratory personnel, were aware of the group (DES or placebo) to which the male offsprings belonged.

Anatomical abnormalities of the male genital tract were sought by: (1) meticulous physical examination, (2) urine cytology (cytology of urine pre- and post-prostatic massage or ejaculation), prostatic fluid, and aspirated epididymal cysts, and (3) biopsies, where feasible (epididymis and testis ...).

Functional abnormalities of the male genital tract were searched for by: (1) evaluation of the medical history (age puberty, first ejaculation, first intercourse, urinary tract infections, venereal infections, and fathering of children), (2) determination of the level of hormones in the blood, follicle stimulating hormone (FSH) and interstitial cell stimulating hormone = luteinizing hormone (LH), by means of radioimmunoassay[5] and plasma testosterone,[6] and (3) semen analysis [7,8] on ejaculates produced during the clinic visits

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after sexual abstinence of 3 days or more. The volume of the whole ejaculate was measured, and sperm count and percent motile spermatozoa were determined between 1 and 2 hours after ejaculation (double or triple determinations). Smears were fixed with methanol and stained with Giemsa solution (20 drops of Giemsa stain [Fisher Scientific Company] per 5 ml of distilled water); a differential count of normal (oval) and abnormal forms was performed, including primitive cells of the germinal epithelium. The semen quality was estimated by a score system for sperm concentration, percent motile sperm, motility grade, and morphology (percent normal forms). The sum of the scores was used for the following classification: normal ≤ 1; doubtful 2 - 4; pathological 5 - 10; 10 > severely pathological (Eliasson).[8]

In all female patients, abnormalities were searched for by evaluation of medical history, meticulous general physical examination, gynecologic and colposcopic examinations, and laboratory studies which included cytologic specimens from the cervix, endocervix and vaginal walls, urine cytology, and FSH and LH determinations (random samples). Patients who were having problems achieving pregnancy also had studies done of progesterone, total estrogens, and testosterone. Biopsies from the vagina and cervix were performed when indicated.

RESULTS

ANATOMICAL ABNORMALITIES OF THE MALE GENITAL TRACT

TABLE I summarizes the abnormal physical findings in these men two and a half decades after in utero exposure to placebo or DES. The DES-exposed offspring had a highly significant increased incidence of epididymal cysts and testicular hypoplasia (testis length $\leq$ 3.5 cm), as compared to the unexposed controls. The exact nature of abnormal physical findings in the testes and epididymal areas was difficult to ascertain without biopsies. However, nine DES-exposed patients allowed their epididymal masses to be aspirated which revealed a straw-colored fluid that did not contain spermatozoa in six cases, and a slightly milky fluid that did contain sperm in three cases. Aspirates of two control males contained spermatozoa in only one case. Cytologic examination revealed only epithelial cells and amorphous precipitates without any material suggestive of malignancy.

One epididymal cyst that was excised showed a thin wall structure which contained clear, straw-colored fluid, and was lined by columnar epithelium without apparent secretory cells. Since no spermatozoa were found in the fluid, the criterion for spermatocele was absent, and a diagnosis of an epididymal cyst was made.

No malignancies have been found in any of these men, to date, on histological examination of one excised epididymal cyst and nine cytological evaluations of aspirated epididymal cysts. Cytologic examinations of the urine specimens (pre- and post-prostatic massage or ejaculation) and prostatic fluids were negative for tumor cells in all of the DES-exposed, as well as the control males.

A history of cryptorchidism was obtained in 17 patients (65%) of the hypoplastic testes in the DES-exposed group, which theoretically raises the

risk of testicular carcinoma.[9] Three of the DES-exposed hypoplastic testes were associated with varicoceles, one with mumps orchitis, and one with trauma.

FUNCTIONAL ABNORMALITIES

The results of circulating blood hormone assays are summarized in TABLE II. There were no significant differences demonstrable in the averages of the circulating blood hormones assayed, except a mild increase in luteinizing hormone in the DES-exposed males which, however, was in the normal range. In the subsets with hypoplastic testes, the FSH average levels were higher in both groups as compared to the total group averages, and the LH average level was higher in the DES group with testicular hypoplasia. Two individuals with bilateral hypoplastic testes had FSH and LH levels above the normal limits of 400 and 150 ng/ml respectively. One of these individuals also had a plasma testosterone level below the lower limit of normal, 270 ng%.

The results of sperm counts are shown in TABLE III. In the DES-exposed males, the average sperm density was somewhat decreased as compared to the placebo-exposed control males. The subsets of hypoplastic testes in both groups showed considerable depression of the average sperm concentration as compared to the total group. The azoospermics and oligospermics also show a positive correlation with testicular hypoplasia.

The distribution of spermatozoa analyses is given in TABLE IV by Elaisson's method which combines the sperm count, the sperm motility, the motility grade, and the sperm morphology into one quantitative number. Note that the average Eliasson Score was significantly higher in the DES-exposed group as compared to the placebo-exposed controls. Further, the hypoplastic testes subset had a considerable further increase (departure from normality) in the average Eliasson Score. TABLE IV also shows a further clustering of patients with abnormal semen (Eliasson Scores $5 \leq$ pathological semen, and $10 \leq$ "severely pathological semen") in the DES-exposed males. Also, the hypoplastic testis subgroups had an increased incidence in the pathological semens.

ANATOMICAL ABNORMALITIES OF THE FEMALE GENITAL TRACT

The medical histories of the DES-exposed and control females were similar in many respects: marital status (54% married vs. 48%), onset of menstruation (average, 12 ± 1.3 years), presence (88%) or absence (12%) of sex experience, usage of birth control pills (35% at the time of examination and 71% in the past), usage of other birth control methods, breast discharge between menarche and first pregnancy.

Fifteen percent of the DES-exposed females had irregular menstrual cycles, as compared to 10% of the control females (TABLE VI). With the exception of one patient who had secondary amenorrhea, all patients with menstrual irregularities (50) in the DES-exposed group had oligomennorrhea. One patient in this group had primary amenorrhea. In the control group the menstrual irregularities consisted of two cases of polymenorrhea and 31 cases of oligomenorrhea. In reference to menstrual flow duration, there was a higher percentage (61%) of menstrual flows lasting 1 to 4 days in the

DES-exposed subjects than in the controls (44%), who presented a higher percentage of flows lasting 5 to 7 days (53% versus 36%) ($p < 0.0005$).

The history of pregnancy for all patients in the control group was 31%, in contrast to 21% for all patients in the DES-exposed group ($p < 0.01$). Similarly, the percentages of live births and miscarriages in the control group were 22% and 8%, respectively, as compared to 13% and 7% in the DES-exposed group.

Ridges of the vagina and cervix (TABLE VII) were seen in 131 or 38% of the DES-exposed females (of these, 56 or 43% were complete circumferential, 41 or 31% were incomplete circumferential, and 34 or 26% were of irregular type), but in none of the controls ($p < 0.0001$). No correlation was found between the presence of ridges and the rate of miscarriages in the DES-exposed group.

Colposcopic findings in the vagina reflecting the presence of adenosis showed statistically significant differences between the two groups. Adenosis was found in 218 or 63% of the DES-exposed group (39 or 11.3% discrete, and 179 or 51.7% confluent), and in 4 or 1.3% of the control group (3 or 0.9% discrete and 1 or 0.3% confluent) ($p < 0.0005$). A breakdown of the colposcopic findings showed columnar epithelium in 59 or 17.1% of the DES-exposed females, in contrast to 4 or 1.3% of the control females ($p < 0.0005$), white epithelium in 178 or 51.4% versus 1 or 0.3% ($p < 0.0005$), leukoplakia in 5 or 1.4% versus one or 0.3% (p not significant), mosaic in 52 or 15% versus 1 or 0.3% ($p < 0.0005$), and punctation in 25 or 7.2% versus 2 or 0.6% ($p < 0.0005$). The epithelial changes were localized in the anterior wall of the vagina in 39 or 17.9% of the patients; anterior and posterior wall in 93 or 42.6%; anterior, posterior, and one of the lateral walls in 32 or 14.7%; and in all four quadrants in 54 or 24.7%. In the DES-exposed subjects with adenosis, 202 or 92.7% had lesions in the upper third of the vagina, 13 or 5.9% in the middle third, and three or 1.4% in the lower third.

Colposcopic findings in the cervix showed statistically significant differences in that transformation zone, white epithelium, mosaic, and punctation more frequently occurred in the DES-exposed females. Columnar epithelium was found in 249 or 72% of the DES-exposed females compared to 207 or 64.9% of the control females (p not significant), transformation zone in 267 or 77.2% versus 171 or 53.6% ($p < 0.0005$), white epithelium in 147 or 42.5% versus 56 or 17.6% ($p < 0.0005$), leukoplakia in 7 or 2% versus 3 or 0.9% (p not significant), mosaic in 100 or 28.9% versus 51 or 16% ($p < 0.0005$), and punctation in 51 or 14.7% versus 20 or 6.3% ($p < 0.0001$).

DISCUSSION

The present study clearly indicates that transplacental effects of DES on the human males do occur. Administration of DES during pregnancy appears to be followed by effects on the fetal male genital tract that have shown up in the form of structural and functional changes that may well impair fertility in a certain number of patients. With the delays in family planning prevalent today, it will probably be another decade before the actual infertility rate is known. However, since semen analyses give insight into the probability of male infertility,[8] one needs to carefully follow these and

expanded numbers of patients with regard to the association of DES-exposure and subnormal fertility.

Although our study has not demonstrated carcinogenesis in human males to date, it has been demonstrated that prenatal exposure to DES produces detectable anatomical changes in the male reproductive tract. Epididymal cysts, hypoplastic testes, cryptorchidism, and induration of the testicular capsule have all been found in a greater incidence in the DES-exposed males. Our findings in humans are quantitatively in agreement with old and new studies in animals (TABLE V). In 1940, Green, Barrill, and Ivy reported that in rats the effects of in utero exposure to estradiol or diethylstilbestrol were cryptorchidism, hypoplastic testes, and underdeveloped epididymides.[10] Dunn and Green (1963) reported the occurrence of cysts of the epididymis in male mice and cancer of the cervix in female mice after subcutaneous DES injections to these mice at birth.[11] McLachlan and Newbold have recently published data on reproductive tract lesions in male mice exposed prenatally to DES.[12] These workers found that of the male mice studied: 60% were sterile, 33% had epididymal cysts, 25% had undescended testes, 25% had nodular enlargement of the seminal vesicles and/or coagulating glands, which were associated with squamous metaplasia. Nomura and Kanzaki have shown that DES given to mice only later in pregnancy produced undescended testes and testicular hypogenesis in addition to lung tumors.[13]

The following factors raise the question of potential malignancy: (1) the 72% incidence of cryptorchidism in the unilateral hypoplastic testes raises the possibility of an increased incidence of testis carcinoma as reported in other series of non-DES related cryptorchidism and testicular carcinomas,[9] (2) the prostatic utricle which is the Müllerian duct remnant in the male which is homologous to part of the female vagina and has been found to be the site of endometrial adenocarcinoma in older human males,[14] and (3) the natural history of the most common carcinoma of the male sexual organs, prostatic carcinoma, occurring largely in the seventh and eighth decades.

A 25-year-old man, who was recently treated by us for testis carcinoma (anaplastic seminoma and embryonal carcinoma), had a history of an undescended testis until school age and exposure to DES in utero[15] It should be emphasized that this case was not one of the Dieckmann's study patients which is the source of the data for this report. Private communications with two other physicians have found associations between DES-exposure in utero and cryptorchidism and adult testicular carcinomas (private communication).

In the adult females, both adenosis and ridging were very significantly greater in those exposed to DES in utero. However, no vaginal or cervical carcinomas have been found to date in these patients. Although the history of pregnancy was significantly greater in the control group (31% versus 21% with $p < 0.01$), and would suggest an effect of DES-exposure in utero on subsequent female fertility, the data and time bases need to be expanded before the fertility aspect can be thoroughly documented. No correlation was found between the presence of ridges and the rate of abortions in the DES-exposed group.

SUMMARY

Epididymal cysts and/or hypoplastic testes have been found in 31.5% of 308 adult males who had been exposed to DES in utero as compared to a 7.8% incidence in 307 placebo-exposed control males. Spermatozoa analyses have revealed severe pathological changes (Eliasson Score > 10) in 18% of 134 DES-exposed males and 8% of 87 placebo-exposed males.

Further investigation of the 26 DES-exposed men with testicular hypoplasia has revealed that 65% had a history of cryptorchidism. Only one of the five placebo-exposed control males with testicular hypoplasia had a history of testicular maldescent.

The results in females have demonstrated vaginal adenosis (63% of 346 DES-exposed versus 1.3% of 319 controls) and ridges (38% of DES-exposed versus 0% of controls) with incomplete findings to date on fertility.

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TABLE I

GENITAL TRACT ABNORMALITIES IN THE ADULT MALES 2½ DECADES AFTER IN UTERO EXPOSURE TO PLACEBO OR DES

	Placebo-Exposed	DES-Exposed	P Values
Total Number of Men	307	308	
Epididymal Cysts	15 (4.9%)	64 (20.8%)	<0.005
Unilateral			
Left	9	29	
Right	3	20	
Bilateral	3	15	
Testicular Abnormalities	9 (2.9%)	35 (11.4%)	<0.005
Hypoplastic Testis	6 (1.9%)	26 (8.4%)	<0.005
Unilateral			
Left	3	14	
Right	1	7	
Bilateral	2	5	
Cryptorchidism*	1	17	<0.005
Capsular Induration	3	9	<0.100
Microphallus	0	4	<0.050
Total Number of Patients With One or More of Above Abnormalities	24 (7.8%)	97 (31.5%)	<0.005

*Cryptorchidism history in patients with hypoplastic testes.

TABLE II

BLOOD HORMONE LEVELS

	Placebo-Exposed		DES-Exposed		
FSH (80 - 400ng/ml)	ng/ml	(n)	ng/ml	(n)	
Hypoplastic Testes $\bar{x}$	233	(4)	273	(22)	} p <0.001
Total Group $\bar{x}$	155	(240)	171	(247)	
Above Normal		(0)		(2)	
LH (20 - 150ng/ml)	ng/ml		ng/ml		
Hypoplastic Testes $\bar{x}$	20	(4)	46.8	(22)	} p <0.001
Total Group $\bar{x}$	29.6	(240)	36.1	(247)	
Above Normal		(0)		(2)	
Testosterone (270 - 1150 ng/dl)	ng/dl		ng/dl		
Hypoplastic Testes $\bar{x}$	705	(4)	675	(22)	
Total Group $\bar{x}$	728	(238)	689	(242)	
Below Normal		(0)		(1)	

TABLE III

SPERM COUNTS

	Placebo-Exposed		DES-Exposed	
Average Sperm Density x 10^6/ml				
Hypoplastic Testes (n)	47 (4)		42 (21)	} p <0.001
Total Group (n)	115 (87)	(p <0.05)	91 (134)	
Azoospermia				
Hypoplastic Testes	0		2	
Total Group	0		3	
≤ 10 Million Sperm/ml				
Hypoplastic Testes	1		5	
Total Group	4		9	
< 20 Million Sperm/ml				
Hypoplastic Testes	2		7	
Total Group	8		20	

TABLE IV

ELIASSON SCORES OF SEMEN ANALYSES

	Placebo-Exposed			DES-Exposed		
Average Eliasson Score						
Hypoplastic Testes (n)	6.2	(4)		10.3	(21)	p <0.001
Total Group (n)	2.5	(87)	p <0.001	4.9	(134)	
Eliasson Score 5 ≤ "Pathological Semen"						
Hypoplastic Testes	2	(50%)		14	(67%)	
Total Group	14	(16%)	p <0.025	43	(32%)	
Eliasson Score 10 < "Severely Pathological Semen"						
Hypoplastic Testes	2	(50%)		7	(33%)	
Total Group	7	(8%)	p <0.050	24	(18%)	

TABLE V

ANIMAL EXPERIMENTS: EFFECTS OF IN UTERO OR NEONATAL EXPOSURE TO ESTROGENS ON MALE OFFSPRING

Authors	Estrogens	Male Offspring Examined
1940		Newborn ♂ Rats
Greene[10] Burrill Ivy	E_2 ∞ * 1-35 mg E_2 di Palm# 1-100 mg DES 10-42 mg Sub Q Preg. Rats Gestation Days 12 - 18 (21)	Cryptorchidism Testicular Hypoplasia Epididymal Hypoplasia Hypospadias Microphallus Seminal Vesicle Inhibition Prostatic Hypoplasia Prostatic Utricle → Vagina Gynecomastia
1963		Adult ♂ Mice (Non inbred Swiss & inbred BALB/c & C_3H_f Strains)
Dunn[11] Green	DES-2mg/0.1 ml saline Sub Q Newborn Mice	Epididymal Cysts (58%)
1975		Adult ♂ Mice (CD-1 Strain)
Mc Lachlan[12] Newbold	100µg DES in corn oil per Kg. B.W. Pregnant Mice Gestation Days 9 - 16	60% Sterile 33% Epididymal Cysts 25% Undescended Testes 25% Ampullary Nodular Masses
1977		Adult ♂ Mice (ICR/JcL Strain)
Nomura[13] Kanzaki	10µg DES/g B.W. Pregnant Mice Gestation Days 17 or 19	70% Undescended Testis "Testicular Hypogenesis" Lung Adenomas

* E_2 ∞ = Estradiol alpha

E_2 di Palm = Estradoil Dipalmitate

TABLE VI

REPRODUCTION DATA IN FEMALE OFFSPRING

	Placebo-Exposed Controls		DES-Exposed
Number Examined	319		346
Irregular Menses	10%		15%
Married	48%		54%
History Pregnancy	31%	(p <0.01)	21%
Live Births	22%	(p <0.01)	13%

TABLE VII

VAGINAL FINDINGS IN FEMALE OFFSPRING

	Placebo-Exposed Controls		DES-Exposed
Number Examined	319		346
Vaginal Adenosis	1.3%	(p <0.0005)	63%
Ridges of Vagina and Cervix	0%	(p <0.0001)	38%

EFFECTS OF LEAD ON REPRODUCTION

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Lead has a profoundly adverse effect on the course of reproduction. Lead may decrease the fertility of a male worker; it may lead to miscarriage or stillbirth in a female worker; it may even affect the wife of a lead worker through his affected sperm.[1] The older medical literature suggesting lead's toxic effect on reproduction amongst workers, animal studies attesting to this problem, chromosomal studies, and more recent studies suggesting that this toxic effect continues will be briefly reviewed.

During the late 19th and early 20th centuries, women in the pottery and white lead industries felt that lead was an abortifacient. Over 100 years ago they knew that women in lead work were more likely to be sterile; that if they became pregnant they were more likely to miscarry; that if the pregnancy went to term it was more likely to end in stillbirth; and that if the child was born living, that death was more likely to come in the first year of life.

Numerous studies chronicle the toxic effect of lead in earlier times:

1. Torelli, in 1930, studied the printers exposed to lead in Milan, Italy. He found the abortion rate for Milan in general to be 4-4.5%, but among the wives of printers the rate was 14% and among the women printers 24%.[2]

2. Tardieu reported to the French Government in 1905 that 608 out of 1,000 pregnancies in lead workers ended in abortion.[3]

3. Legge, in summarizing the reports of 11 English factory inspectors in 1879, found that of 212 pregnancies in 77 females working with lead, only 61 living children were produced. Fifteen had never become pregnant; there were 21 stillbirths; miscarriages occurred 90 times and of 101 children born, 40 died in their first year.[4]

4. In 1911, Sir Thomas Oliver (Chief Factory Inspector) noted that females exposed to lead premaritally had twice as many miscarriages and stillbirths as female mill workers of similar age, and that females exposed to lead after marriage had a threefold increase.[2]

5. Koinuma compared the marital life records of workmen exposed to lead in storage battery plants with the records of those working in non-leaded occupations. 24.7% of the lead group had sterile marriages compared to 14.8% in the controls; 8.2% of the pregnancies in the lead group ended in miscarriage or abortion compared to 0.2% for the controls.[5]

6. Nogaki studied the pregnancy outcome of 104 Japanese women before and after beginning lead work showing an increase in miscarriages to 84/1000

pregnancies from a pre-lead rate of 45.6/1000.[6] The miscarriage rate for 75 comparable employees not exposed to lead was 59/1000 pregnancies. The maternal blood leads were high ranging from .110 to .317 mg%.

7. In 1905, Rudeaux analyzed 442 pregnancies in women married to lead workers; 66 ended in abortion, and 241 in premature birth.[2]

8. Pindborg noted that 60% of 25 pregnancies in the first trimester aborted in women who had ingested lead oxide as an abortifacient, and had mild to moderately severe lead poisoning.[7]

9. Rennert, Chyzzer, and Oliver all reported convulsions and macrocephaly in the offspring of women involved in a cottage industry using lead glazes; the fetus may have a very low tolerance for lead in demonstrating effects on the nervous system.[2]

There is conclusive evidence that lead passes through the placenta. Blood lead levels in cord blood are correlated with blood lead levels in the mother at the time of delivery. Gershanik et al. in examining 98 cord blood pair samples found a coefficient of correlation of 0.63 with maternal samples.[8] Transplacental passage becomes detectable at 12-14 weeks of gestation. Increased elimination of lead through breast milk in combination with previous intrauterine exposure is a reason for concern in regard to the health of the infant.

Lead exposure from public drinking water passing through lead pipes has resulted in intrauterine death of a fetus (with 3 maternal blood leads of 55, 34 and 72 µgm/100ml), congenital defects (maternal blood lead 31 µgm/100ml), and threatened abortion with subsequent premature birth (maternal blood lead of 53 µgm/100ml).[9]

Fahim et al. compared the course and lead values in 249 pregnancies in Columbia, Missouri, with 253 occurring in the center of America's lead belt at Rolla, Missouri.[10] At Columbia, greater than 96% delivered normally at term, 3% were pre-term (defined as a neonate born before 37 weeks of gestation and weighing less than 2500 grams), and less than 1% had premature rupture of the membrane.

At Rolla, only 70% were term, 17% had premature membrane rupture (defined as spontaneous rupture of the membrane before the onset of labor, and when labor does not begin within 12 hours), and 13% were pre-term. (TABLE I) The striking blood lead findings from the Rolla lead belt area were a doubling of maternal blood lead in the premature membrane rupture and pre-term groups, and a fourfold increase of fetal blood lead in these groups. Fahim has also noted an increase in molar pregnancies, (which result from a blighted ovum) in the Rolla region. He also states that women in the lead belt have increased menstrual disturbances--amenorrhea, dysmenorrhea, irregularity of menstrual cycles, and menorrhagia.

In 1972, Z. Panova published a study of 140 females, exposed for 1-12 months to $<7 \mu gm/m^3$ of lead, using a control group of 100 females working in a textile factory.[11] The number of abnormal cycles was studied by repeated vaginal cyto-smears. In 37% of exposed females, and in 22% of controls, menstrual cycle disturbances were observed ($p < .02$). Particularly in the

20-25 year age group there was an increased prevalence of anovular cycles, and of cycles with a disturbed lutein phase.

The reproductive ability of men occupationally exposed to lead is interfered with by altered spermatogenesis. Lancranjan et al. reported a significant increase in teratospermia amongst lead-poisoned workmen (blood lead mean 74.5 µgm/100ml) and workmen with moderately increased absorption (blood lead mean 52.8 µgm/100ml).[12] (TABLE II) Hypospermia and asthenospermia were increased not only in both preceding groups, but also those with only slightly increased absorption (blood lead mean 41 µgm/100ml). They concluded that lead has a direct toxic effect upon the male gonads. Abnormal spermatogenesis may be the cause of the high fetal wastage rate amongst the wives of lead workers.

Experimental investigations using various animal species have revealed toxicity by lead on fertility, embryogenesis, and development of the offspring.

1. Varma et al., in 1974, fed 2% lead acetate to male Swiss mice for 4 weeks noting that their fertility index subsequently decreased by 50%.[13]

2. Schroeder et al., in 1971, administered 25ppm lead in the drinking water to pregnant mice and rats, observing runting, reproductive failure, and shortened life span.[14]

3. Stowe et al., in 1972, fed 1.0% lead acetate to white leghorn female hens from age 4 weeks onward.[15] The lead-toxic hens grew more slowly, reached sexual maturity later, and laid significantly fewer eggs than control hens. One-fourth of the eggs laid by the lead-toxic hens had soft and malformed shells.

4. Sharma and Buck, in 1976, exposed 12 sheep to sublethal doses of powdered metallic lead in their feed during their entire period of gestation.[16] Their rate of lambing was 18% compared to 100% in nine unexposed controls. Abortions occurred at a rate of 27% in the exposed group, but did not occur in the control group.

5. McLellan et al., in 1974, fed pregnant mice low doses of lead chloride such that the pregnancies went to term.[20] The subsequent litters showed a lower growth rate and lower gross motor activity compared to controls.[17]

6. Hubermont et al., in 1976, reported on female rats fed drinking water at .1, 1, and 10ppm lead for 3 weeks before mating, during pregnancy, and 3 weeks after delivery.[18] Newborns from the 10ppm group had lowered ALAD activity in blood and elevated tissue prophyrins compared to their mothers indicating an increased susceptibility of the newborns.

7. Stowe and Goyer, in 1971, found that either paternal-only or maternal-only lead exposure in rats resulted in a reduction of the number of pups/litter, a reduction in mean pup birth weight, and a reduction in pup survival.[19] These effects were magnified however, in offspring whose parents had both been exposed to lead.

8. Hildebrand et al., in 1973, fed lead acetate at doses of 5 and 100 micrograms orally to 80 male and female rats for 30 days.[20] At the end of

the study period, the blood leads of the females were higher than the males. They noted impotence and prostatic hyperplasia in the males at the lower dose, progressing to testicular damage in those reaching blood leads of 50 µgm/100ml. In the females, they noted irregularity of the estrus cycle at both doses.

9. Maisin et al., in 1975, fed lead acetate in the diet to male mice; with increasing exposure the percentage of abnormal spermatozoa increased.[21] Lead acetate fed to pregnant mice resulted in an increase in the number of embryos dying after implantation.

10. Brady et al., in 1975, studied the influence of parental lead exposure on subsequent learning ability of offspring of rats. Offspring of either a leaded father-normal mother, or vice versa, or both parents leaded were tested at 30 days of age using a black-white discrimination water T maze.[22] The three leaded groups made more errors and had longer swimming times than controls, with the offspring of both leaded parents faring the worst.

11. Carson et al. showed that subclinical prenatal exposure to maternal blood lead levels of 34 µmg/100ml did slow learning of a visual discrimination task in lambs when they were 10-15 months old.[23]

12. Sauerhoff and Michaelson found that newborn rats that suckled mothers eating a diet containing 4% lead carbonate displayed hyperactivity, aggressiveness, and stereotyped repetitive behavior as manifested by excessive self-grooming starting at 4 weeks of age.[24] There was a significant increase in brain lead in the exposed rats beginning 5 days after onset of exposure. There were no changes in brain norepinephrine levels, but there was a significant decrease in dopamine content in exposed rats compared to controls at 21 and 29 days.

Studies of hyperactive children have found a higher incidence of lead exposure compared to controls; in addition, these children had a 15% higher blood lead than in controls, and following chelation with penicillamine, 60% excreted toxic levels of lead.[25]

13. Teratogenic effects have been cited in three reports: (a) Ferm and Carpenter produced congenital skeletal malformations in hamster embryos following the treatment of the pregnant hamster with various salts of lead;[26] (b) Karnofsky and Ridgway produced central nervous system injury by injecting lead nitrate into the yolk sac of the chick embryo;[27] and (c) Gilani demonstrated congenital cardiac anomalies by studying 8-day-old chick embryos which had been administered lead acetate on the second day of incubation.[28]

Fifty percent of human abortions have chromosomal abnormalities; 10% of stillbirths have chromosomal abnormalities. Chromosomal abnormalities may result in teratogenic effects, e.g., mongolism or Turner's Syndrome. Certain malignancies, e.g., chronic myelogenous leukemia may be characterized by a chromosomal defect. Numerous studies have documented chromosomal abnormalities amongst lead workers.

1. Muro and Goyer analyzed chromosomes from leukocyte culture of mice fed a diet of 1.0% lead acetate. They observed an increased number of

gap-break type aberrations, largely involving single chromatids.[29] The chromosomal abnormalities included 28 (16.9%) gaps versus 4 (1.3%) in the controls, 9 (5.4%) breaks versus none in the controls, and 20 (12%) fragments versus none in the controls.

2. Schwanitz et al. reported a three- to fourfold increase in chromosomal observation in human lymphocytes treated in vitro with lead acetate as compared with control cells exposed to sodium acetate.[30]

3. Lehnert, in Germany, found an increase in gap-break chromosomal changes in lead workers having a blood lead in the range of 62-89 µgm/100ml.[31] He found a positive correlation with an increased urinary ALA and the percent of abnormal mitoses seen.

4. The data of Lehnert and Schwanitz have been corroborated by Forni and Secchi in Italy.[32] They analyzed chromosomes from cultures of blood lymphocytes from 65 workers occupationally exposed to lead, and in 65 unexposed controls matched for age. They had three groups: (1) 15 preclinical with blood lead mean 64 ± 16 µgm/100ml, (2) 37 clinically poisoned with blood lead mean 78 ± 25 µgm/100ml, and (3) 13 with past lead poisoning no longer exposed for 18 months with blood lead mean 56 ± 16 µgm/100ml. There was a significantly increased rate of chromatid aberrations and of unstable chromosome changes (mostly of the one-break type) in both groups 1 and 2, when compared to controls, but differences were not significant for group 3. Thus, chromosomal abnormalities have been found in lead-exposed workers; however, there are two negative studies by O'Riordan and Evans[33] and Bauchinger et al.[34] Forni et al. then carried out a prospective study in 11 subjects beginning employment in a storage battery plant.[35] They found a significant increase in the percentage of abnormal metaphases at the end of the first month that increased again in the second month levelling off by the sixth month. Lead absorption was mild to moderate, and chromosomal rearrangements were scanty. Chromosomal abnormalities (particularly chromatid changes) do not have a clearly defined biological significance, but may have a relation to reproductive failure.

In summary, the fetus is particularly sensitive to the toxic effects of lead. This may be manifest in miscarriage, stillbirth, birth defect, or increased perinatal mortality. Subsequent behavioral effects from intrauterine lead exposure may also occur. Adverse reproductive effects may occur from lead exposure to the male worker (through altered spermatozoa) as well as to female workers. There may be no threshold limit at which adverse effects could not occur in the course of development of the human fetus.

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TABLE I

RELATIONSHIP OF PREGNANCY OUTCOME AND MATERNAL AND FETAL BLOOD LEAD BETWEEN MISSOURI'S LEAD BELT (ROLLA) AND A NON-LEAD REGION (COLUMBIA)

	#pregnancies	I		II		III	
Columbia	249	96.4%		3%		0.4%	
		M Pb	F Pb	M Pb	F Pb*	M Pb	F Pb*
		13.1±.1	4.3±.1	30.1 (one sample)	12.0	26.0±.84	9.6±.7
Rolla	253	70%		17%		13%	
		M Pb	F Pb	M Pb	F Pb*	M Pb	F Pb*
		14.3±.16	4.6±.08	25.6±1.7	14.2±.8	29.1±.5	17.5±1.1

SOURCE: Reference #10

I Term

II Term with premature membrane rupture

III Preterm (<37 weeks and <2500 gms.)

*P<.001 Maternal and fetal blood Pb II and III different than I.

M Pb = maternal blood lead

F Pb = fetal blood lead

TABLE II

FREQUENCY OF ALTERATIONS IN SPERMATOGENESIS
IN LEAD-EXPOSED WORKMEN AND CONTROLS

	N	PbB µg/100ml	N Semen Analysis:	Spermato-genesis	Alterations in Astheno-spermia	Hypo-spermia	Terato-spermia
A. (a) Lead-poisoned workmen	23	74.5±26	16	15(93%)	8(50%)*	8(50%)*	14(86%)*
(b) Moderate increase lead absorption	42	52.8±21	29	22(68%)	15(51%)*	13(44%)*	17(58%)*
(c) Slight increase lead absorption	35	41±12	19	12(63%)	8(42%)**	8(42%)**	6(31%)
B. Physiological lead absorption in a polluted environment	50	23±14	25	7(28%)	6(24%)	7(28%)	4(16%)
C. Controls	50		50		6(12%)	5(10%)	7(14%)

SOURCE: Reference #12

*p <0.001
**p <0.01

PbB = blood lead

A REVIEW OF THE METHODOLOGIC APPROACHES IN THE ASSESSMENT OF AN ASSOCIATION BETWEEN VINYL CHLORIDE EXPOSURE AND REPRODUCTIVE HAZARDS

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As early as 1930 the first adverse toxic effects of vinyl chloride (VC) were observed. Since then, numerous clinical observations and epidemiologic studies have indicated a wide range of toxicity involving the central nervous system, the liver, the bones of the fingers, and the lungs attributed to VC. In 1971, the toxicity of vinyl chloride broadened to include carcinogenesis. Viola et al.[1] reported the induction of tumors of the skin, lungs, and bones in rats exposed by inhalation to 30,000 ppm of VC for 12 months. In 1974, Maltoni and Lefemine[2] reported the induction of angiosarcoma of the liver in animals following exposure by inhalation to VC. Shortly thereafter, Maltoni and Lefemine[3] and Keplinger et al.[4] demonstrated that VC induced adenomas and adenocarcinomas of the lung, lymphoma, neuroblastoma of the brain, angiosarcoma of the liver, and various other tumors in a variety of animal species. On the basis of the VC bioassay findings, Waxweiler et al.[5] undertook an epidemiologic study to assess the magnitude and spectrum of oncogenic effects in individuals occupationally exposed to vinyl chloride. This study demonstrated that two major causes of death were in excess among workers occupationally exposed to VC-PVC, i.e., for nonneoplastic respiratory disease (6 observed deaths versus 3.4 expected) and for all malignant neoplasms combined (35 observed deaths versus 23.4 expected). Furthermore, when malignant neoplasms were analyzed according to organ system and interval since onset of exposure, an excess of cancer of four organ systems was found: brain and central nervous system, respiratory system, hepatic system, and lymphatic and hematopoietic systems. With regard to types of tumors, Waxweiler et al.[5] reported that of 14 histologically confirmed cases of biliary and liver cancer among workers from the four study plants, 11 cases of angiosarcoma of the liver were diagnosed; of 10 cases of brain cancer, 9 were histologically classified as glioblastoma; and of the 8 out of 14 cases of primary lung cancer which were histologically reviewed, 5 were large-cell undifferentiated and 3 were adenocarcinoma. The distribution of histologic types of tumors for each of these three cancer sites was unusual. More recently, several additional studies[6,7,8] have corroborated this excess of multi-organ carcinogenicity among individuals occupationally exposed to vinyl chloride. This observation of the carcinogenicity of vinyl chloride, first in animals and subsequently in humans, had a profound positive effect on public health, first in terms of the recognition of the need for rapid regulatory control of VC in the industrial setting and second in terms of an increased acceptance and awareness of the value of experimental bioassay.

At the same time, however, Maltoni and Lefemine[3] had demonstrated the induction of angiosarcoma in four pups of pregnant rats exposed to VC ranging from 6,000-10,000 ppm. Unfortunately, this observation led to confusion in the United States as industry and some governmental agencies concluded that women of child-bearing capacity should be excluded from working in industries manufacturing or using vinyl chloride, in lieu of total containment of this toxic material. For an objective scientific evaluation of the reproductive toxicity of VC, one must assess not only the effects of VC as transmitted through the female, but also the potential for any adverse effect that may be transmitted through the male.

What evidence is there bearing on the teratogenicity or of embryo lethality of vinyl chloride? One report[9] addresses the issue of the effects of maternally inhaled vinyl chloride on fetal development. While maternal toxicity was observed, VC alone was reported not to cause significant embryonal or fetal toxicity and not to be teratogenic in several species tested.

What can we say about the potential for teratogenic response to VC in humans? Four epidemiologic studies have addressed this issue. In 1975, Infante[10] observed a significant excess of total birth defects in three communities in northern Ohio (Ashtabula, Painesville, and Avon Lake) containing vinyl chloride polymerization facilities. As reported, the total malformation rate per 1,000 live births in each of those three communities and in all three communities combined was significantly higher than expected on the basis of the malformation rate for the total State. This association persisted when contrasts were made between the study communities and the remainder of the counties within which those cities were located. In addition, this study demonstrated that malformations involving the central nervous system in those three communities were particularly high, both among the stillbirths and among the live births, as contrasted with the State rates. Recognizing that "many underlying factors could be responsible," the author indicated the need for further study.

In 1975, Edmonds et al.[11] reported the results of a hospital based case-control study involving one of the communities previously studied by Infante.[10] Although a significant excess of children born with central nervous anomalies was confirmed, the authors reported no association between parental occupational vinyl chloride exposure and malformations of the central nervous system. The authors also reported no association by residential proximity to a point source of VC exposure in Painesville. The absence of an association between VC exposure (direct or indirect) and central nervous system anomalies in this study must be viewed in the context of limitations in methodology. For example, residential proximity to a VC polymerization facility was measured only with reference to the Painesville facility, whereas, 2 of the 15 index cases, on this hospital based study, were both of a mother who resided in Ashtabula, Ohio, a city having another VC facility. In addition, whereas the authors reported that no parents of either cases or controls lived within 2 miles of the Painesville VC facility, no mention was made of the fact that few people lived within this 2-mile limit due to the industrial zone nature of the area.

Because of the continued concern about the teratogenic potential of VC, Edmonds[12] undertook and reported another study of CNS malformations in Kanawha County, West Virginia, the site of another VC polymerization facility. This

study demonstrated 59 infants born between 1970 and 1974 with CNS defects as contrasted with only 37 expected on the basis of U.S. rates.

Analyses by residential proximity to the VC polymerization facility demonstrated proportionately more ($p < 0.02$) cases born within a 3-mile radius of the facility. Furthermore, cases of CNS anomalies were reported to be concentrated to the northeast of the VC facility, as contrasted with controls which clustered to the southwest. In this area the wind was reported to blow predominantly from the southwest direction.

Subsequently, data were also analyzed from another county (Midland County, Michigan) containing a vinyl chloride polymerization or monomer facility. As seen in TABLE I, the rate of total malformations in Midland County, Michigan, during 1970-1974 was 21.3/1,000 live births as contrasted with 9.5/1,000 live births for the entire State of Michigan. This elevated rate of malformations in Midland County was evident for each of the years 1971-1974. With regard to specific congenital malformations, 34 urogenital defects were observed, whereas only 7 were expected; 12 heart anomalies were observed, whereas only 4 were expected; and 15 cases of cleft lip and cleft palate were observed, whereas only 5 were expected. These observations were reported as statistically significant.

Obviously there are limitations to each of these studies. One such limitation is that all studies were undertaken in communities subject to facilities engaged in heavy industrialization or facilities engaged in the production or use of multiple chemicals making it difficult to determine whether any one chemical acting alone is associated with the excess of congenital anomalies observed in these communities. In order to address this limitation, data bearing on the carcinogenicity of VC may be helpful. VC is known to induce a rare form of liver cancer, angiosarcoma, both in experimental animals and among individuals occupationally exposed. Is there data that would suggest that this rare form of cancer would be found among individuals residing in communities having VC or PVC facilities, who were never employed at these facilities?

Brady et al.,[15] in a study of 26 confirmed cases of angiosarcoma of the liver, reported that of 10 female cases (no direct occupational exposure to arsenic, VC, or therapeutic exposure to thorium dioxide), 5 lived within 1 mile of a VC polymerization plant (1 case lived within 1,700 ft. for 62 years) or of a PVC fabrication plant (4 cases lived from 500 to 4,500 ft. for 8 to 27 years), whereas none of their matched controls lived as close. This latter observation, according to the authors, added support to the hypothesis that indirect modes of exposure to VC-PVC might be important in the etiology of liver angiosarcoma.

These same observations lend support to the public health concern that indirect modes of exposure to VC/PVC may be important in the etiology of congenital anomalies. In spite of these suggestive findings, the definitive answer to the human teratogenicity of VC may not be possible to ascertain due to the insensitivity inherent in the epidemiologic method.

What is the evidence bearing on the mutagenicity of vinyl chloride? Several studies[14] have indicated that vinyl chloride is mutagenic in microbial test systems, i.e., E. Coli, S. Typhimurium, and S. Pombe, and in a

plant system, Tradescantia. Vinyl chloride metabolites also have been shown to induce mutations in mammalian cells. In terms of the potential for transmissibility of these genetic effects, studies have demonstrated the induction of sex-linked recessive lethal mutations in Drosophila exposed to VC. In contrast, a single study of dominant lethal effects of vinyl chloride in the mouse was reported as negative, though no data were presented in tabular form.

In terms of the human experience, at least four studies have demonstrated an excess of chromosomal aberrations in circulating lymphocytes of males occupationally exposed to vinyl chloride when contrasted with controls not so exposed.[14]

Additional human evidence in support of the mutagenicity and potential reproductive hazard through male exposure is a study of miscarriage among wives of males occupationally exposed to vinyl chloride.[15] The data on pregnancy outcome were obtained by interviewing males currently employed in the polymerization of vinyl chloride and a "control group" consisting of males currently engaged in the fabrication of polyvinyl chloride or the manufacture of rubber goods.

No interviews were conducted with the workers' wives, and no data were obtained concerning the maternal age at the time of each pregnancy. However, maternal age was estimated through the use of paternal age.

As may be seen in TABLE II, among pregnancies occurring prior to the husband's exposure, the fetal death rate taken as any product of conception not born alive, was 6.9 for the "control group" as contrasted with 6.1% for the VC exposed group, following direct age-adjustment of rates in the VC exposure group to the paternal age-distribution of the "control group." This difference was not statistically significant. In contrast, among pregnancies occurring subsequent to the husband's exposure, the fetal death rate was 8.8 for the "control group" versus 15.8 for the study group, a difference significant at the $p < 0.05$.

To determine whether women who had experienced abortions chronically might have weighted the results in favor of a higher fetal mortality rate in the primary VC exposure group, data for the pregnancies of women who had two or more spontaneous abortions were eliminated. The data were then reanalyzed to determine whether or not the trend of a greater miscarriage rate could be maintained in the primary VC exposure group.[16] As shown in TABLE III, the trend was maintained for each analysis. As reported previously,[15] the significant excess in fetal mortality after the husband's exposure to VC could not be explained on the basis of bias from interviewers nor from respondents.

With regard to the amount of evidence needed to indicate that an agent poses a reproductive hazard through male occupational exposure, are the above cited studies sufficient? Or, among human populations is it necessary or even possible to undertake studies using congenital anomalies as the biological endpoint? In reference to laboratory studies is it necessary to demonstrate that vinyl chloride or any other mutagen does indeed reach gonadal tissue? If data were not available presently for the carcinogenicity of VC, what would be the public health posture regarding the mutagenic and genetic effects of VC? In the case of VC, the question of what evidence would be considered as sufficient has not been answered by either the scientific or regulatory community.

The array of evidence both male and female related for reproductive hazards associated with industrial chemicals is increasing. The questions now facing the scientific and regulatory communities are: (1) What endpoints are methodologically possible to assess from an epidemiologic approach? (2) And, what evidence is to be considered necessary for public health decisions?

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TABLE I

CONGENITAL ANOMALIES PER 1,000 LIVE BIRTHS AMONG RESIDENTS OF MIDLAND COUNTY, MICHIGAN, AND THE ENTIRE STATE OF MICHIGAN, 1970-1974

	Midland County			State of Michigan
Year	Number of Births	Number of Defects Apparent At Birth	Rate/1,000	Rate/1,000
1970	1,285	11	8.6	8.8
1971	1,269	29	22.8	9.6
1972	1,096	35	31.9*	9.7
1973	1,024	29	28.3*	10.1
1974	1,062	18	16.9	9.6
1970-74	5,736	122	21.3	9.5

*Highest rate in state.

TABLE II

MEAN PATERNAL AGE, NUMBER OF PREGNANCIES, AND
AGE-ADJUSTED FETAL DEATH RATES
ACCORDING TO HUSBAND'S VC EXPOSURE

	"Controls"[a]	Primary VC Exposure[b]
	Prior to husband's exposure	
Number of families	95	70
Mean paternal age at conception (years)	23.0	26.4
Number of fetal deaths among wives	11	15
Number of pregnancies	159	148
Age-adjusted fetal deaths/ 100 pregnancies[c]	6.9	6.1
	Subsequent to husband's exposure	
Number of families	113	62
Mean paternal age at conception (years)	30.4	30.2
Number of fetal deaths among wives	24	23
Number of pregnancies	273	139
Age-adjusted fetal deaths/ 100 pregnancies[c]	8.8	15.8[d]

SOURCE: Reference #15

a. Rubber and PVC fabrication workers.
b. VC polymerization workers.
c. Rates age-adjusted to "control" group paternal age distribution.
d. Subsequent to husbands' exposure, the frequency of fetal deaths among wives was significantly greater in the primary VC exposure group as compared to the "controls" ($P < 0.05$).

TABLE III

NUMBER OF PREGNANCIES AND AGE-ADJUSTED FETAL DEATH RATES ACCORDING TO HUSBAND'S VC EXPOSURE EXCLUDING PREGNANCIES FOR WOMEN WITH ≥2, 3, OR 4 FETAL DEATHS

	Controls		Primary VC exposure	
	Number of pregnancies	Fetal death rate	Number of pregnancies	Fetal death rate
	≥2 Fetal deaths excluded			
Before husband's exposure	155	5.8%	126	1.7%
After husband's exposure	255	4.7%	111	6.2%
	≥3 Fetal deaths excluded			
Before husband's exposure	159	6.9%	141	3.1%
After husband's exposure	265	6.8%	120	10.8%
	≥4 Fetal deaths excluded			
Before husband's exposure	159	6.9%	142	5.8%
After husband's exposure	265	6.8%	127	11.8%

Rates for the primary VC exposure group are age-adjusted to the control group.

BIRTH DEFECTS AND PARENTAL OCCUPATION: PRELIMINARY RESULTS FROM METROPOLITAN ATLANTA

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INTRODUCTION

In North America, interest in the effects of the workplace on reproductive outcome has been quite intense recently. Little is known about occupationally-induced adverse pregnancy effects; very few examples of known hazardous agents or conditions readily come to mind. Perhaps the most widely known is lead poisoning which increases the rate of stillbirth and abortion.[1] Other effects are less well established, and the field is obviously ripe for investigation.[2-5]

We report here some preliminary results from the Center for Disease Control's Metropolitan Atlanta Congenital Defects Surveillance Program (CDSP). Some of these results were presented previously.[6]

Use of these data from Atlanta should be regarded as nothing more than casting a net with a very coarse mesh. If we are lucky, we may catch some real associations, but most are likely to get away. The numbers involved are small, and the type of adverse pregnancy outcome limited. Therefore, this sort of exploration can do virtually nothing to help us in pronouncing an occupation or industry "safe" for reproducing humans. On the other hand, utmost caution in the interpretation of those associations which do appear is in order. We made a large number of comparisons, and many of the associations we found might be expected to result from chance alone. Our approach is less than ideal, but it does represent a start in a rather sparsely studied field.

MATERIAL AND METHODS

The Metropolitan Atlanta CDSP attempts to ascertain all babies with structural congenital malformations born to mothers residing within a five-county area including and surrounding the city of Atlanta.[7] Multiple sources of case ascertainment are used, but the primary source is personal contacts of CDC staff with the nursing personnel of the local hospitals. From the total roster of affected babies, the mothers of a subset with certain defects are selected for interview. At the time of this writing, some 1,100 interviews had been completed, and the occupational histories recorded on 989 have been reviewed. TABLE I shows a list of the regularly selected defects. The numbers in TABLE I are the counts of babies with each defect, and these counts are not mutually exclusive. For example, a baby with cleft palate and reduction deformity would contribute one count to the cleft palate category and one count to the reduction deformity category. In addition, mothers of babies who

had defects other than those on the list have been interviewed. Most interviews were conducted in the homes of the parents and completed within 6 months of birth, and generally the mother has been the sole informant. Roughly 85% of designated mothers were interviewed; the remaining 15% either refused permission, moved away from the area, or their attending physician refused permission. Information gathered during the interview included the employment history of both mother and father; mothers were asked for their history beginning 2 years prior to conception until birth, while the fathers' employment history was limited to occupation at the time of conception. We are now gathering information on paternal occupation in the same way that we have always gathered that for mothers.

The occupational coding system used was that devised by the U.S. Census Bureau for the 1970 census.[8] This scheme is quite comprehensive, and provides for both an industry as well as an occupational code. The occupational classification includes 417 categories, and the industry code, 215 categories. There are codes for jobs ranging from accountants and architects, to sheriffs and bailiffs, and industries ranging from agricultural production to wholesalers of scrap and waste material. The Census Bureau has compiled lengthy lists of job and industry titles (about 23,000 and 19,000 entries respectively) which are cross-referenced with the codes.

In conducting surveys, the Census Bureau asks several simple, specific questions about occupation and industry. For the data at hand, the questions asked were not the same as those of the Census Bureau. However, it was possible to code the vast majority of respondents using the available information. For fathers, only the occupational and industry codes associated with employment at the time of conception were used. For the mother, there are codes for each job held beginning 2 years prior to conception until birth.

In the analyses presented here, the occupational and industry codes were combined into several groups (TABLES II-IV). These roughly correspond to the Census Bureau's major categories except that certain occupations and industries which seemed to be of current interest were kept separate.[9] We also added some groups (e.g., housewives) for which there was no provision in the census code.

Since only parents of affected babies were interviewed, and not control parents of unaffected babies, the occupational distribution of parents of babies with one malformation is compared to that of parents of babies with all other malformations.

The computer was programmed to display only those malformation-occupation (industry) 2 x 2 tables which include at least two parents in the occupation (industry) group, where the odds (cross-product) ratio was at least 2.0, and where the χ^2_c was at least 3.84. These χ^2_c values are not used in a "testing" sense; rather they are used (in conjunction with the odds ratio) as a guide to the strength of the association between a particular malformation and a particular occupation (industry). Comparisons were made for maternal exposure during the first trimester, and prior to, or at the time of, conception; paternal comparisons were made for each malformation and each occupation, each industry, and each occupation-industry cross-classification.

RESULTS

TABLE II shows the occupational distribution of all Atlanta women ≥ 16 years at the time of the 1970 census, and of the 989 interviewed mothers. The time of employment in this table for our case mothers is the first trimester of pregnancy, so it is not surprising that the overall percentage "employed" is lower than for all Atlanta females. Among employed case mothers, the distribution is quite like that of all Atlanta women except for the category of "health worker." This may represent a selection bias whereby malformed babies born to health workers are more likely to be ascertained by our surveillance system, and/or health workers are more likely to grant an interview. They may also tend to work during pregnancy more than other women. Finally, it may be that these mothers are more likely to have babies with malformations. The occupational distribution for case fathers is quite similar to that for all Atlanta males (TABLE III). Unfortunately, Atlanta area sex-specific statistics for industry were not available, but TABLE IV compares interviewed case fathers with all Atlanta-employed persons.

TABLE V shows the selected classifications for maternal exposures during the first trimester. The most prominent malformation is cleft lip, with or without cleft palate, and three of the employment categories are health-related while the other two are the business and repair services industries. Further exploration of the latter two classifications indicated that there was no apparent pattern as to specific type, or place, of employment. On the other hand, the associations in the health field appear to be due to an excessive number of registered nurses among mothers of babies with cleft lip. Out of a total of 16 nurses, six were mothers of cleft lip babies (odds ratio = 3.2; $\chi^2_c = 4.0$); four of the clefts were isolated malformations, while one was associated with anencephaly and the other was associated with ear, heart, and intestinal defects. No pattern was apparent among the mothers who were sales workers and had babies with central nervous system defects, nor was there any discernible pattern among the mothers of babies with reduction deformities. Selected maternal exposures prior to, or at the time of, conception are shown in TABLE VI. Two mothers of Down's Syndrome children were managers in printing firms. Three mothers of babies with omphalocele and gastroschisis were also employed in the printing industry--two operated printing presses, while one worked both as a binder and a press operator. The remaining mother employed in the printing industry operated a press, and her baby was affected by microcephaly, cleft palate, and limb defects. Not shown in TABLE VI are associations of cleft lip with health workers, and the business and repair services industries which were quite similar to the associations shown in TABLE V.

Selected paternal exposures are shown in TABLE VII. Two fathers of babies with cleft palate were clerical personnel in the printing industry. Both were mailers, and handled freshly printed material. The other occupation/industry category associated with cleft palate was that of painters working in the construction industry.

For cleft lip with or without cleft palate, several associations are shown. The category for male health workers is shown even though the χ^2_c is < 3.84 because of the maternal associations found (TABLE V). There is no apparent occupational clustering within this category: One father was a

pharmacist, one a supervisor of an emergency room (a nonphysician), one a medical technician, and one a psychiatrist. There was no apparent clustering of employment in the remaining occupation/industry categories either for cleft lip or for the remaining malformations listed in TABLE VII.

DISCUSSION

There are fairly large numbers of employment categories which are seemingly overrepresented among parents of babies with particular malformations, but there are a number of difficulties in interpreting these results. First, you are well aware of the heterogeneity within the various occupational and industrial categories. Complicating this is the fact that the major groupings used here are those of the U.S. Census Bureau which are designed primarily for economic tabulations. Nevertheless, the codes assigned to individuals are very specific, and the approach used here was to start with the broad grouping and look further when an unusual association appeared. Second, there is certainly heterogeneity within the various categories of malformations which we have used.

How do we put these findings into perspective? There are so many comparisons that no doubt many associations occurred by chance. In addition, bias may be a factor, as was pointed out for female health workers. It is our strong opinion that these apparent relationships should be used only as guides for future studies. Investigation of the printing industry seems warranted since there are a wide variety of potentially noxious chemicals used in these trades.[10] In addition, further study of the association between cleft lip with or without cleft palate and maternal employment as a nurse would be appropriate. Nurses have a variety of unusual exposure potentials, possibly including infectious diseases, anesthetic gases, and freer access to drugs.

Full exploitation of this approach will have to await the collection of more data. Finally, a comment should be made on the limitations of this approach in contrast to others. One major limitation of this approach is that the type of adverse pregnancy outcome with which we deal is limited. Another is that no information relating to parents of normal children was collected. The assumption made in comparing one malformation category versus all the rest is that any factor which might be responsible for a teratogenic or mutagenic effect would be unlikely to result in a uniform increase in all types of malformations. It is more likely that some particular outcome would result from a given agent or factor. Even so, the lack of normal controls is unsatisfying. A further limitation stems from our location: Metropolitan Atlanta is not a heavily industrialized area, and therefore potentially hazardous employment exposures are not abundant.

The converse approach to ours, that is, beginning with parents exposed in a particular occupation or industry, has the advantage of providing relatively large numbers of exposures. It also makes it possible to deal with a wide variety of adverse reproductive outcomes. On the other hand, this approach has the disadvantage that even among the exposed, the outcomes of interest are likely to be rare.

SUMMARY

The mothers of some 1,100 babies with selected congenital defects have been interviewed in Metropolitan Atlanta. The employment histories of the parents of 989 of these babies have been coded using the occupation-industry classification scheme of the U.S. Bureau of the Census. The frequency of a particular occupation among parents of babies with one type of defect was compared to the frequency among parents of babies with other congenital malformations. Among mothers of babies with cleft lip with or without cleft palate, the nursing profession was more frequent than expected. There was also an excess of "craftsmen" in the printing industry among mothers of babies with omphalocele and gastrochisis. Because of the large number of comparisons made, many associations which appeared unusual could have easily arisen by chance, and the results of this study should be interpreted with caution.

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TABLE I

MALFORMATION DISTRIBUTION IN BABIES OF INTERVIEWED MOTHERS,[1] METROPOLITAN ATLANTA

	Number With Malformation
Major Central Nervous System[2]	233
Cleft Palate	94
Cleft Lip With/Without Cleft Palate	160
Tracheo-esophageal Atresia	31
Intestinal Atresia	96
Reduction Deformity	99
Abnormalities of Autosomal Chromosomes	157
Diaphragmatic Hernia	40
Omphalocele and Gastroschisis	74

1. Total interviewed mothers = 989

2. Anencephaly, spina bifida, encephalocele

TABLE II

OCCUPATION DISTRIBUTION
METROPOLITAN ATLANTA, FEMALES

	Interviewed Case Mothers, First Trimester		All Atlanta Females ≥16 Years, 1970	
	Number	Percent[1]	Number	Percent[1]
Health Workers	37	9.9	12,137	5.1
Public School Teachers	27	7.3	14,526	6.2
Other Professional Workers	15	4.0	16,041	6.8
Managers	8	2.2	8,779	3.7
Sales Workers	14	3.8	17,708	7.5
Clerical Workers	180	48.4	99,220	42.0
Painters	1	0.3	4,060	1.7
Other Craftsmen	3	0.8		
Gas and Garage Attendants	0	0.0	22,406	9.5
Other Operatives	22	5.9		
Laborers	5	1.3	2,745	1.2
Airline Attendants	1	0.3	38,628	16.4
Other Service Workers	59	15.9		
Total "Employed"	372	37.6*	236,250	46.8*
Other Categories				
Housewife	538	54.4*	-	-
Student	38	3.8*	-	-
Armed Forces	0	0.0*	-	-
Unknown	41	4.1*	-	-
Total	989	100.0	504,578	100.0

1. Percent of total "employed" except where marked with asterisk; asterisk signifies percent based on total

TABLE III

OCCUPATION DISTRIBUTION
METROPOLITAN ATLANTA, MALES

	Interviewed Case Fathers, at Conception		All Atlanta Males ≥16 Years, 1970	
	Number	Percent[1]	Number	Percent[1]
Health Workers	9	1.1	4,894	1.4
Public School Teachers	8	0.9	3,200	0.9
Airline Pilots	6	0.7	1,681	0.5
Other Professional Workers	111	13.1	45,617	13.0
Managers	123	14.5	51,590	14.7
Sales Workers	60	7.1	35,428	10.1
Clerical Workers	83	9.8	35,797	10.2
Painters	13	1.5	3,736	1.1
Other Craftsmen	182	21.5	68,124	19.4
Gas and Garage Attendants	5	0.6	2,878	0.8
Other Operatives	136	16.1	52,666	15.0
Laborers	69	8.1	22,518	6.4
Service Workers	42	5.0	23,329	6.6
Total "Employed"	847	85.6*	351,458	78.6*
Other Categories				
Househusband	22	2.2*	-	-
Student	26	2.6*	-	-
Armed Forces	15	1.5*	-	-
Unknown	79	8.0*	-	-
Total	989	99.9	447,414	100.0

1. Percent of total "employed" except where marked with asterisk; asterisk signifies percent based on total.

TABLE IV

INDUSTRY OF EMPLOYMENT, METROPOLITAN ATLANTA

	Interviewed Case Fathers, At Conception		All Employed Persons ≥16 Years, 1970	
	Number	Percent[1]	Number	Percent[1]
Agriculture, Forestry, Fisheries	10	1.4	4,316	0.7
Mining	2	0.3	925	0.2
Construction	88	12.5	37,824	6.4
Manufacturing, Durable Goods	66	9.4	64,872	11.0
Printing and Publishing	11	1.6	9,375	1.6
Manufacturing, Chemical, Petroleum, Rubber	14	2.0	6,790	1.2
Manufacturing, Other Non-durable Goods	35	5.0	34,845	5.9
Transportation	75	10.6	35,274	6.0
Communication	20	2.8	11,865	2.0
Utilities and Sanitation	23	3.3	11,085	1.9
Wholesale, Drugs, Chemical, Petroleum	4	0.6	40,486	6.9
Other Wholesale Trades	22	3.1		
Gasoline, Retail	9	1.3	97,725	16.6
Other Retail Trades	93	13.2		
Finance, Insurance, Real Estate	41	5.8	43,369	7.4
Business and Repair Services	35	5.0	23,503	4.0
Personal Services, Laundering and Cleaning	2	0.3	32,544	5.5
Personal Services, Beauty and Barber	2	0.3		
Other Personal Services	5	0.7		
Entertainment	13	1.8	4,271	0.7
Health Services	14	2.0	25,765	4.4
Other Professional and Related Services	50	7.1	67,000	11.4
Public Administration, Including Armed Forces	71	10.1	35,874	6.1
Total "Reported"	705	71.3*	587,708	100.0
Other Categories				
Home	22	2.2*	-	-
Student	26	2.6*	-	-
Not Reported	236	23.9*	-	-
Total	989	100.0	587,708	100.0

1. Percent of total "reported" except where marked with asterisk; asterisk indicates percent of total.

TABLE V

MATERNAL EXPOSURE DURING FIRST TRIMESTER

	Occupation (Industry)		All Other Occupations (Industries)		Odds Ratio	χ^2_c
	Malf+	Malf-	Malf+	Malf-		
A) Major Central Nervous System Anomalies						
1) Sales Worker	7	7	226	749	3.3	4.1
B) Cleft Lip With/ Without Cleft Palate						
1) Health Worker/ Health Industry	10	24	150	805	2.2	3.6
2) Health Worker	11	26	149	803	2.3	4.2
3) Health Industry	16	40	144	789	2.2	5.8
4) Clerical Worker/ Business & Repair Services Industry	4	1	156	828	21.2	10.7
5) Business & Repair Services Industry	4	3	156	826	7.1	6.0
C) Reduction deformity						
1) Other Service Worker/Other Retail Trades Industry	7	13	92	877	5.1	11.5

TABLE VI

MATERNAL EXPOSURE AT OR BEFORE CONCEPTION

	Occupation (Industry)		All Other Occupations (Industries)		Odds Ratio	χ_c^2
	Malf+	Malf-	Malf+	Malf-		
A) Abnormalities of the Autosomal Chromosomes						
1) Manager/Printing Industry	2	0	155	832	-	5.3
b) Omphalocele and Gastroschisis						
1) Other Craftsmen/ Printing Industry	3	1	71	914	38.6	17.6
2) Other Craftsmen	3	5	71	910	7.7	6.6
3) Printing Industry	3	5	71	910	7.7	6.6

TABLE VII

PATERNAL EXPOSURE AT CONCEPTION

	Occupation (Industry)		All Other Occupations (Industries)		Odds Ratio	χ^2_c
	Malf+	Malf-	Malf+	Malf-		
A) Major Central Nervous System Anomalies						
1) Manager/Business & Repair Services Industry	4	2	229	754	6.6	4.1
B) Cleft Palate						
1) Clerical Worker/ Printing Industry	2	0	92	895	-	10.0
2) Painter/Construction Industry	3	5	91	890	5.9	4.4
C) Cleft Lip With/ Without Cleft Palate						
1) Sales Worker/Durable Goods Industry	3	1	157	828	15.8	6.4
2) Sales Worker/ Business & Repair Services Industry	2	0	158	829	-	5.1
3) Health Worker	4	5	156	824	4.2	3.5

TABLE VII (Cont'd)

	Occupation (Industry)		All Other Occupations (Industries)		Odds Ratio	χ^2_c
	Malf+	Malf-	Malf+	Malf-		
4) Business & Repair Services Industry	11	24	149	805	2.5	5.1
5) Clerical Worker/ Transportation Industry	5	7	155	822	3.8	4.1
C) Reduction Deformities						
1) Business & Repair Services Industry	8	27	91	863	2.8	5.3
D) Abnormalities of the Autosomal Chromosomes						
1) Manager/Non-durable Goods Industry	3	0	154	832	-	10.3
2) Other Craftsmen/ Durable Goods Industry	4	4	153	828	5.4	4.7
3) Other Craftsmen/Non-durable Goods Industry	3	2	154	830	8.1	4.4
4) Non-durable Goods Industry	11	24	146	808	2.5	5.4

TABLE VII (Cont'd)

	Occupation (Industry)		All Other Occupations (Industries)		Odds Ratio	χ^2_c
	Malf+	Malf-	Malf+	Malf-		
E) Diaphragmatic Hernia						
1) Other Operatives/ Construction Industry	2	5	38	944	9.9	5.5
F) Omphalocele and Gastroschisis						
1) Other Operatives/ Other Retail Trades Industry	4	9	70	906	5.8	7.2
2) Clerical Worker/ Durable Goods Industry	2	1	72	914	25.4	7.9

WASTE ANESTHETIC GASES AND REPRODUCTIVE HEALTH IN OPERATING ROOM PERSONNEL

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Our first awareness that waste anesthetic gases significantly contaminated the operating room environment undoubtedly coincided with the introduction of inhalation anesthesia into clinical practice. This had to be so since the earliest techniques for administration of chloroform and ether consisted of simply vaporizing the liquid anesthetic onto several layers of gauze placed over the patient's nose and mouth. One's sense of smell was thus sufficient to indicate detectable amounts of anesthetic contamination. Although easily recognized, this pollution generated little serious concern, and few efforts were made to reduce the waste anesthetic concentration.

The first suggestion that a health concern might be associated with waste anesthetic gas pollution followed publication of a report by Vaisman, in 1967, presenting results of a study which investigated the working conditions of Russian anesthetists.[1] Of some 354 anesthetists surveyed, many reported multiple complaints of itching, headache, and fatigue. Unfortunately, the data were uncontrolled, and in the light of the vague polysymptomatology, it was tempting to dismiss the findings entirely. What commanded attention was her observation indicating that 18 of 31 pregnancies among the female anesthetists terminated in spontaneous abortion, and only 7 of the pregnancies were without complication. During the next few years, similar disturbing findings were noted in several small-scale surveys conducted in Denmark,[2] the United States,[3] and the United Kingdom.[4] Although none of these studies were able to establish a precise etiology, the implication was that the waste anesthetic gases were in some way causal.

There is no doubt that waste anesthetic gases pollute the atmosphere of all operating rooms. In 1969, Linde and Bruce quantified these concentrations and found levels of waste halothane and nitrous oxide to average 10 ppm and 130 ppm, respectively.[5] Several years later, a similar study was conducted at Stanford University in which the concentration of halothane in the operating room was precisely measured with a portable mass spectrometer.[6] The concentrations of halothane were found to vary between 5-9 ppm depending on the anesthesia circuit used. It was also observed that waste anesthetic gases were distributed widely throughout the operating suite.

Of interest, subsequent experiments demonstrated that placement of a gas scavenging trap over the escape valve on the anesthesia machine would significantly reduce waste anesthetic concentrations (FIGURE 1). Removal of the waste gases was readily accomplished through a length of plastic tubing which provided a conduit to the outside via the nonrecirculating air conditioning system. This approach demonstrated a high level of efficiency (TABLE 1).

In the above study, the mass spectrometer was also used to measure end-tidal concentrations of halothane present in physicians and nurses working in the operating room (TABLE II). Results indicated that a significant

amount of waste anesthetic remained in the body, providing a carryover concentration from 1 day's exposure to the next. Similar data have been shown to obtain for other highly lipid soluble halogenated anesthetics.[7]

What is the experimental evidence that exposure to anesthetics produces harmful effects? In fact, significant data exist in the experimental animal indicating teratogenic effects in both the chick embryo and rat following exposure to clinical anesthetic concentrations during critical periods of gestation. For example, Shepard and Fink have demonstrated that marked skeletal changes are found in fetuses of rats exposed to 50% nitrous oxide of 1% halothane during the 8th to 11th day of gestation.[8]

It is important to keep in mind that these animal studies were conducted at clinical anesthetic concentrations which are 500-1,000 times greater than the trace levels found in an unscavenged operating room. On the other hand, information exists indicating that measurable toxic effects are also demonstrable following exposure to trace concentrations of anesthetics. For example, Corbett et al. have shown a toxic effect of low concentrations of nitrous oxide upon fetal resorption in the rat which corresponds to spontaneous miscarriage in the human.[9] In these studies concentrations of only 0.1% nitrous oxide (1,000 ppm) resulted in a significant increase in resorptions. Unfortunately, much of the animal data are variable and inconclusive. Other workers studying trace concentrations of ethrane, halothane, and nitrous oxide have been able to confirm the above results.

Although the effect of anesthetics on spontaneous miscarriage (fetal resorption) is an important observation, of greater concern is their potential effect on the production of fetal abnormalities. Knill-Jones and Spence were the first to suggest such a possibility as a result of their survey of the health conditions of practicing female anesthetists in the United Kingdom.[4]

Another area of concern is the possibility of an increased cancer rate among anesthetists. Several years ago Bruce et al. suggested the possibility of a small increase in cancer of the lymphoid system among members of the American Society of Anesthesiologists.[10] Although Bruce[11] was unable to confirm these findings in a second prospective study, Corbett et al.[12] subsequently published data indicating a significant increase in cancer rates in female Michigan nurse anesthetists.

Finally, the question of an increased incidence of liver disease among patients anesthetized with the halogenated anesthetics has, by implication, been directed to individuals chronically exposed to trace concentrations of halothane, methoxyflurane, enflurane, etc. in the operating room. This possibility is further supported in the studies by Cascorbi et al. suggesting that anesthetists, by virtue of chronic exposure to anesthetics in the operating room, have induced their liver enzymes.[13] As a consequence, anesthetists were shown to be able to metabolize halothane more efficiently than a control group of pharmacists.

This was the general background of concern against which the American Society of Anesthesiologists Ad Hoc Committee, with the support of the National Institute for Occupational Safety and Health, undertook a national study on the effects of trace anesthetics on the health of operating room

personnel.[14] The study began, late in 1972, with distribution of a questionnaire to all operating room personnel in the United States. Two mailings were made which included, in the exposed group, the combined membership of the American Society of Anesthesiologists (ASA), the American Association of Nurse Anesthetists (AANA), and the Association of Operating Room Nurses and Technicians (AORNT). The membership of the American Academy of Pediatrics (AAP) served as a control group for the physicians, and a segment of the membership of the American Nursing Association (ANA) provided a control for the operating room-exposed nurses and technicians (TABLE III).

Analysis of the available data included over 24,000 pregnancies which were equally divided between female respondents and wives of male respondents. In conducting statistical analyses associated with the outcome of these pregnancies, it proved necessary to apply an adjustment for the mother's age and her smoking habits, since both factors independently influenced the data (FIGURE 2). As anticipated, there was an increase in spontaneous abortion rates with increasing age of the mother. However, heavy smokers had significantly more miscarriages than did nonsmokers at each age level. The effect of smoking also turned out to be important in the incidence of congenital abnormalities. Women who smoked heavily during pregnancy (one pack or more per day) showed almost two times the number of congenital abnormalities in their children (FIGURE 3).

In analyzing the spontaneous miscarriage rate for female members of the American Society of Anesthesiologists actively working in the operating room, this rate was found to be twice that of the unexposed pediatrician group (TABLE IV). By definition, exposed females included women who were in the operating room during the first trimester of pregnancy, as well as the year preceding pregnancy. The control group consisted of women members of the American Academy of Pediatrics who had no operating room exposure. Increases in spontaneous abortion rates, but to a slightly lesser extent, were also found among nurse anesthetists, operating room nurses, and technicians.

Once again, although the question of increased spontaneous abortion is of considerable interest, of greater concern is the question of a possible increase in congenital abnormalities (TABLE V). The incidence of congenital abnormalities in the children of exposed female physician anesthetists was found to be approximately two times that of the unexposed pediatricians, and the membership of the American Association of Nurse Anesthetists showed a 60% increase.

An alternative method to examine these data is to perform an intragroup analysis by comparing exposed and unexposed respondents within the same society. If one contrasts female anesthetists who worked in the operating room during their first trimester of pregnancy, and for at least 1 year preceding, with female anesthetists who were away from the operating room during this same period, the increase in congenital abnormalities is of the same magnitude as that observed with an intergroup analysis.

Of particular interest is the observed increase in congenital abnormalities in children born to wives of operating room-exposed male anesthetists (TABLE VI). Since these women had no direct exposure to the operating room, the findings suggest an effect on spermatogenesis. In this connection, recent evidence in the rat by Kripke et al. indicates that young male rats exposed

to 20% nitrous oxide show a decrease in sperm production and an increase in sperm abnormalities.[15] The effects appear within 2 to 3 days of exposure, and are reversible upon removal of the animal from the contaminated environment. Most recently, data has originated from the Hazelton Laboratories indicating that male rats exposed to 1 part per million of halothane and 50 parts per million of nitrous oxide for a period of 1 year show significant chromosomal aberrations in bone marrow cells and spermatogonia.[16]

A serious problem is the question of cancer among exposed operating room personnel. If we compare exposed and control groups, there appears to be twice the incidence of cancer in exposed female anesthetists (TABLE VII). There are also significant increases for the nurse anesthetists and operating room nurses and technicians. These data suggest that the effect may be exposure related. If we break down the individual cancer cases according to histology or to anatomic site, we find no particular differentiation, with but one exception (TABLE VIII). The combined incidence of lymphoma and leukemia in exposed female respondents is three times that of the control, while the normal three to one sex differential in male versus female is maintained.

Although we have no satisfactory explanation, the increase in cancer rates appears to be limited to the female. There is no increase in male cancer (TABLE IX). Incidentally, this observation has been verified recently in a preliminary report by the American Cancer Society investigating causes of death among members of the American Society of Anesthesiologists.[17]

The incidence of liver disease appears higher in both males and females exposed to the operating room. The data exclude serum hepatitis, since anesthetists and other operating room personnel have increased exposure to blood and to blood products (TABLE X).

In considering the overall data, it would appear that significant health problems are associated with occupational exposure to the operating room. However, certain caution is needed in their interpretation. Although the national U.S. study analyzed information from over 40,000 exposed male and female respondents, and compared the health problems of these individuals, both intragroup and with over 10,000 selected controls, this study was subject to the problems associated with all retrospective surveys. In particular, there remains the possibility of bias, or misinterpretation, by the respondent who completed the questionnaire. There was no personal contact with the respondents, and the study depended entirely upon written replies.

Reassuring to the above results is comparative analysis of the U.S. data with that of the two large recent epidemiologic studies conducted in the United Kingdom.[18] Despite differences in methods of sampling and analysis, the studies reveal a remarkable degree of confirmation. Examination of data from female anesthetists in the U.S. and the U.K. shows similar increases in rates of spontaneous abortion and congenital abnormalities (TABLE XI). Unfortunately, the U.K. studies did not have data on female cancer or liver disease. Data obtained from the study of male anesthetists in the United Kingdom also show remarkable agreement (TABLE XII). In both countries, the incidence of spontaneous abortion in wives of the male anesthetists is unchanged from control. However, both indicate significant increases in the incidence of congenital abnormalities in offspring of

exposed males. Liver disease among male anesthetists was also significantly increased in both countries. In both the U.S. and U.K. the incidence of male cancer remained unchanged from control.

A question of practical importance relates to the effect of length of exposure to the operating room upon the incidence and severity of these health problems. Such data is difficult to come by, and in the case of cancer may involve exposure periods of 20 years or longer. However, analysis of the obstetrical data does provide some useful information.

Exposure of the pregnant woman during the first trimester of her pregnancy results in only a slight increase in spontaneous abortion and congenital abnormality rates. The increase is not statistically significant. However, if we add to this first trimester exposure the additional factor of long-term exposure for at least 1 year in the operating room preceding pregnancy, the risk increases markedly (FIGURE 4). Thus, both first trimester exposure and long-term exposure are contributory, with the long-term exposure apparently being the more significant factor.

An important corollary to the above question is whether a reduction in health hazard follows the woman's removal from the operating room. Fortunately, the data suggest that both spontaneous miscarriage and congenital abnormality rates tend to return to normal with time (FIGURE 5). Return to a normal spontaneous miscarriage rate occurs more rapidly than does the return rate for congenital abnormalities. The latter requires absence from the operating room of at least 2 years to reachieve baseline level.

In summary, it would appear that data from several large-scale studies are confirmatory in their findings that work in the operating room is associated with significant health risks to exposed operating room personnel and their offspring. Although a cause-effect relationship to the waste anesthetic gases remains to be proven, there would appear to be considerable support for such a possibility. Other potential contributions must of course be considered, including stress, chemicals peculiar to the operating room, the presence of oncotic viruses, etc.

There is, however, little question as to the appropriate wisdom for minimizing exposure risk in the operating room by removing the waste anesthetic gases. Such recommendation has indeed been made by the American Society of Anesthesiologists Ad Hoc Committee, and regulations for its implementation are included in the Criteria Document published by the National Institute for Occupational Safety and Health.[19]

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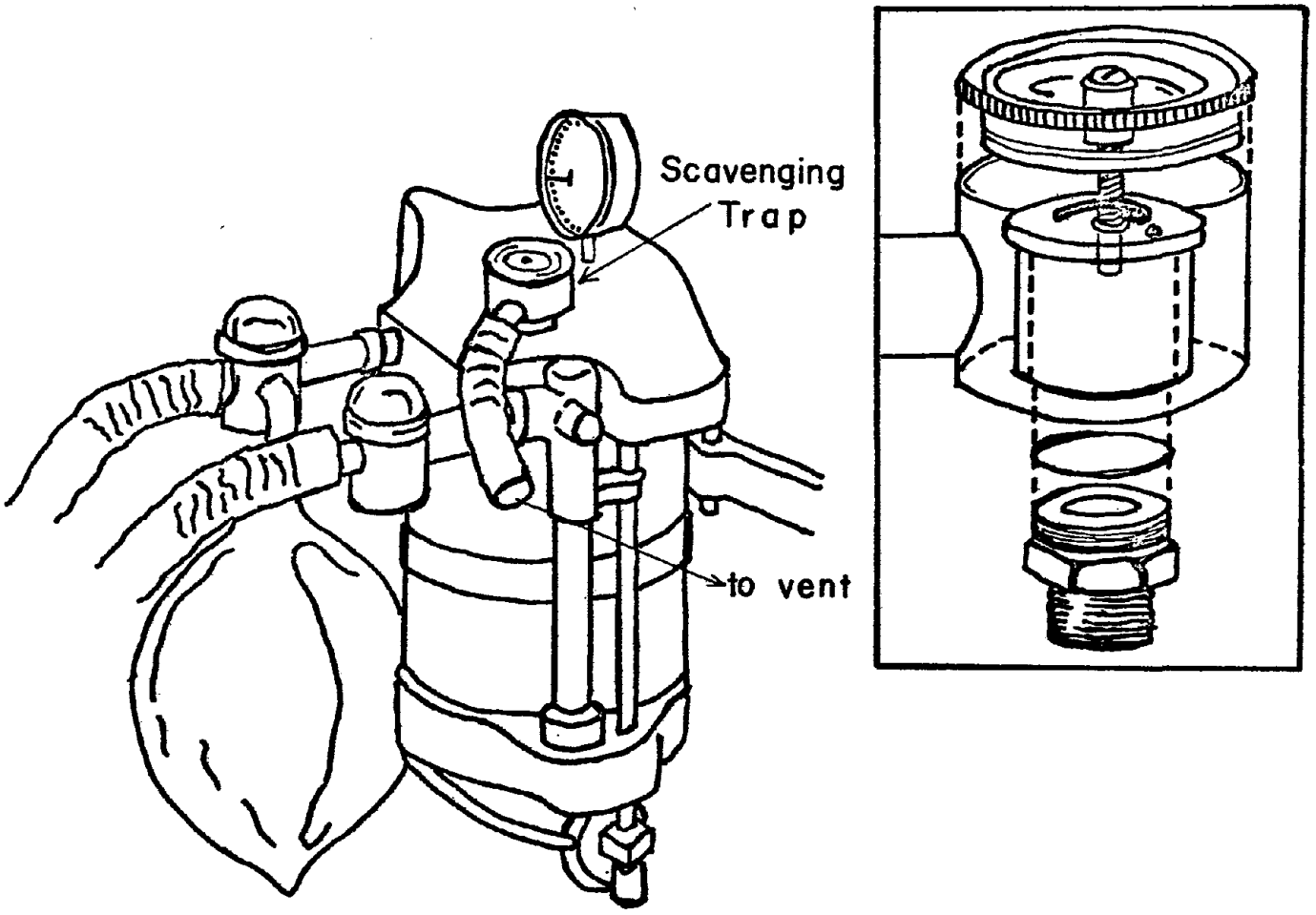

FIGURE 1

Anesthetic gas scavenging trap

FIGURE 2

Risk of spontaneous abortion according to age in smokers versus nonsmokers

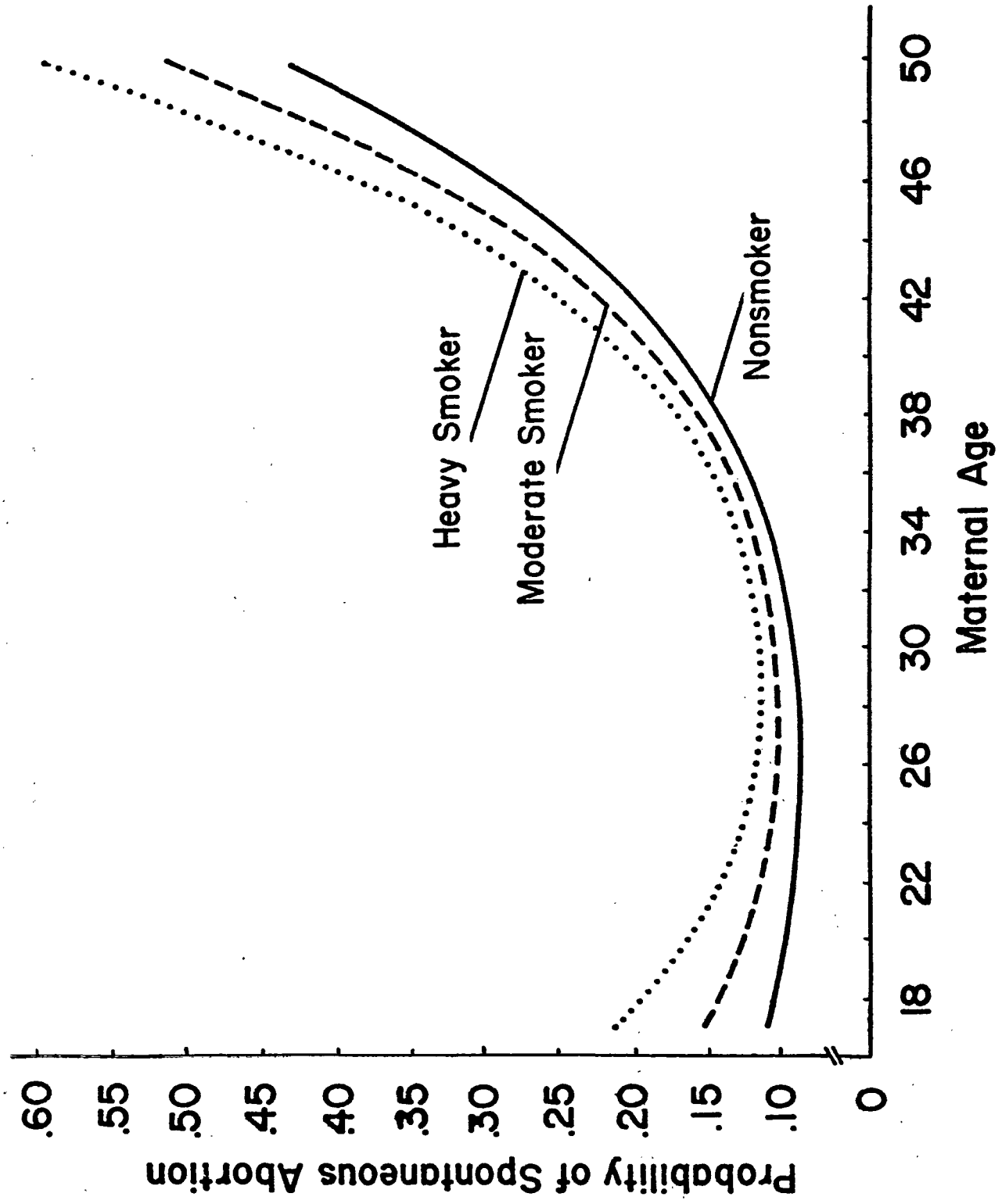

FIGURE 3

Risk of congenital abnormality in children according to age and smoking habit of mother

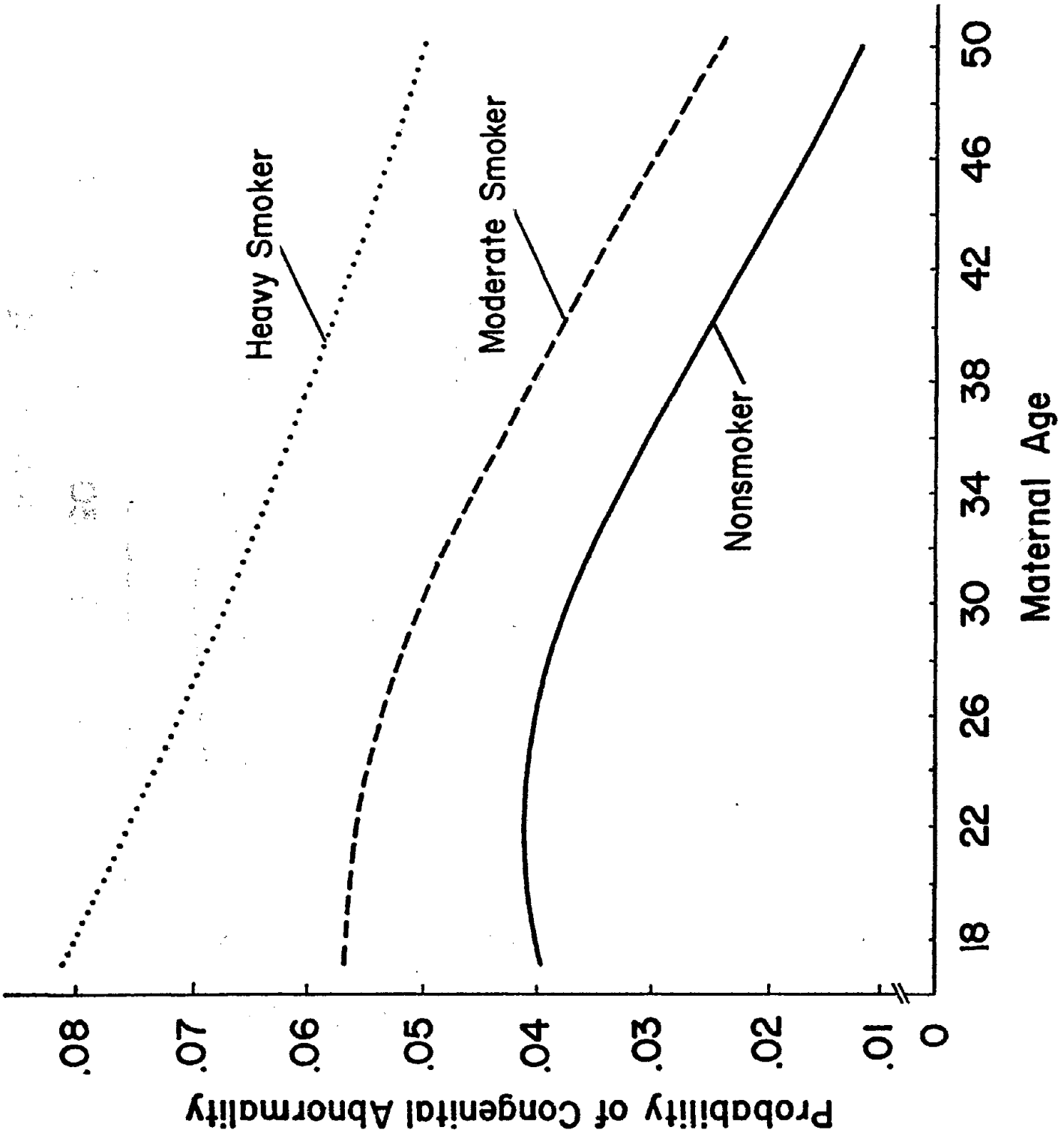

FIGURE 4

Risk of spontaneous miscarriage and of congenital abnormality in their children for women anesthetists. Exposed women have worked in the operating room during their first trimester and the year preceding.

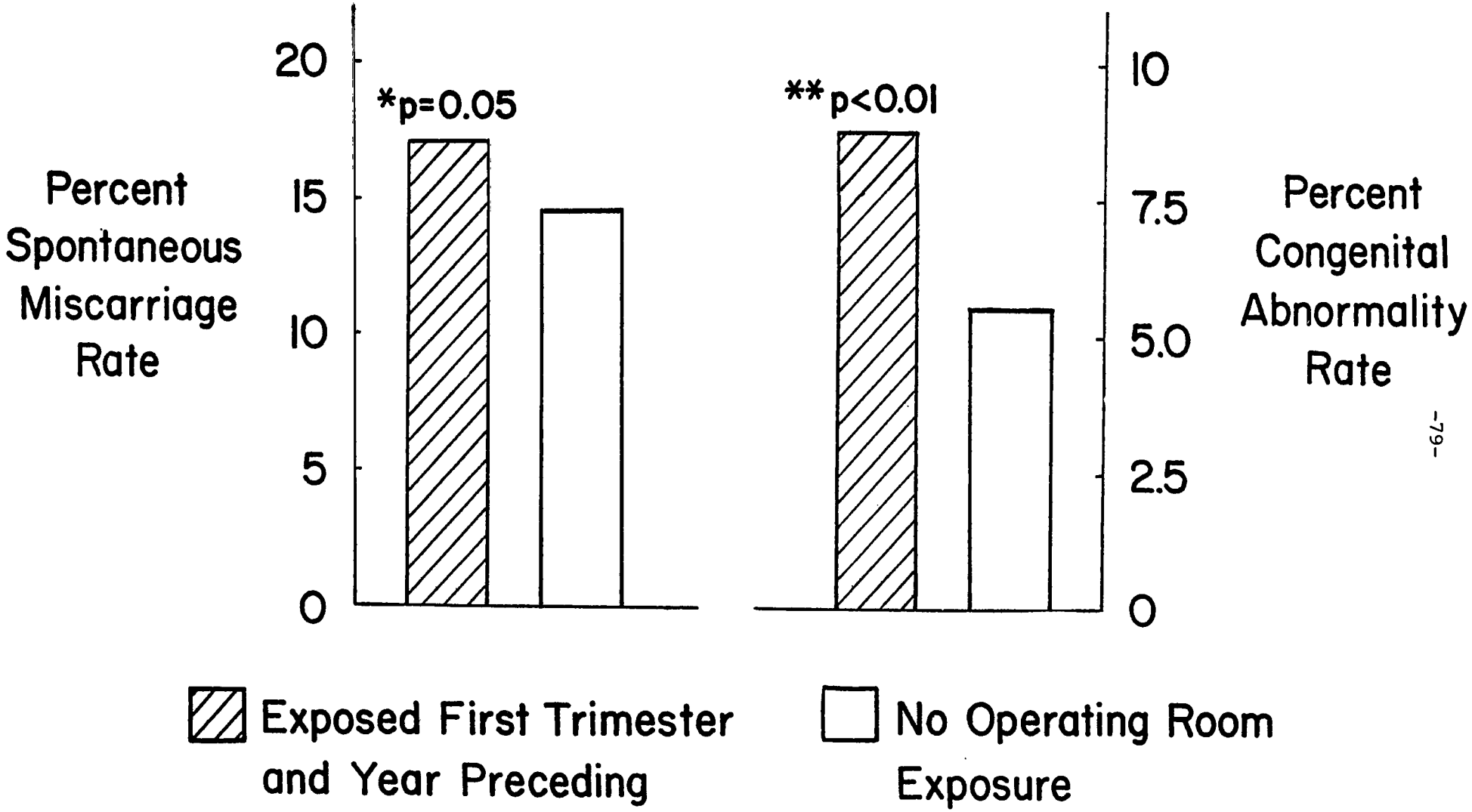

FIGURE 5

Decrease in risk of spontaneous miscarriage and of congenital abnormality in their children for women anesthetists upon their absence from the operating room

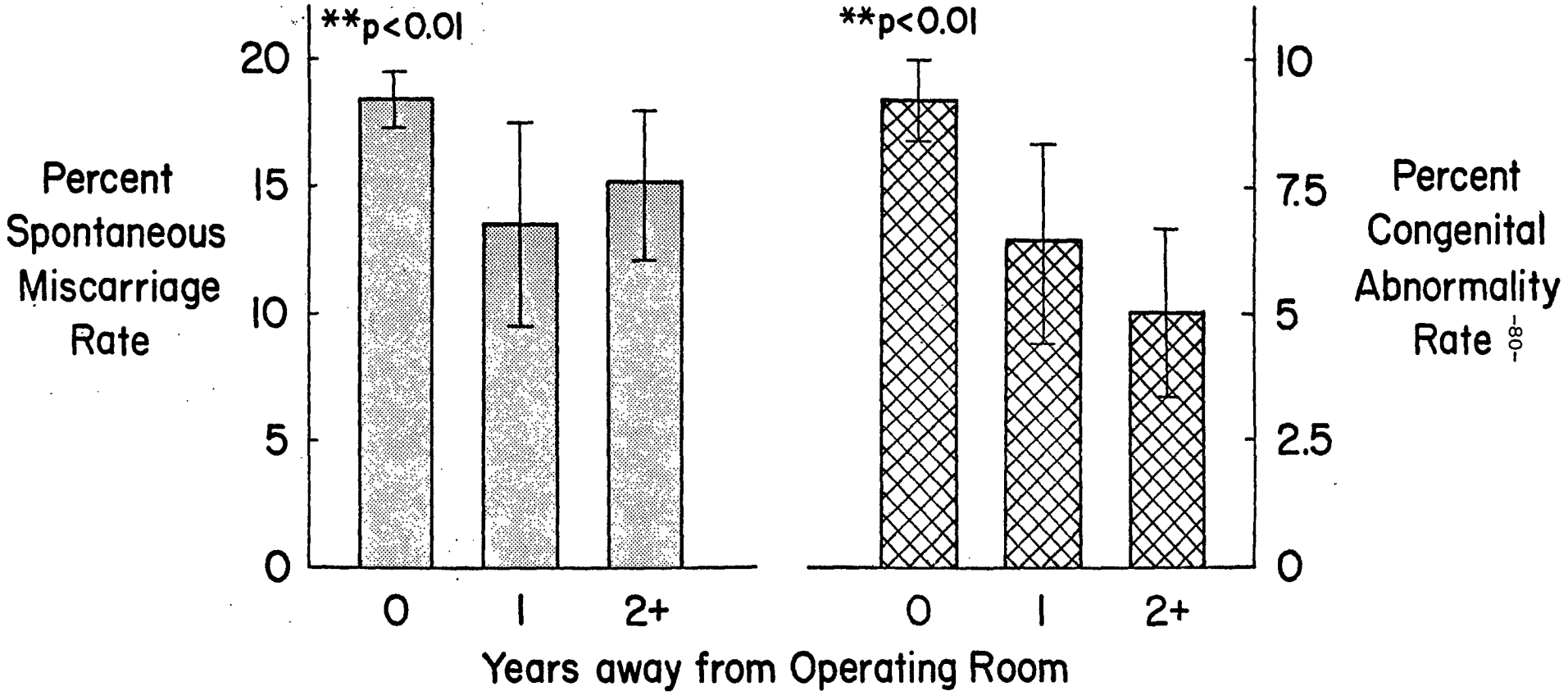

TABLE I

CONCENTRATIONS OF WASTE HALOTHANE IN THE O.R. USING VARIOUS ANESTHESIA CIRCUITS, WITH AND WITHOUT SCAVENGING

	ppm Halothane (± S.E.)	Percent Reduction
Non-rebreathing system (no scavenging)	8.69 ± 0.91	-
Non-rebreathing system (with scavenging)	0.79 ± 0.15	91%
Semi-closed circle system (no scavenging)	4.93 ± 0.96	-
Semi-closed circle system (with scavenging)	0.73 ± 0.10	85%

TABLE II

MEAN END-TIDAL CONCENTRATIONS OF HALOTHANE IN NURSES AND ANESTHETISTS

	Initial Halothane Concentration (ppm ± S.E.)	Mean Halothane Concentration During Work Day (ppm ± S.E.)
Nurses (n = 27)	0.01 ± .006	0.21 ± .04
Anesthetists (n = 9)	0.08 ± .03	0.46 ± .07

TABLE III

QUESTIONNAIRE RESPONSE RATES FOR THE VARIOUS PROFESSIONAL SOCIETIES

	Membership Survey	Total Returns	Percent of Total
ASA	11,192	8,071	72.1
AAP	7,910	3,759	47.6
AANA	14,594	8,991	61.6
AORN-T	23,799	13,449	56.5
ANA	16,001	6,887	43.0
	73,496	41,157	

TABLE IV

AGE AND SMOKING STANDARDIZED RATES FOR SPONTANEOUS ABORTIONS IN FEMALE PHYSICIANS

ASA	17.1 ± 2.0 (468)	AAP	8.9 ± 1.8 (308)

$p < 0.01$

TABLE V

AGE AND SMOKING STANDARDIZED RATES FOR CONGENITAL ABNORMALITIES IN CHILDREN OF FEMALE PHYSICIANS

ASA	5.9 ± 1.4 (384)	AAP	3.0 ± 1.1 (276)

$p = 0.07$

TABLE VI

AGE AND SMOKING STANDARDIZED RATES FOR CONGENITAL ABNORMALITIES IN CHILDREN OF EXPOSED MALE PHYSICIANS

ASA	5.4 ± 0.4 (2,988)	AAP	4.2 ± 0.5 (1,714)
	p = 0.04		

TABLE VII

AGE STANDARDIZED CANCER INCIDENCE AMONG FEMALE RESPONDENTS IN THE VARIOUS PROFESSIONAL SOCIETIES

ASA	3.0 ± 0.6 (1,008)	AAP	1.6 ± 0.5 (566)	p = 0.05
AANA	2.6 ± 0.2 (6,407)	ANA	1.8 ± 0.2 (5,400)	p <0.01
AORN-T	2.3 ± 0.2 (11,843)	ANA	1.8 ± 0.2 (5,400)	p = 0.07

TABLE VIII

INCIDENCE OF LYMPHOMA AND LEUKEMIA AMONG MALES AND FEMALES IN THE VARIOUS PROFESSIONAL SOCIETIES

	Exposed	Unexposed	
Males	0.078 ± 0.028 (8,942)	0.076 ± 0.052 (2,603)	p = 0.49
Females	0.062 ± 0.002 (19,258)	0.017 ± 0.017 (5,928)	p = 0.05

TABLE IX

AGE STANDARDIZED CANCER INCIDENCE AMONG MALE PHYSICIANS

ASA	0.7 ± 0.1 (6,233)	AAP	0.7 ± 0.2 (2,495)
	p = 0.49		

TABLE X

AGE STANDARDIZED LIVER DISEASE RATES AMONG FEMALE MEMBERS OF THE VARIOUS PROFESSIONAL SOCIETIES

ASA	4.9 ± 0.7 (924)	AAP	2.9 ± 0.8 (512)	p = 0.04
AANA	3.8 ± 0.3 (5,178)	ANA	1.7 ± 0.2 (4,512)	p <0.01
AORN-T	2.1 ± 0.2 (9,741)	ANA	1.7 ± 0.2 (4,512)	p = 0.08

TABLE XI

COMPARATIVE RATES FOR OCCUPATIONALLY ASSOCIATED DISEASES IN THE U.S. AND U.K. IN FEMALE PHYSICIAN ANESTHETISTS AND CONTROL GROUPS

	Anesthetists		Control Physicians	
	U.S.	U.K.	U.S.	U.K.
Spontaneous Abortion	17.1** (1,061)	17.5* (1,034)	8.9 (642)	13.9 (1,330)
Congenital Abnormalities	5.9 (384)	5.5* (883)	3.0 (276)	4.2 (1,817)
Liver Disease	4.9* (924)		2.9 (512)	
Cancer	3.0* (1,008)		1.6 (566)	

*p = <0.05
**p <0.01

TABLE XII

COMPARATIVE RATES FOR OCCUPATIONALLY ASSOCIATED DISEASES IN THE U.S. AND U.K. IN MALE PHYSICIAN ANESTHETISTS AND CONTROL GROUPS

	Anesthetists		Control Physicians	
	U.S.	U.K.	U.S.	U.K.
Spontaneous Abortion (Spouses)	11.6 (3,416)	10.4 (5,891)	12.6 (1,982)	10.2 (7,296)
Congenital Abnormalities	5.4* (2,988)	3.9* (5,175)	4.2 (1,714)	3.2 (6,442)
Liver Disease	4.3** (4,218)	3.8** (1,796)	2.7 (2,327)	2.3 (4,533)
Cancer	0.7 (6,233)	1.1 (1,796)	0.7 (2,495)	0.8 (4,533)

* $p = <0.05$
** $p < 0.01$

CHLOROPRENE: ADVERSE EFFECTS ON REPRODUCTION

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Chloroprene 2-chlorobutadiene is a colorless, flammable, volatile liquid with a pungent, ethereal odor. An estimated one billion pounds of chloroprene are produced annually in the United States.[1] All of the chloroprene produced is subjected to polymerization, either into polychloroprene, a synthetic rubber marketed in the U.S. under the trade name Neoprene, or into a liquid polymer, polychloroprene latex. An estimated 2,000 workers have direct exposure to this agent in the U.S. during the manufacture of chloroprene monomer and its polymerization into polychloroprene. The number of workers having indirect exposure through working with polychloroprene rubber or latex is not known. Because of the structural similarity of chloroprene to vinyl chloride, a known carcinogen and mutagen, a search and synthesis of chloroprene-related research addressing carcinogenesis and mutagenesis was undertaken. This review will emphasize the data bearing on potential mutagenic or reproductive hazards.

With regard to carcinogenesis, one experimental study has been reported as negative.[2] However, because of limited presentation of information and inadequate study design and methodology, interpretation of the study results is not possible.[3] In terms of epidemiologic observations, two studies have concluded that workers exposed to chloroprene are at an increased risk of developing lung and skin cancer.[4,5] A third study also is suggestive of an increased risk of lung cancer among a subset of maintenance mechanics exposed to chloroprene.[6] These studies, however, have not given adequate consideration to job classification, environmental concentrations, intensity and duration of exposure, or latency--factors known to influence the risk of cancer.[7] In two studies, no mention is made of age adjustment procedures nor of the criteria used to diagnose the cancers.[4,5] On the basis of these studies, it is difficult to develop valid inferences regarding the carcinogenicity of chloroprene.

With regard to mutagenicity or adverse effects on reproduction, as far back as 1936, Von Oettingen et al. reported infertility associated with chloroprene exposure to male mice.[8] The fertility rate (number of animals pregnant) in mice exposed to chloroprene levels ranging from 12 to 150 parts per million was 43% (6/14) versus 83% (5/6) for controls. Most chloroprene-exposed mice received doses at the relatively lower end of the dose range. In rats exposed to higher levels of chloroprene, the fertility rate was 32% (6/19) as compared to 100% (5/5) for the control group.

TABLE I shows the effects of chloroprene on testicular weight and on the sperm of rats. At atmospheric concentrations of 0.5 parts per million (ppm), five of the eight animals were reported to have atrophied testicles.[9,10]

Total embryonic mortality was 40.9% in the chloroprene-exposed group as compared to 9.9% in the control group. The results were reported as statistically significant. The authors stated that the embryonic mortality was due to preimplantation loss, not postimplantation loss. Also the number of dead spermatozoa for animals with non-atrophied testicles was reported to be 85% as compared to 32% in the control animals. Spermatozoan motility time in minutes was 91 for the chloroprene-exposed rats, as compared to 333 for control animals.

TABLE II shows the results of a dominant lethal test by Davtian et al.[11] Following male rat exposure to chloroprene at 11 ppm, and also at 1.1 ppm, a significant excess of total embryonic mortality was observed. Again, most of the excess is a reflection of preimplantation loss.

The authors state that 36 animals were used, but it is not possible to determine whether there were 36 total animals or 36 in each group. There also does not appear to be an increase with an increase in the dosage of chloroprene in this particular study.

TABLE III shows the study results of a dominant lethal test in the mouse.[12] Following 1.0 ppm atmospheric chloroprene exposure to male mice, preimplantation loss was reported to be 32%, as compared to 3% in controls. At 0.5 ppm, 27% preimplantation loss was observed versus 11% for controls. Total embryonic mortality was in significant excess at both chloroprene exposure levels.

TABLE IV shows data from analyses of bone marrow cells of some of the same mice that were exposed to chloroprene and used for the dominant lethal test.[12] At concentrations of 1 ppm or 0.5 ppm, a significant excess in the percentage of aberrant cells was observed. Ten percent of the cells had aberrations in each exposure group as compared to 2% and 3% in the control animals. In this study, it also appears that an average of 80-100 cells were analyzed per animal.

TABLE V shows a summary of experimental studies indicating cytogenetic, mutagenic, or reproductive effects of chloroprene. In 1972, Davtian reported a dominant lethal effect, effects on the sperm, and testicular atrophy in the rat.[9] In 1973, Davtian et al. reported dominant lethality and chromosomal aberrations in bone marrow cells in the rat.[11] Again, in 1974, Bagramyan and Babayan reported chromosomal aberrations to be in significant excess in bone marrow cells in the rat.[13] Davtian[9] and Volkova[10] also reported dominant lethality and effects on the sperm. In 1976, Sanotskii reported dominant lethality and chromosomal aberrations in mice.[12] In 1975, Bartsch et al. reported that chloroprene was mutagenic in Salmonella Typhimurium, TA-100.[14] Brusick has also indicated that chloroprene is slightly mutagenic in Salmonella Typhimurium.[15] Vogel has demonstrated sex-linked recessive lethal mutations in Drosophila.[16]

Thus, reports indicate that chloroprene is mutagenic in bacteria, is sex-linked recessive lethal in Drosophila, and causes both dominant lethality as well as chromosomal aberrations in bone marrow cells in the mouse and rat. In 1936, chloroprene had been associated with sterility and later with decreased numbers and motility of sperm as well as testicular atrophy in mice and rats at very low concentrations.

With regard to cytogenetic or reproductive effects in humans, TABLE VI shows data for the frequency of chromosomal aberrations in lymphocyte cultures from workers in the Soviet Union.[12] The control group contained nine subjects that were not exposed to chloroprene, while the study group consisted of 18 workers indicated as having an average chloroprene exposure concentration of 5 ppm. The percentage of aberrant cells with chromosomal aberrations was 4.9% in the study group versus 0.65% in the concurrent control group and 1.2% in the historical control group. The frequency of gaps per 100 cells is 3.7 in the exposed workers versus 1.1 in the control group. This observation also was indicated as statistically significant.

In terms of epidemiologic considerations, from the data in TABLE VI, it is noteworthy that there is an age difference of 6 years between the study and control group. Data not shown in TABLE VI indicate that the number of cells analyzed per individual in the study group ranged from 45-185 cells while the number of cells analyzed per individual in the control group ranged from 19-43 cells. Also, 5 out of 18 in the study group were women, while 1 out of 8 in the control group was a woman.

TABLE VII shows data for the frequency of chromosomal aberrations in lymphocytes of female workers exposed to chloroprene at two concentrations as contrasted with historical controls. A significant excess of cells with aberrations is demonstrated for the group of 20 women whose age ranged between 19-23 years. A second group of eight women exposed to lower levels of chloroprene and whose ages ranged between 19-50 years also had a significantly elevated number of cells with aberrant chromosomes. The mean number of cells analyzed per exposure group is 87 and 81, respectively.

TABLE VIII shows study results by Bochkov.[17] Again, a significant excess of cells with aberrant chromosomes is apparent for workers exposed to chloroprene. The mean number of cells analyzed per individual was 100 for the chloroprene-exposed group, while an average of 137 cells was analyzed for subjects in the control group.

TABLE IX shows a summary of human studies indicating cytogenetic or reproductive effects of chloroprene. In addition to chromosomal aberrations, Sanotskii has reported that the examinations of chloroprene workers revealed functional disturbances in spermatogenesis after 6 to 10 years of work in chloroprene production, and morphological disturbances after 11 or more years.[12] The reproductive function in the wives of 143 workers exposed to chloroprene indicated a threefold excess of spontaneous abortion, as compared to wives of 118 controls consisting of factory and office workers in an electrical engineering plant.

Although any single study cited in the summary of the literature does not allow one to definitively conclude that chloroprene is mutagenic, the consistency of positive response, and the number of test systems indicating a positive mutagenic response, as well as additional observations indicating that chloroprene affects the sperm, testicles, and reproductive outcome as a result of male exposure only, it would seem that a prudent approach would be to control chloroprene as an agent which possesses a potential mutagenic risk to humans.

In view of the data presented in this summary of reports on the mutagenicity of chloroprene, several points need to be addressed initially in terms of methodologic considerations. They are as follows: How sensitive is the dominant lethal test, and which is the most sensitive species for use in conducting the test? What is the significance of preimplantation versus postimplantation loss? In cytogenetic studies, what is the significance of gaps versus chromatid or chromosome breaks? How many cells should be analyzed per individual in order to identify with reasonable probability an aberrant cell? What is the appropriate sample size of individuals needed? (These latter two questions are dealt with in two recent publications.)[18,19] Are sex, race, and age variables that are related to the prevalence of cytogenetically abnormal cells?

In addition, when conducting human studies, is it possible to measure either the agents or metabolites of the agents being studied in human tissue, in order to biologically verify that those individuals with presumptive exposure were, in actuality, exposed to the agent under study? It is not uncommon to review the results of studies where there is no confirmation that individuals in the study group were exposed to the agent under study, while subjects in the control group were exposed to other known mutagens or carcinogens. This is particularly the situation when the control group consists of other industrially exposed workers. Such methodology would presumably result in an underestimate or dilution of the effects of the agent being studied.

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TABLE I

Effects on testicular weight and sperm
of rats exposed to chloroprene

Atmospheric chloroprene concentration (ppm)	No. male animals exposed	No. with atrophied testicles	Total embryonic mortality	No. dead spermatozoa[a] (%)	Spermatozoan motility time (min.)
0.5	8	5	40.9±11.5*	85±13.8**	91±44*
0	8	1	9.9±1.8	32±9.9	333±15

SOURCE: Davtian (1972); Volkova et al. (1976).

[a]For animals with non-atrophied testicles

*p<0.05.
**p<0.01.

TABLE II

Frequency of embryonic mortality following male rat exposure to chloroprene

Atmospheric chloroprene concentration (ppm)	Preimplantation loss (%)	Postimplantation loss (%)	Total embryonic mortality (%)
11.0	26.0±4.4[a]	8.4±3.4	32.01±7.4[b]
1.1	27.2±6.0[b]	4.7±1.5	30.08±5.7[b]
0	11.7±2.9	2.2±1.1	12.88±2.7

SOURCE: Davtian, Fomenko, and Andreyeva (1973). (Number of animals in each group not stated.)

[a] All values are means ± S.E.

[b] $p<0.05$.

TABLE III

Frequency of dominant lethal mutations following male mice exposure to chloroprene

Atmospheric chloroprene concentration (ppm)	No. of animals M	F	Fert. capacity (%)	Preimplantation deaths (%)	Postimplantation deaths (%)	Total embryonic mortality (%)
1.0	15	30	52±8[a]	32±10*	35±3	63±10*
0	14	31	54±8	3±2	26±10	28±10
0.5	11	31	80±10	27±4*	27±4	42±6**
0	10	25	80±11	11±4	10±?	19±6

SOURCE: Sanotskii (1976).

[a]Values are means ± S.E.

*p<0.05.
**p<0.01.

TABLE IV

Metaphase analysis of bone marrow cells
of mice exposed to chloroprene
for two months

Atmospheric chloroprene concentration	No. of animals	No. cells analyzed	Aberrant cells (%)
1.0	8	799	10.0±0.7*
0	8	750	3.0±0.5
0.5	10	910	10.9±1.3*
0	6	488	2.0±0.6

SOURCE: Sanotskii (1976).

*$p<0.001$.

TABLE V

Laboratory studies indicating cytogenetic, mutagenic, or reproductive effects of chloroprene

Laboratory test system	Observation	Investigators	Reference
Mice & rats	Sterility	Von Oettingen, Hueper, Deichmann-Gruebler, Wiley (1936)	
Rat	1. Dominant lethal 2. Effects on sperm 3. Testicular atrophy	Davtian (1972)	10
Rat	1. Chromosomal aberrations 2. Dominant lethal	Davtian, Fomenko, Andreyeva (1973)	
Rat	Chromosomal aberrations	Bagramian and Babaian (1974)	
Rat	1. Dominant lethal 2. Effects on sperm	Davtian (1972); Volkova (1976)	
Mice	1. Dominant lethal 2. Chromosomal aberrations	Sanotskii (1976)	
S. Typhimurium	Mutagenic	Bartsch, Malaveille, Montesano, Tomatis (1975)	
S. Typhimurium	Mutagenic	Brusick (unpub. 1977)	
Drosophila	Recessive lethal	Vogel (1975)	

TABLE VI

Frequency and types of chromosomal aberrations in lymphocyte cultures from workers in the Soviet Union

Group	Subjects studied	Mean age (yrs.)	No. cells analyzed	Aberrant cells (%)	Rate per 100 cells	
					Aberrations	Gaps
Chloroprene workers[a]	18	39	1,666	4.77±0.57+	4.9	3.71±0.59*
Controls[a]	9	33	572	0.65±0.56	1.0	1.14±0.43
Spontaneous[b]	181	--	28,386	1.19±0.06	1.2	

Average atmospheric chloroprene concentrations = 5.0 ppm.

[a]Data from L. D. Katosova (1973).

[b]Data from N. P. Bochkov (1972) reported by Sanotskii (1976).

+$p<0.001$ all values are means ± S.E.

*$p<0.01$.

TABLE VII

Frequency of chromosomal aberrations in lymphocyte cultures from workers in the Soviet Union

Atmospheric chloroprene concentration	Duration employment (years)	Subjects studied (women)	Age range (years)	No. cells analyzed	Aberrant cells (%)
0.8-1.9[a]	1-4	20	19-23	1,748	3.49±0.51+
0.3-1.1[b]	1-20	8	19-50	648	2.50±0.49*
0[c]	----	181	-----	28,386	1.19+0.06

[a]Volkova et al. (1976).

[b]Data from Fomenko and Katosova (1973) reported by Sanotskii (1976).

[c]Data from N. P. Bochkov (1972) reported by I. V. Sanotskii (1976).

+$p<0.001$ all values are means ± S.E.

*$p<0.01$.

TABLE VIII

Frequency of chromosomal aberrations in lymphocyte cultures from workers in the Soviet Union

Group	Subjects studied	No. cells analyzed	Aberrant cells (%)
Chloroprene workers	57	5,720	2.90
Controls	437	60,020	1.19

SOURCE: N. P. Bochkov (1976).

TABLE IX

Human studies indicating cytogenetic or reproductive effects of chloroprene

Human studies	Observation	Investigators
Workers	Chromosomal aberrations	Katosova (1973)
Workers	Chromosomal aberrations	Bochkov (1976)
Workers	Chromosomal aberrations	Sanotskii (1976) reporting data of Fomenko and Katosova (1973)
Workers	Chromosomal aberrations	Volkova et al. (1976)
Workers	1. Decrease in motility and number of sperm 2. Three-fold excess of miscarriages in wives of male workers	Sanotskii (1976)

DIBROMOCHLOROPROPANE (DBCP)

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I would like to discuss the use, toxicology, human experience, and some unanswered questions regarding 1,2-dibromo-3-chloropropane (DBCP). This soil fumigant has been commercially produced since 1956, and has been the compound of choice against nematodes on the following types of plants. In California, it is used on grapes, peaches, citrus fruits, tomatoes, and some other plants; in Hawaii, it is used primarily on pineapples; in the Carolinas, it is used on peaches and soybeans. In Central America and Israel it is used on bananas.

In California it is applied in one of two ways: either it is added to the irrigation water--or it is metered into the ground where the farmers have a tractor-like wheel that disperses the material into the ground. A few farmers have used sprinklers, but if one is interested in eliminating nematodes this means of application is not very effective, since the nematodes are not on the leaves. As a fumigant, DBCP is much safer to apply than some of the other commonly used gaseous fumigants, e.g., methyl bromide or hydrocyanic acid (hydrogen cyanide), primarily because DBCP is a liquid. Also, the acute effects of methyl bromide and hydrogen cyanide are much more severe than are the acute effects of DBCP.

The toxicology of DBCP was first reported in 1961 by Torkleson, et al. who observed hepatic and renal effects, plus testicular atrophy in rats.[1] Testicular atrophy was noted at the lowest exposure level tested, i.e., five parts per million. On the basis of extrapolation, the report also stated that one part per million was presumed to be a safe exposure level. Testicular effects also were observed in guinea pigs and rabbits--essentially every species tested developed testicular atrophy.

Faidysh,[2] from the Soviet Union, reported in 1970 that DBCP had demonstrated hepatic, renal, and testicular effects, and that these effects were reversible. Olson reported that mice and rats fed DBCP by oral gavage developed gastric carcinoma.[3] The female rats also developed mammary cancer. Rosenkranz reported that DBCP was mutagenic in bacterial systems.[4]

With that background, I will discuss what has occurred in man.

The Occidental Chemical Company is located in Lathrop, California, in the central valley and farming heartland of the State. The Occidental Chemical Company formulates pesticides. In the formulation of pesticides, technical grade material is taken from the manufacturers and mixed and diluted with solvents, or several compounds may be mixed together in preparation for use by farmers. Most technical mterials are not suitable for farmers' use.

The DCBP was handled in one particular area of the plant, the agricultural chemical division. From 1968 to 1977, the Occidental Chemical Company formulated approximately from 1.5 to 4 million pounds of DBCP per year. This was about 10% of the total produced in the United States. DBCP comprised about 20% of the total product from the Agricultural Chemical Division (ACD).[5] This Division produces an additional 100 products.

For several years there had been a rumor among the agri-chem workers that if you worked in this area of the plant, you did not have children. The wives of most of the men who worked in this area were in the childbearing age. It had been observed, however, that no one had children after starting to work in the ACD.

In July of 1977, the union asked some individuals to volunteer for semen analysis. The laboratory agreed, but added that results would only be sent to a physician. Based on my previous relationship with the union, as a consultant to the university program in which I work, the results were sent to me. In July 1977, an envelope containing the results of lab studies arrived in the mail. When I examined the contents, the first slip had a name and sperm count. Result: no sperm seen. The following one read, sperm count, result: no sperm seen. The next one: sperm count, result: no sperm seen; likewise for about five or six of them.

Having never heard of the town or the laboratory, I was a little suspicious, and decided that the most reasonable course of action would be to reconfirm the results. Whenever the results of laboratory tests cannot be readily explained, there is a tendency to repeat the procedure.

I wondered what would happen if I went to this small town to talk to these men and told them it looked like they were all sterile, probably as a result of something to which they were exposed at the plant, and left. I thought maybe we should also inform the company.

The union leader contacted the company and announced that a physician was going to talk to the workers, as well as to the company management. He informed them of the reasons for this.

On July 17, 1977, a visit was made to the Occidental Chemical Plant. We first talked to the management.

In order to reconfirm the results for the five workers with zero sperm count, they were asked to remain continent for 3 days prior to producing a new sample. Four were azoospermic (zero sperm count), and one was severely oligospermic (low sperm count). A complete history and physical for each of the individuals, as well as extensive laboratory tests, were made. Based on these results, a meeting was held with both the union and the management, and it was decided to evaluate the rest of the employees who worked in this one particular area of the plant. During the next few weeks, an additional 34 individuals were examined.

Of the initial 39 people who worked in the agri-chem area, or who worked with ACD chemicals, three were women. One was a production worker, who had been taking birth control pills and the remaining two were secretaries.

Of the 36 men, 11 were vasectomized. Of the 25 remaining, 11 were severely oligospermic or azoospermic, 3 had intermediate counts (between 10 and 30 million), and 11 had sperm counts above 40 million.

In all 36 men (both those affected and those not affected) there were no changes in libido, erectile ability, or sexual performance. There was no history of impotence, and no evidence of gynecomastia. In essence, they had no abnormal histories or physical findings.

Other than abnormalities of the semen and the gonadotropins (FSH and LH), laboratory evaluation did not reveal any hematological, renal, hepatic, or thyroid function abnormalities.

TABLE I shows data for nonvasectomized DBCP-exposed workers who had very low (group A) or normal (group B) sperm counts. As indicated in the table, those with normal sperm counts had worked a relatively shorter period of time.

The mean sperm counts for groups A and B were 0.2 and 93 million, respectively. FSH in mIu/ml for group A was 11.3; for group B it was 2.6. In this laboratory, the upper limit of normal is 6.0. Thus, this difference between groups A and B is highly significant. LH for group A was 28.4 mIu/ml as compared to 14 for group B. This difference is also significant at $p < 0.01$. For testosterone no differences were observed between groups A and B.

The two primary manufacturers of DBCP, Dow Chemical Company and the Shell Chemical Company, also studied some of their workers. The Dow Arkansas plant had been producing DBCP for approximately 2 years.

TABLE II shows percentage distributions for workers in the Dow Arkansas facility for those who were exposed to DBCP versus those who were not exposed. Almost 33% of those exposed were azoospermic as compared to 4% for those categorized as not exposed to DBCP.

The Shell Oil Company has two plants, one in Mobile, Alabama, and one in Denver, Colorado. The Alabama plant had been making DBCP for at least 15 months. The Denver facility had ceased manufacturing DBCP in June 1976.

TABLE III shows data for DBCP-exposed versus non-DBCP-exposed employees from the Shell Mobile facility. A significant difference between the groups was observed for those with sperm counts of less than 20 million. Essentially, 20% of the DBCP exposed, as compared to 0% of the controls had sperm counts of less than 20 million.

TABLE IV shows data for the Denver plant, where DBCP had not been made for the past 2 years. Of those exposed to DBCP, 7.1% were azoospermic as compared to zero for controls. There has been some difficulty in determining exposure in this plant.

In California, subsequent to our initial data gathering, NIOSH contracted with us to do a larger study at the plant. The company's personnel records did not allow one to determine who worked there over any period of time. Thus, anybody who wanted to participate in the study was examined.

A total of 196 men were evaluated. This included the 36 previously described: 142 produced semen samples; 107 had histories of exposure to DBCP, and 35 gave a history of no exposure to DBCP; 42 men had vasectomies, and 9 DBCP-exposed men did not produce a semen sample.

FIGURE 1 shows a cumulative percentage distribution of the DBCP-exposed workers as compared to those not so exposed. As indicated, 14% of the exposed were azoospermic as compared to 3% for the controls. Also, there is a difference in the curves as well as a difference in the medians. The median sperm count for the exposed (a) was 45 million as compared to 78 million for those never exposed (b).

TABLE V shows data indicating the ratio of what was initially defined as normospermic (> 40 million per ml) and oligospermic (< 40 million per ml) men by months of potential for exposure to DBCP. Exposure duration to DBCP is grouped as no exposure (control), 1 to 6 months, 7 to 24, 25 to 42, and greater than 42 months.

For the 35 controls of normospermic to oligospermic, the ratio was 8 to 1; for those who had 1 to 6 months exposure, the ratio became 3 to 1; for those who had 7 to 24 months exposure, the ratio became 1 to 1; for those who had 25 to 42 months exposure, the ratio became 1 to 2; and for those who had more than 42 months exposure, the ratio became 1 to 5.

For the three men with sperm counts greater than 40 million with exposure of more than 42 months, all had been away from DBCP for a period of time, whereas for those with less than 40 million/ml, all were still exposed to DBCP at the time of the study.

We were interested to determine whether there was a method, other than a sperm count, for predicting testicular function. Thus, FSH, LH, and/or testosterone levels were observed in relation to sperm count.

FIGURE 2 shows FSH in relation to sperm count. As indicated, the break point is at a sperm count of less than 1 million per CC. Thus, FSH may be useful only for prediction of those who are azoospermic. This is not a very useful test for sperm counts between 1 and 20 million.

Unfortunately, at present the only adequate testicular function test for DBCP is the semen analysis.

Ten individuals with azoospermia also volunteered for testicular biopsies. When we did these we had no idea about the pathology, i.e., was the azoospermia due to a blockage or testicular damage, etc.

Photomicrograph I represents an individual who was exposed for 10 years. There is not much cellularity in the seminiferous tubules. As Sertoli cells are within the seminiferous tubules, this lack of cellularity represents a Sertoli cell only syndrome. There is an absence of inflammation. There is not much of an increase in fibrosis as compared to the normal testes. This apparent increase in stroma is probably a reflection of collapsed tubules.

Photomicrograph II represents an individual who was exposed for 3 years and had a sperm count of about 1 million. Again, severe loss of spermatogenesis can be observed although there are some focal areas in which spermatogenesis is occurring.

Many questions regarding the toxicity of DBCP remained unanswered. Data indicate that DBCP is indeed a human testicular toxin. At the dose to which workers were exposed, other effects have not been obvious. For some of the severely affected men, the result appears to be a chemical vasectomy. The dose of DBCP required to produce this effect in humans is not known. Workers were exposed intermittently to atmospheric concentrations of 300 parts per billion. However, the amount of DBCP that might have been absorbed through the skin could not be determined.

With regard to reversibility, some evidence suggests that the effect in some people is reversible. Two individuals who had been exposed for 3 and 4 years, and had subsequently not been exposed for 3 years and 4 years respectively, have normal sperm counts. An additional DBCP-exposed individual who terminated employment 3 years ago had a sperm count of 50,000 at that time. He now has a sperm count of 28 million. On the other hand, two individuals who have not been exposed for 9 to 13 years are currently azoospermic. Both had children prior to exposure to DBCP. The effects on the testes for these individuals are probably permanent. Testicular pathology with a Sertoli cell only syndrome most likely is also permanent.

The mechanism of action for the toxic response is not known. DBCP fed to animals has not been found in the urine or the feces. DBCP obviously affects germinal tissue and has been shown to be mutagenic in bacterial systems. Whether it is mutagenic in humans is not known. Are the spermatogonia that survive "superior cells," or are they damaged? No one knows. DBCP also has been shown to be a strong carcinogen in two animal species. The carcinogenicity in humans has not been studied.

DBCP continues to be used. It is still the best single agent against nematodes. As compared to other fumigants, it has much less acute toxicity than ethylene bromide. At the present time, it is not being used in California, although it will probably be used in the future for specified crops. Currently no U.S. producer is manufacturing it. DBCP is being shipped to the United States from Mexico by two different companies.

Finally, some comments about doing industrial studies for testicular function. First, it is not easy. Inherent in such a study is a semen sample. Obtaining a semen sample is not as easy as drawing a blood sample. There are two ways of obtaining a semen sample: by masturbation or by coitus interruptus. In order to accomplish this, it is important that the physician in charge of the study talk to each worker, individually, prior to commencement of the study in order to explain what is wanted and why. It does not work very well to pass out memos or notices saying that a semen sample is needed, do not have intercourse for 3 days and bring us a sample. It is also better to have the samples collected at home and then brought in. In the first group of men examined, samples were collected at the office early in the morning. Although this was relatively satisfactory, several of the men were distressed by the atmosphere. Thus, it is better to have the

samples collected at home. There are also potential religious or social problems. In some societies and in some segments of the United States, this can be a very major problem.

The final consideration before one sets up a study is to arrange for appropriate followup for men who are discovered to have low sperm counts. This must be done before anyone is examined. Not every gynecologist nor every urologist is either interested in, or cares about, infertility. One is well advised to make certain that the referral physician has an interest in, or specializes in, infertility. Those with problems of infertility are going to want more than to just be informed of their problem.

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TABLE I

COMPARISON OF NON-VASECTOMIZED DBCP WORKERS WITH VERY LOW (GROUP A) AND NORMAL (GROUP B) SPERM COUNTS

Group	No. of Subjects	Age (Yr)	Length of Exposure (Yrs)	Sperm Count ($x10^6$/ml)	FSH (mIu/ml)	LH (mIu/ml)	Testosterone (ng/dl)
A	11	32.7 ± 1.6#	8.0 ± 1.2*	0.2 ± 0.1**	11.3 ± 1.8*	28.4 ± 3.3#	459 ± 35
B	11	26.7 ± 1.2#	0.08 ± 0.02*	93 ± 18	2.6 ± 0.4*	14.0 ± 2.8#	463 ± 31

SOURCE: Reference #6

All results given as mean ± standard error of mean.

#Difference between Groups A and B significant at $p < 0.01$.

*Difference between Groups A and B significant at $p < 0.001$.

**Nine workers with 0 sperm/ml, two with 1×10^6/ml.

TABLE II

DOW CHEMICAL COMPANY
DISTRIBUTION BY PERCENTAGE OF MEAN SPERM COUNTS
MAGNOLIA PLANT

Sperm Count in Millions	Percentage of Mean Sperm Counts	
	Exposed	Not Exposed
0	32.8	4
<50	44.3	52
>50	23.0	44
N = 61		25

SOURCE: Reference #7

TABLE III

SHELL CHEMICAL COMPANY
DISTRIBUTION BY PERCENTAGE OF MEAN SPERM COUNTS
MOBILE PLANT

Sperm Count in Millions	Percentage of Mean Sperm Counts	
	Exposed	Not Exposed
0	2.3	0
>0-10/ml	8.0	0
10.1-20/ml	8.0	0
20.1-40/ml	21.8	29.4
40.1-100/ml	41.4	41.4
>100/ml	18.4	29.4
N = 87		17

SOURCE: Reference #8

TABLE IV

SHELL CHEMICAL COMPANY
DISTRIBUTION BY PERCENTAGE OF MEAN SPERM COUNTS
DENVER PLANT

Sperm Count in Millions	Percentage of Mean Sperm Counts	
	Exposed	Not Exposed
0	7.1	0
>0-10/ml	3.6	7.1
10.1-20.0/ml	12.5	7.1
20.1-40/ml	17.9	14.3
40.1-100/ml	30.4	46.4
>100/ml	28.6	25.0
	N = 57	28

SOURCE: Reference #8

TABLE V

RATIO OF NORMOSPERMIC* MEN TO OLIGOSPERMIC** MEN
BY MONTHS EXPOSURE TO DBCP

	Exposure Duration (Months)				
	None	1-6	7-24	25-42	>42
Normospermic	8	3	1	1	1
to	"	"	"	"	"
Oligospermic	1	1	1	2	5
N	35	48	14	12	17

SOURCE: Reference #9

*Normospermia = $<40 \times 10^6$ (44 men are in this category).
**Oligospermia = $>40 \times 10^6$ (82 men are in this category).

N = 126

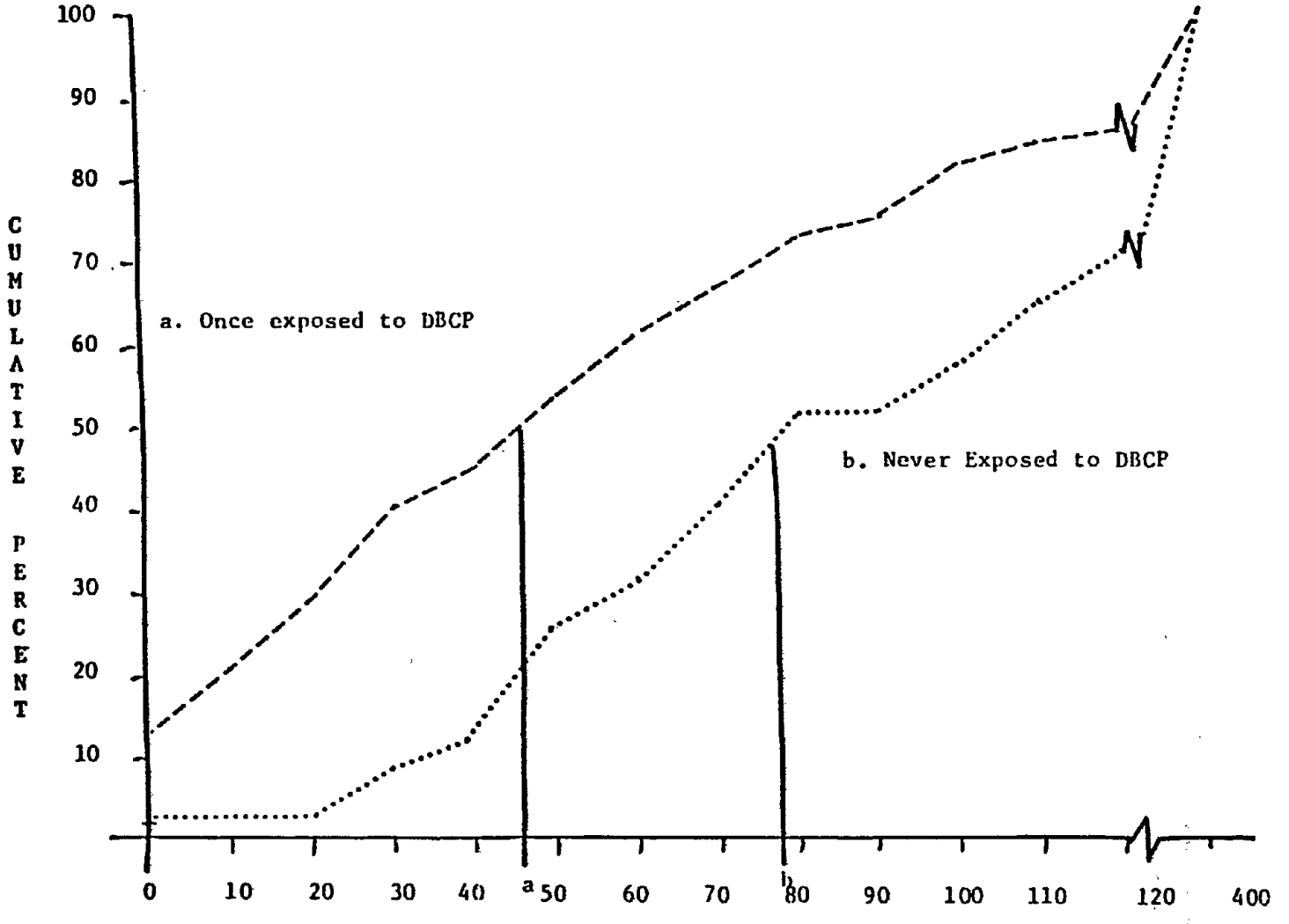
Ratios are rounded to nearest whole number

FIGURE 1

Cumulative percentage distributions for sperm count for two groups:

a. Exposed to DBCP (N = 107)

b. Never exposed to DBCP (N = 35)



Sperm Count in Millions:

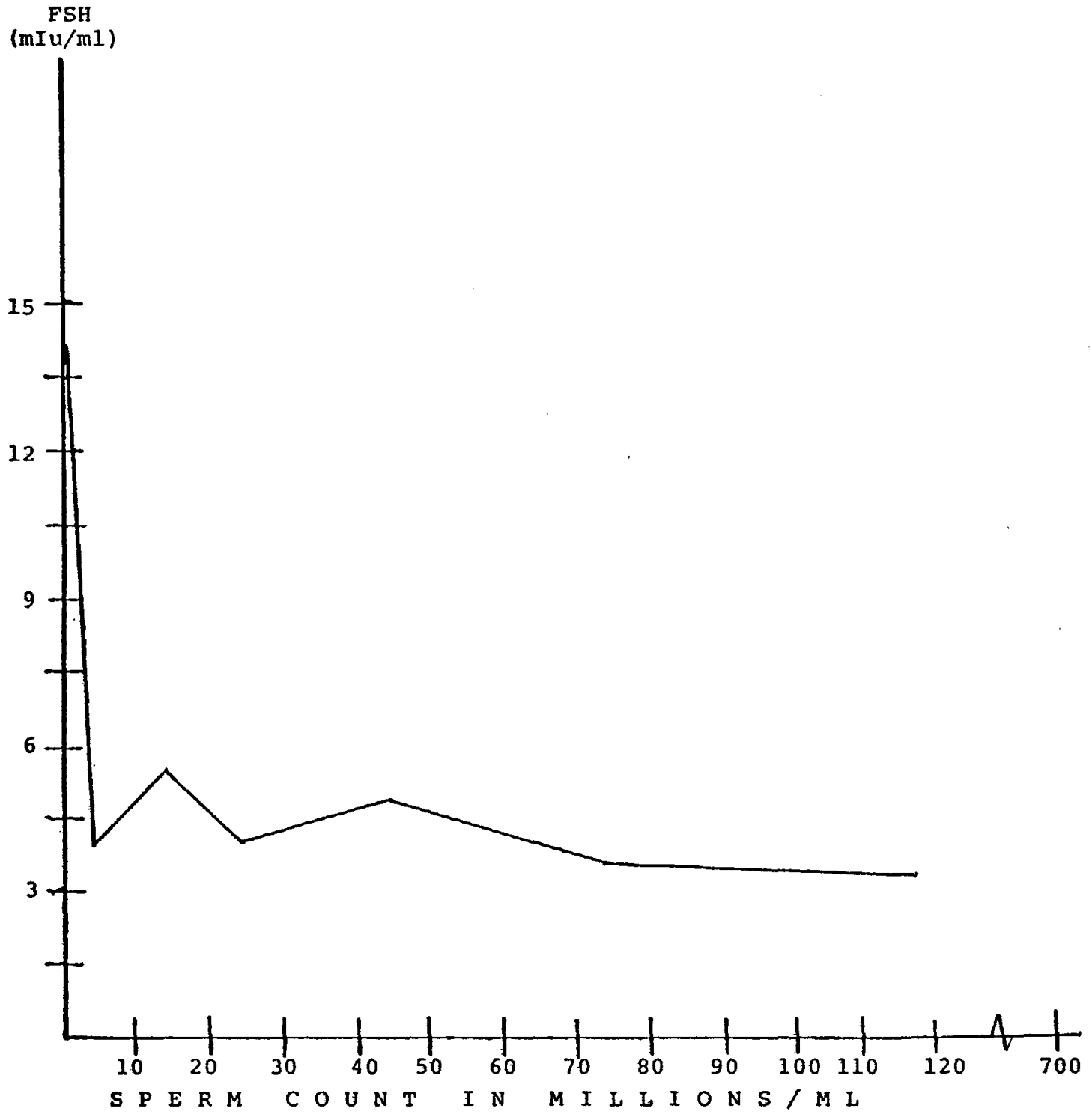
a. Median Sperm Count = 45.6×10^6/ml

b. Median Sperm Count = 78.7×10^6/ml

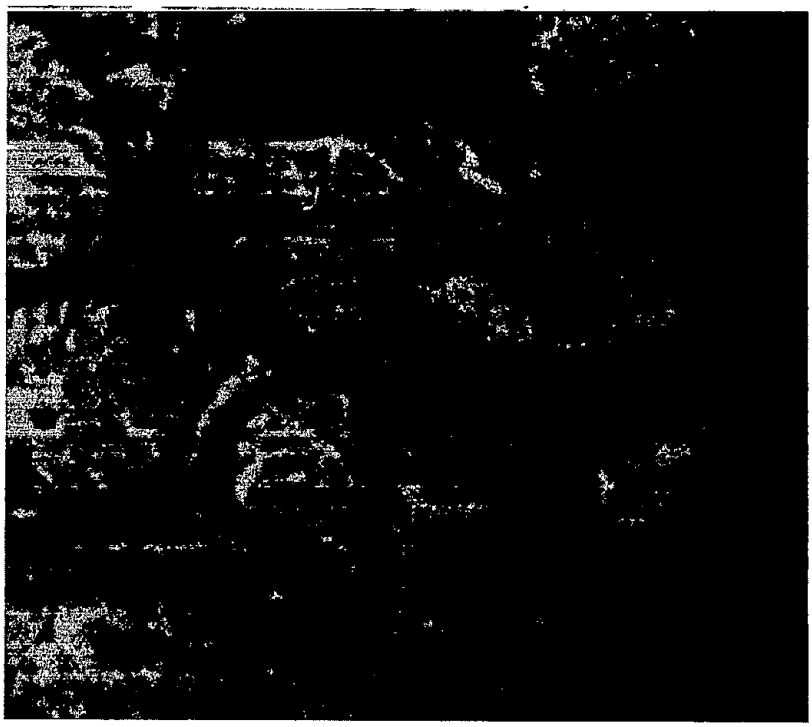
SOURCE: Reference #9

FIGURE 2

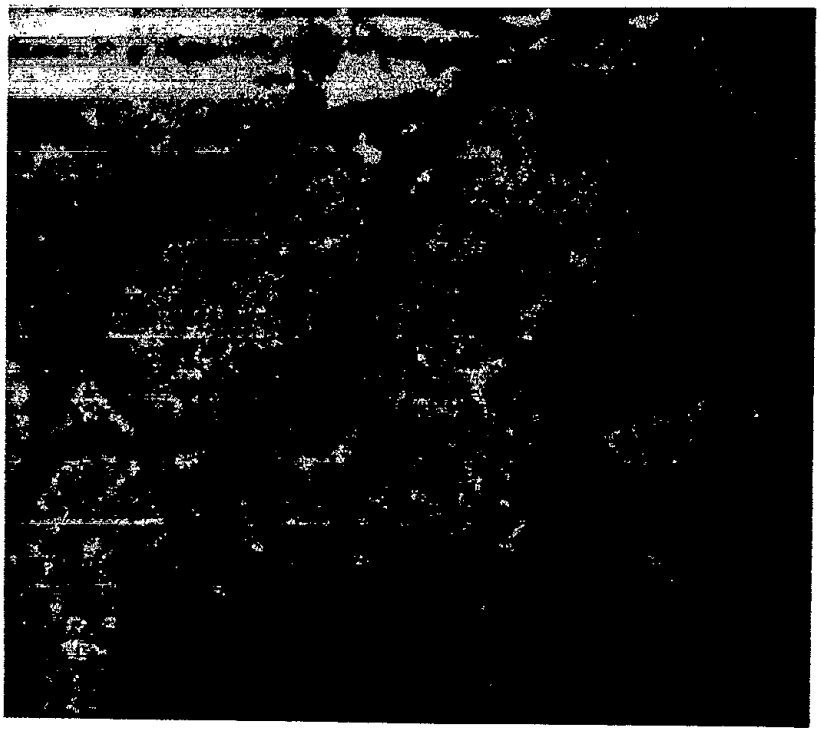
Mean FSH by sperm count group for
105 DPCP-exposed employees



SOURCE: Occidental Chemical Company, 1977



Photomicrograph I



Photomicrograph II

AN EVALUATION OF IN VITRO TESTING FOR MUTAGENS

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INTRODUCTION

Chemicals exhibiting mutagenic activity pose a potential hazard to their users. Mutations in gametic cells can lead to spontaneous abortion and a host of genetic diseases whose symptoms and consequences for the afflicted offspring can range from subtle and seemingly innocuous, to salient and pathetically debilitating. Mutations in somatic cells during embryogenesis can lead to various embryopathies due to mosaicism and anomalies that will be evident during the lifetime of the afflicted offspring. Likewise, somatic mutation is generally believed to be an early event in the genesis of cancer. Consequently, mutagenicity testing is receiving increasing acceptance as an integral part of the premarketing, toxicological evaluation of chemicals.

The evaluation of a chemical for mutagenic activity is essentially a two-phase process. First, there is the qualitative identification of the mutagenic activity. The testing in this phase is designed primarily to answer the question: is the chemical mutagenic, yes or no. Subsequently, qualitative mutagens are assessed quantitatively, the ultimate goal being to understand the potential risk to user populations. Quantitative testing thus answers the question: What is the dose response in a mammalian system? This general principle of qualitative followed by quantitative testing has been the topic of several papers, and is popularly known as the tier approach to mutagenicity testing.[1-10] Keeping in mind that no matter how many tiers are proposed, the evaluation process still remains biphasic and sequential, the crucial importance of the initial phase of testing dictates that several criteria should be satisfied.[4,11-13] The qualitative testing should be capable of detecting the entire spectrum of mutagenic events; it should account for the importance of metabolism in activating and deactivating mutagens; it should produce no false negatives unnecessarily, and only a nominal amount of false positives; and it should provide reproducible results within any given laboratory, and among different laboratories. Obviously, this last criterion necessitates the use of standardized protocols in order to judge reproducibility. Also, from a regulatory agency's viewpoint, standardized protocols for testing would be essential to the evaluation process. It would also be desirable, if not essential, that the qualitative testing make use of short-term test procedures as opposed to long-term animal studies.

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It is generally accepted that no single test procedure can satisfy these criteria and, consequently, a collection, a so-called battery, of test procedures will be necessary. However, there are differences of opinion over the exact prescription of test procedures that should comprise the qualitative testing. The differences in opinion essentially concern whether approval for conditional use of a chemical should be granted on the basis of satisfactory (negative) results with both *in vitro* and *in vivo* systems or only with *in vitro* systems. It will be the purpose of this paper to argue that qualitative testing for mutagenic activity of the sophistication described by the aforementioned criteria cannot be accomplished by any battery of *in vitro* test procedures. Such qualitative testing is best accomplished by a battery of *in vitro* and *in vivo* test procedures.

CHEMICAL MUTAGENESIS IN MAMMALIAN SYSTEMS

From a consideration of the complexity of the mammalian system, several points that relate to mutagenicity testing can be demonstrated. Classical or presumed modes of action of mutagens illustrated in FIGURE 1 are discussed below.

Luteoskyrin inhibits DNA synthesis and causes chromosomal aberrations in Ehrlich ascites tumor cells *in vitro*, presumably by its binding to DNA in some unknown manner. *In vitro* studies of this binding indicate the formation of two different types of complexes with DNA in the presence of magnesium ions.[14,15]

Planar compounds consisting of multiple, fused rings can intercalate between bases of DNA. Intercalating agents cause a mutation that is characterized by addition or deletion of base pairs during DNA synthesis (replication or repair). The altered DNA template thus codes for a mRNA with a shifted reading frame.[16-18] The potency of some intercalating agents as frameshift mutagens is increased manyfold by the addition of a reactive side group like nitrogen mustard.[19] Some intercalating agents are also potent clastogens.[20]

Bleomycin causes strand scission presumably at an alkalilabile linkage in DNA.[21] Chromosomal aberrations and transformation are also evident in cells treated *in vitro*.[22]

Three types of adduct formation with DNA can be distinguished: alkylation, arylation, and acylation. The formation of a covalent bond with DNA is enough of a perturbation to the DNA template to make it more prone to miscode during its replication.[23] While such a model would seem to predict that the resulting mutations would be limited to base substitutions, frameshift mutations are also observed with aromatic amines[24] and aryl epoxides.[25] The frameshift mutation may result from intercalation comparable to that observed with acridines having nitrogen mustard side groups[19] or from displacement of the modified DNA base out of the double helix, so-called base displacement.[26] Unlike DNA alkylated or arylated *in vivo*, DNA acylated *in vivo* is difficult to isolate due to the instability of acylamine groups under the acidic conditions used for degrading the DNA into its nucleic acids. However, acylation may be inferred if deamination resulting from acid hydrolysis alters the base composition of the DNA (e.g., deaminated cytosine is uracil).[27]

Strand linking is an example of adduct formation in which a polyfunctional agent convalently reacts with both strands of the DNA helix. This crosslinking of complementary strands prevents the DNA helix from denaturing; it therefore prevents DNA synthesis and probably leads to cell death. In the attempted removal of the crosslinks, mutations may arise from error-prone excision repair or repair that is unfinished at the critical time of replication.[28] Mitomycin C and some of the pyrrolizidine alkaloids are examples of chemicals known to crosslink DNA strands.[29]

Unique to eukaryotes are the packaging of DNA into several chromosomes, and the segregating of these chromosomes during cell division by an intracellular system of microtubules. Therefore, also unique to eukaryotes are mutations that arise from disfunctioning of this "mitotic apparatus." The mitotic poisons colchicine and podophyllotoxin bind to microtubule protein at a site that is different from the binding site of the vinca alkaloids.[30] However, griseofulvin inhibits mitotis without affecting microtubule assembly.[31] The halogenated anesthetics halothane, enflurane, and methoxyflurane also induce segregational errors, but not chromosomal breakage, in mammalian cells treated *in vitro*, and in chick embryos treated *in vivo*; the nature of this antimitotic activity remains to be elucidated.[32]

Finally, there are the mutations that arise from antireplication effects. Theoretically, agents will exist that can decrease the accuracy of DNA synthesis by affecting the DNA polymerases involved in replication or repair. Analogs of purines and pyrimidines that are structurally related enough to be anabolized and incorporated into DNA will still show a propensity to mispair and introduce base substitutions during DNA synthesis. Nucleic acid deficiency during replication is also known to lead to mutation. Thymidine starvation of thymine auxotrophs of Salmonella induced what appear to be deletions.[33] Clastogenicity is also produced in mammalian cells *in vitro* that are depleted of thymidine reserves by treatment with the antifolate Methotrexate.[22]

At this point, let us make a couple of observations based on this conservative outline of mutational events that should be important to qualitative testing. Obviously, eukaryotic systems are needed to detect mitotic poisons, whereas prokaryotic systems would suffice theoretically for detecting mutagens that directly modify the DNA template. Furthermore, the intuitive urge to use mammalian systems in testing is further supported by the need to identify chemicals which break chromosomes or cause nondisjunction, both of which cannot be detected in prokaryotic or eukaryotic microbial systems at the present state-of-the-art. Finally, there is no reason why a chemical cannot cause mutation by more than one mode of action. For example, the fungicide methyl 2-benzimidazolylcarbamate behaves as a base mutagen in Salmonella and an antitubulin agent *in vitro* and apparently *in vivo*.[34] Consequently, more information about a mutagen can be gained by developing a profile of testing im multiple systems.

FIGURE 2 also depicts the involvement of metabolism in chemical mutagenesis. First, one can consider the metabolic activation of proximate mutagens to their ultimate, reactive species. This activation can take place in the same cell that is later mutated, or it can occur at sites distant to the target cell. In the case of the latter, transport of the ultimate species to the target cell occurs. Similarly, the actual activation

of the proximate mutagen may proceed in several steps, the final one(s) being completed in the target cell. The activation of many proximate mutagens which covalently react with DNA is generally attributed to the mixed-function oxidases of the endoplasmic reticulum. Recent work also suggests that the mixed-function oxidases embedded in nuclear membrane may play a heretofore unrecognized role in mutagenesis.[35] In particular, these nuclear enzymes may provide a nuclear location for the production of radicals from reductive dehalogenation and electrophilic sulfur from oxidative desulfurization, both of which are presumably too short-lived to migrate from the endoplasmic reticulum to the nucleus to react with the DNA.

A second level of metabolism to be considered in chemical mutagenesis is that of hormones. The biological action of steroidal hormones is mediated by their binding to a cytoplasmic protein in target cells and the translocation of this steroid-protein complex into the nucleus for association with a nuclear receptor.[36] In contrast, thyroxine, another lipophilic hormone, simply diffuses to its chromosome receptor without any intervening protein carrier although one apparently exists.[37] Nonlipophilic hormones bind to a receptor in the plasma membrane of target cells. This binding triggers the production of cAMP which, in turn, complexes with a cytoplasmic protein. In bacteria, a cAMP-protein complex acts directly at the gene level.[38] Conceivably, this activity is descriptive of what occurs in mammalian cells, assuming the activity has been conserved throughout evolution.

Given that the chromosome is the site of action for both hormones and mutagens, there is at least some basis to suspect that hormones, antihormones, and chemicals which induce oversecretion of endogenous hormones can act as mutagens. Several investigators have reported that some steroids are cytogenetically active *in vivo* during meiosis in both female mice[39,40] and male dogs[41] and during mitosis in bone marrow cells of rats.[42] L-throxine has been reported to increase the incidence of aneuploidy in bone marrow cells of mice treated *in vivo*.[43] The antiestrogen DES behaves like the mitotic poison colchicine in that it causes polyploidy in cells treated *in vitro*.[44] Reports on the mutagenicity testing of nonlipophilic hormones in the literature are scarce and seemingly all negative. While the former suggests a general lack of interest in testing of these hormones, the latter may reflect a form of organotropism in which the membrane receptors of target cells confer specificity to the biological as well as mutagenic action of these hormones. Hence, demonstration of mutagenicity (e.g., antimitotic effects) would depend on a proper pairing of hormone and cell type. Similarly, this type of specificity of mutagenic action could be associated with lipophilic hormones. However, the ease with which the lipophilic hormones can cross any cell membrane, and the possibility that, at high enough concentrations, these hormones can saturate the intracellular environment may mask this specificity with a nonspecific mutagenic effect. Presumably, this masking would be more likely to occur with *in vitro* than with *in vivo* testing.

Thirdly, one can consider metabolism as it relates to antimetabolites and DNA synthesis. As mentioned previously, depletion of nucleic acid reserves during replication can cause mutations. Hence, all of the enzymes involved in the anabolism of purines and pyrimidines which support DNA synthesis are potential targets for inhibition by chemicals that are generally (though not necessarily) markedly similar to natural substrates.

Finally, the cell membrane is presented as a separate level of complexity. In a sense, the cell membrane is another aspect of metabolism in that what determines a chemical's entrance into a cell is the chemical's lipophilicity or the presence of an enzyme system in the membrane that allows for transport into the cell. Like the other aspects of metabolism, membrane specificity which is mediated by so-called permeases will vary with different organisms. Consequently, when test systems that are nonmammalian are used to detect mutagens active in mammalian systems, membrane specificity may cause erroneous results (i.e., false negatives). For example, non-folic-acid-requiring bacteria are relatively insensitive to the antifolates as measured by growth inhibition because these bacteria are unable to assimilate them. However, these bacteria's dihydrofolate reductases, the target enzyme of the antifolates, are just as sensitive to these drugs as those enzymes of folic-acid-requiring bacteria.[45] This may explain why Methotrexate which is clastogenic to hamster cells treated in vitro,[22] presumably by causing thymidine deficiency, is not mutagenic in the Salmonella tester strains,[46] which do not require folic acid in their medium in order to grow.[46] On the other hand, actinomycin D is apparently too large to pass through the outer membrane of the Salmonella tester strains to be bactericidal or mutagenic.[46] This contrasts with its clastogenicity in hamster cells treated in vitro.[22]

Let us extend the earlier observations on qualitative testing which were made solely on the basis of the types of mutational events that should be monitored. Again, the need for mammalian systems in qualitative testing is indicated, this time by the relationship between metabolism and chemical mutagenesis. It was recognition of the importance of metabolism that originally led to the innovation of S-9 for in vitro testing.[47] However, such metabolic complexity cannot be adequately, if at all in some respects, simulated by just using the solution of liver enzymes popularly known as S-9. One must be aware of the inability of in vitro procedures to detect proximate mutagens activated by routes other than the hepatic mixed-function oxidases isolated from the endoplasmic reticulum. For instance, chemicals that are activated or detoxified by the intestinal bacteria include: carcinogens (e.g., 1,2-dimethylhydrazine);[48] drugs (e.g., 4-isothiocyanato-4'-nitrodiphenylamine);[49] food additives (e.g., cyclamate);[50] food dyes (e.g., Citrus Red No. 2);[51] and endogenous chemicals (e.g., bile acid).[52] As previously suggested, some mutagens may be activated to highly reactive species (e.g., radicals) by the mixed-function oxidases in nuclear membrane. Presumably, a nuclear location for the actual activation would allow these short-lived species to reach the DNA and thereby cause mutations and induce cancer. If this is the case, the use of liver microsomes to provide this metabolism may produce the ultimate species, but the mutagenicity may not be detected with the indicator organism due to competing reactions with other constituents in the test system.

Also, one must appreciate the variability associated with in vitro activation. The fact that this variability exists argues against standardized testing which was presented earlier as a criterion for qualitative testing. Rather, test conditions (e.g., amount of liver enzymes);[53] composition of S-9;[54] the liver donor's sex, species,[55] and strain;[56] and other variables[57] should be optimized on an individual basis for each chemical before ultimately concluding that a chemical is not mutagenic. However, when a chemical is shown to be mutagenic in vitro, the qualitative nature of this finding

is illustrated by the experience with the trichomonacide Metronidazole. A highly mutagenic metabolite isolated from the urine of patients and mice administered the drug apparently is produced by oxidative metabolism in the liver; mice pretreated with hepatotoxic carbon tetrachloride do not excrete this metabolite in their urine. This same metabolite is not produced in either the Phenobarbital-induced or Aroclor-induced Salmonella/S-9 system although the drug is mutagenic with or without S-9 activation.[58] In this case, the tester organism itself carries out the in vitro activation through the action of its nitroreductases.[59] Thus, the S-9 though hepatic in origin still lacks the enzymes, cofactors, or conditions necessary to perform that metabolism that occurs in vivo. More importantly, what mutagenic response that is observed is not entirely representative of the mutagenic potential of this drug in vivo.

Consequently, both in vitro and in vivo systems will be needed in the qualitative testing for mutagenicity. The problem with qualitative testing that only utilizes in vitro systems, as suggested by some,[4,5,7] is primarily the false negatives that surely will be incurred. This is not to suggest that all chemicals capable of causing somatic or gametic mutations will be detected by in vivo testing. Actually, genetic toxicology is still too young a field to have amassed complete profiles of mutagenicity testing on all the chemicals known to cause mutations in experimental systems. However, what is intuitively true--that some mutagens will need in vivo metabolism to be detected--has been borne out by examples. For instance, 6-chloropurine is mutagenic to Salmonella tester strain G46 in the host-mediated assay but not in direct plate testing.[60] The antipurine is also clastogenic to mouse bone marrow cells in vivo but not to human lymphocytes in vitro.[61] While it is correctly argued that in vivo testing is statistically handicapped by virtue of its limited number of experimental units, the use of isotopically labeled chemicals can be expected to provide that kind of data (e.g., on the formation of an adduct with DNA or the incorporation of nucleic acid analogs into DNA) that is necessary to judge statistical insensitivity.

IN VITRO TESTING: STRUCTURE-ACTIVITY TRENDS*

To discuss in terms of chemical types those mutagens that may and may not be detected by in vitro testing, let us consider the correlation studies done with Salmonella. If one accepts that many mutagens are also carcinogens and vice versa, these correlation studies provide a data base for illustrating some points about in vitro testing for mutagenicity, even though the actual evidence for mutagenicity in a mammalian system is weak, if not lacking, for many of these chemicals that will be considered. Previous correlation studies (TABLE I) generally have been interpreted as indicating that a high percentage of all carcinogens will be mutagenic in the Salmonella/S-9 system. That the false negatives that have been observed may be associated with certain chemical types has been discussed only very briefly.[63,67]

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*The following is a brief review of material that will be published in its entirety elsewhere.

However, these interpretations are equivocal, and their practical value is limited due to the small number of chemical types that were considered.

It should be noted that interest in an overall correlation between carcinogenicity and mutagenicity of chemicals in a test system like the Salmonella/S-9, one presumes that all categories of chemical carcinogens exhibit the same satisfactorily high correlation within each category. Despite how high an overall correlation may be, it is essential to determine if certain categories of chemical carcinogens are not detected as well as mutagens in that test system. Consequently, for a heterogeneous population, like the set of all carcinogens, it is necessary to overstratify (i.e., overcategorize) in constructing the categories. The use of overstratification will help elucidate the heterogeneity of mutagenic reponse if it does exist.

TABLE II presents an analysis of 477 presumable carcinogens. About 57% have been tested for mutagenicity in the Salmonella/S-9 system for an overall correlation of 77%. The most salient commonality among the 210 carcinogens that are positive in Salmonella is the electrophilicity that is intrinsic to the molecule, or introduced by enzymatic modification. The former can be thought of as ultimate mutagens and include known or presumable alkylating and acylating agents: diazo compounds, nitrosamides, nitrosoureas, dimethylcarbamic chloride, diaryl alkynyl carbamates, aziridines, oxiranes, thiirane, strained lactones, halogenated methanes and ethanes, ultimate mustards, sulfates, sulfonates, sultone, and phosphate. Only a few intrinsically electrophilic carcinogens are not mutagenic in Salmonella: penicillic acid, succinic anhydride, and maleic anhydride. The latter two are apparently too bactericidal to test.

Those carcinogens that are proximate mutagens in Salmonella are chemicals which S-9, or the bacteria themselves, metabolize to reactive species like alkylating and arylating agents. They include: triazenes, azoxy compounds, N-nitrosamines, aromatic amines, polyaromatics, heteroaromatics, and proximate mustards. The nitroaromatics, though mutagenic without S-9, are known to be metabolized by the bacterial nitroreductases to their reactive forms.[59]

The failure of 64 carcinogens to be mutagenic in Salmonella demonstrates the shortcomings of *in vitro* testing, in general, and of bacterial testing, in particular. These shortcomings are summarized quickly as such: An inability to devise an *in vitro* activation system that can be standardly and reliably employed; and an inability to detect the entire spectrum of mutational events that can lead to the induction of cancer. Proximate mutagens whose activation may not be accomplished by S-9 include: Those mutagens whose activations entail the formation of short-lived species (e.g., electrophilic sulfur from thiocarbamyls and radicals from polyhalogenated cyclics and aliphatics) or benzene oxide (i.e., phenyls); and those mutagens whose activations involve several enzymatic steps (e.g., symmetrical hydrazines, azonaphthols, urethane). In the case of chemicals that are probably converted to several mutagenic metabolites *in vivo* (e.g., Maneb, Monuron), there must be some accounting for how faithfully the S-9 activation corresponds to the *in vivo* metabolism. Other chemical categories of carcinogens that have not exhibited high correlations include benzodioxoles, steroids, and antimetabolites. Carcinogens that are toxic to bacteria (e.g., DES,

anhydrides) and agents that crosslink complementary DNA strands (Mitomycin C) may require optimizing rather than standard procedures to be detected as mutagens. Of course, carcinogens which affect the proper functioning of the mitotic apparatus (griseofulvin) cannot be detected in Salmonella.

SUMMARY

This paper has emphasized three main points about in vitro testing. Firstly, from a consideration of the chromosomal organization of the mammalian organism, it is clear that eukaryotic organisms, in particular mammalian cells in culture, will be needed among the test systems used in qualitative testing. Not to use mammalian systems is to preclude the identification of mutagens which cause nondisjunction and possibly those which break mammalian chromosomes.

Secondly, while in vitro activation using S-9 has added a whole new dimension to in vitro testing, it would be unwise to presume that this activation is an accurate representation of what occurs in vivo. This shortcoming will express itself by the production of false negatives, and presumably false positives, when only in vitro procedures are used in the qualitative testing for mutagens. By necessity, in vivo systems must be included in the qualitative testing so as to have a chance of detecting those proximate mutagens whose activation is too involved to be simulated in vitro. In this respect, the host-mediated assay and body-fluid analysis using microbial indicators are an attempt to combine the sensitivity of these indicators with the in vivo metabolism on an intact host. However, even this procedure has its limitations and therefore cannot serve as a substitute for strictly in vivo procedures.

Thirdly, the suggestion that a high percentage of all carcinogens will be detected in the Salmonella/S-9 system, the generally recognized premier in vitro system, is not warranted by a re-analysis of the correlation studies. More importantly, there is reason to suspect that as many as eight chemical categories of carcinogens exhibit individual correlations that are unsatisfactorily low by any standard. Hence, a more sophisticated way of interpreting Salmonella results is indicated. It is necessary to see negative findings in Salmonella in light not of the overall sensitivity of the test, but of that individual chemical category's sensitivity. In some cases, the category's sensitivity will be so low as to discourage assuming that a chemical is not genotoxic because it has failed to mutate Salmonella. The assumption of no effect must come from a battery of in vitro and in vivo procedures which maximize the chances of detecting mutagenicity if it is indeed a property of the chemical under screening.

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TABLE I

REPORTED CORRELATIONS BETWEEN CARCINOGENICITY AND MUTAGENICITY IN THE SALMONELLA/S-9 SYSTEM

Reporter	Percent Positive in Salmonella	Number of Carcinogens Tested	Alleged Number of Chemical Categories
Odashima[a] (63)	63%	27	13[b]
Poirier and Simmon[c] (64)	72%	∿70	5[b]
Sugimura et al. (65)	92%	98	NS[d]
McCann et al. (66; see also 67)	88%	178[e]	11
Heddle and Bruce (68)	68%	37[f]	NS
Purchase et al. (69)	93%	58	4[b]

a. Not all of the chemicals were tested with S-9 activation. N-nitrosodimethylamine and N,N-dimethyl-4-(3-methylphenyl)azo benzamine have been counted as being mutagenic; this was noted in the discussion of the results, but not in the accompanying tables, of reference 63.

b. This total includes a miscellaneous category.

c. Strains TA100 and TA98 were not employed in the study.

d. Not stated.

e. Cigarette smoke condensate which was mutagenic is not included in this total.

f. X-rays which were not mutagenic are not included in this total.

TABLE II

477 PRESUMABLE CARCINOGENS AND THEIR TESTING IN THE SALMONELLA/S-9 SYSTEM

Sensitivity*	Categories	Number of Chemicals	Tested	Positive
Not Evaluable	Cyanamide, Cyclohexylamine, Substituted Diphenylethane, Stilbenediol, Dioxane, Pyrazolinone, Pyrrolizidine, Haloalkyl Ether, Sulfanilamide, Polysaccharide, Polymer, Metal Complex, Miscellaneous.	37/477 (8%)	11/37 (30%)	1/11 (9%)
High	Triazene, Diazo, Azoxy, N-Nitroso, Diaryl Alkynyl Carbamate, Aromatic Amine, Nitroaromatic, Polyaromatic, Aziridine, Oxirane/Thiirane, Heteroaromatic, Halomethane/Haloethane, N-, S-, or O-Mustard, Sulfate/Sulfonate/Sultone, Phosphate.	318/477 (67%)	200/318 (63%)	188/200 (94%)
Medium	Hydrazine, Lactone, Chloroethylene, Inorganic.	34/477 (7%)	23/34 (68%)	13/23 (57%)
Low	Azo, Carbamyl/Thiocarbamyl, Phenyl, Bendioxole, Anhydride, Polychlorinated Cyclic, Steroid, Antimetabolite.	88/477 (18%)	40/88 (45%)	8/40 (20%)
	40 Categories (including a miscellaneous category).	477/477 (100%)	274/477 (57%)	210/274 (77%)

*Sensitivity comments on the relative ability of the Salmonella/S-9 system (53) to detect carcinogens in the given categories. "Not Evaluable" indicates either a small number of chemicals in the category or a lack of testing; "High," "Medium," and "Low" indicate that individual category correlations are in the upper, middle, and lower 33rd percentiles, respectively.

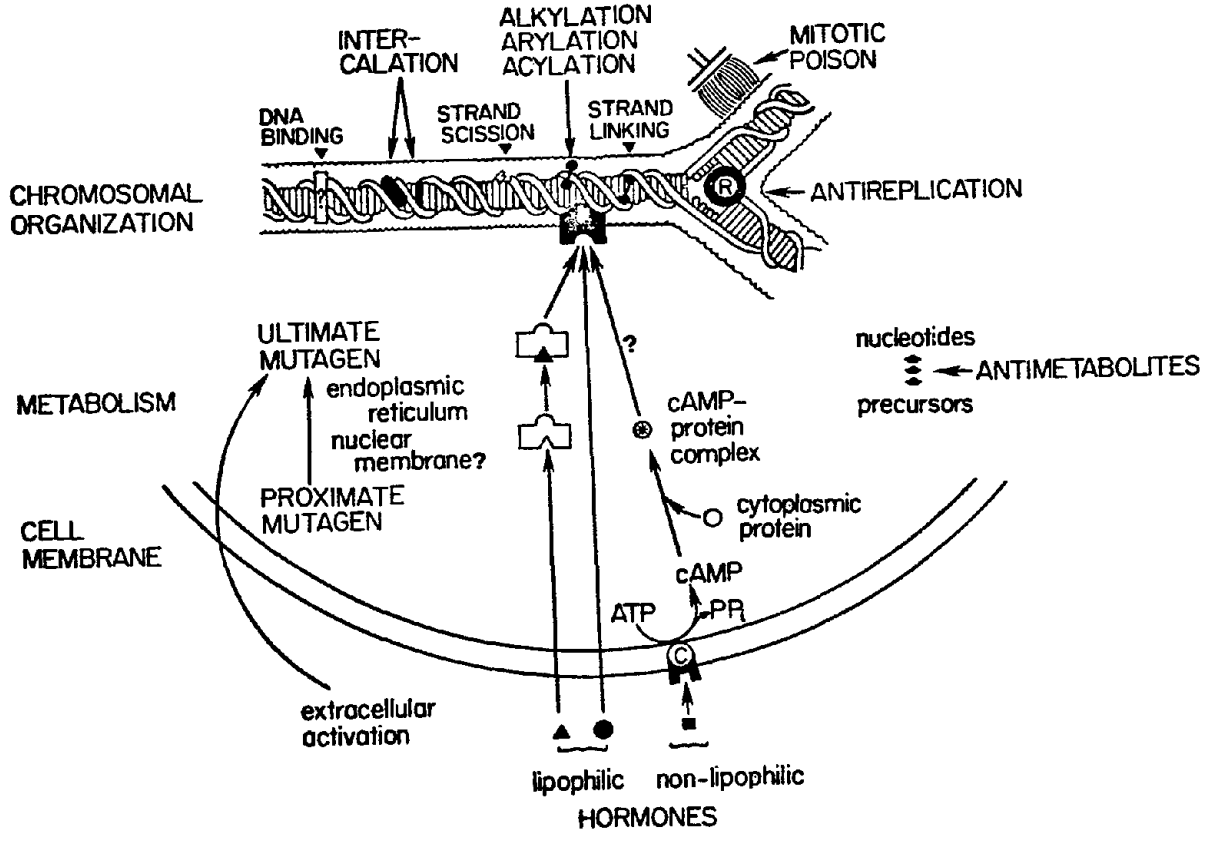

FIGURE 1. The complexity of chemical mutagenesis in mammalian systems. R = DNA replicating enzyme; C = adenyl cyclase. (Modified from Figure 5.1 of reference 62 with permission.)

STATE OF THE ART: CHEMICALLY INDUCED MAMMALIAN CELL MUTAGENESIS AND TRANSFORMATION

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ABBREVIATIONS

AAF:	acetylaminofluorene	FAA:	fluorenylacetamide
8-AG:	8-azaguanine	FUdR:	5-fluorodeoxyuridine
ARA-C:	1-β-D-arabinofurano-xylcytosine	IUdR:	iododeoxyuridine
		MAM:	methylazoxymethanol
BA:	benzo(a)anthracene	3-MC:	3-methylcholanthrene
BaP:	benzo(a)pyrene	MMS:	methyl methanesulfonate
BCdR:	5-bromodeoxycytidine	MNNG:	N-methyl-N'-nitro-N-nitrosoguanidine
BUdR:	5-bromodeoxyuridine		
DBA:	dibenz(a,h)anthracene	MNU:	methyl nitrosourea
DEN:	diethylnitrosamine	4-NQO:	4-nitroquinoline-1-oxide
DMBA:	dimethylbenz(a)anthracene	OUA:	ouabain
DMN:	dimethylnitrosamine	6-TG:	6-thioguanine
EMS:	ethyl methanesulfonate		

INTRODUCTION

Each year thousands of new chemicals are synthesized, and hundreds of which significantly affect the population are introduced into the environment with little knowledge of their potential adverse effects upon the population. The need for the detection of potential mutagens and carcinogens has long been recognized, but until recently extensive mutagenic and carcinogenic testing has been relatively limited.

In the last 10 years, an active effort has been under way to develop test systems to detect the ability of chemicals to produce cellular alterations which result in mutagenic and neoplastic phenotypes. Currently, such tests are performed both *in vivo* and *in vitro*. *In vitro* tests provide a rapid, economical means of detecting potentially mutagenic and carcinogenic compounds. *In vitro* mammalian cell systems, such as those using cultured Chinese hamster cells and/or mouse fibroblasts are well-documented. Although the results of such *in vitro* tests may not yield definitive answers about the mutagenicity and/or carcinogenicity of chemicals as they relate to man, they can serve as prescreens for the selection of compounds to be evaluated in long-term, *in vivo* animal bioassays. Furthermore, such bioassays can also give insight into the molecular basis of carcinogenesis. For example, studies can be directed toward evaluating the potential of chemicals to act as initiators and/or promoters of carcinogenesis. Of course, the *in vitro* systems are not infallible, nor can they detect all potentially biohazardous agents. However, published data suggests a high degree of correlation

between test results derived from bacterial and mammalian cell bioassays and those derived from in vivo studies.

1. Cell Culture Assays for Chemically-Induced Phenotypic Transformation.

The pioneering studies of Berwald and Sachs on in vitro neoplastic transformation of mammalian cells by polycyclic carcinogenic hydrocarbons mark the advent of cell culture transformation techniques.[1,2] Subsequent to these studies, numerous important contributions have been made in the area of chemical oncogenesis in culture (see 3,4 for review). A survey of some of the significant studies of chemical carcinogenesis in culture is presented in TABLE I. A selected number of these will be discussed in greater detail. For additional information, the reader is referred to previous reviews by Heidelberger,[3,5] Casto and Hatch,[4] Mishra and di Mayorca,[6] Pontén,[7] and Casto.[8]

The mammalian target cell systems that have been employed in such in vitro bioassays include fibroblastic and epithelioid cells. The fibroblastic cell systems have been generated from various tissue sources derived mainly from rodents such as hamster, mouse, rat, and guinea pig. The epithelioid cell systems have been derived mainly from rodent epidermal and hepatic tissue. TABLE II lists several of the systems employed for in vitro cell transformation and the types of assays used. Several of the criteria that have been established as indices of phenotypic transformation are listed in TABLE III. The most generally used endpoint for transformation of fibroblastic cells relies on a morphological alteration of cells. This is generally recognized as an aberrant colony or focus and a disordered relationship of these cells to one another; member cells are generally misoriented, nonparallel, and piled upon each other. Such morphologically transformed cells will usually colonize in semisolid agar and form tumors in syngeneic hosts.[60,61] Numerous attempts have been made at establishing a similar morphological endpoint using epithelioid target cells, but in most instances morphological aberrations could not be recognized and only recently have any of these effects proven successful (see 82 for review).

The test cells that have been commonly employed for in vitro neoplastic transformation assays have included (1) normal diploid cell strains (usually mixed populations of primary, secondary, or tertiary cultures), (2) established cell lines, which are aneuploid, nontumorigenic, and possess one or more desirable characteristics, such as cellular uniformity, a high degree of contact-inhibition of cell growth and division, high plating and cloning efficiencies, an unlimited in vitro lifetime, and low background spontaneous transformation rate, (3) cell lines initiated from tumor tissues which have karyotypes indistinguishable from those of normal tissues, and (4) cell lines derived from normal donors or those exhibiting genetic abnormalities or a predisposition to cancer.

a. Normal Diploid Rodent Systems.

Syrian hamster embryo cells of early passage have served as a normal diploid target system for in vitro chemically-induced transformation. This system has been used in a variety of ways. In mass culture assays, cells

are treated with the suspect carcinogen and subcultivated at high cell density and surveyed for morphological alterations.[1,62] In the more quantitative colony assays, individual cells are exposed to chemical carcinogens and examined for morphological alterations arising in the colonies formed by each cell. These colony assays have been performed both in the presence and absence of X-irradiated mitotically arrested metabolically active "feeder" cells,[1,2,9-18,52,62-64] which can serve to both enhance cell plating efficiency and metabolize chemical carcinogens (mutagens) to their more proximate or ultimate carcinogenic (mutagenic) forms.[34,124] The results of such an approach were quantifiable and the frequency of transformation was shown to be dependent upon carcinogen dose and duration of treatment. In addition, there has appeared to be a good correlation between the carcinogenicity of the chemical agents tested and the frequency of transformed colonies.

The focus assay approach has also been successfully employed with hamster embryo cells, both in the presence and absence of simian adenovirus SA7. In the viral-chemical cocarcinogenesis studies,[15,48,49,54-56] cells are exposed to chemical carcinogen prior to or postinfection with SA7 ($3 - 4 \times 10^7$ PFU/plate) after which they are plated at an intermediate cell density, grown to confluency and monitored for the formation of multilayered transformed foci on a background monolayer of untransformed cells. The results are scored as chemical enhancement of SA7 focus formation corrected for the number of surviving cells at risk. Agents such as aflatoxin B_1, benzo(a)pyrene (BaP), 3-methylcholanthrene (3-MC), and 7,12-dimethylbenz(a)-anthracene (DMBA) enhanced viral transformation only when chemical treatment preceded virus infection; cytosine arabinoside, caffeine, and 6-acetoxy-BaP enhanced SA7 transformation only when chemical treatment followed treatment with virus; agents such as β-propiolactone, methyl methanesulfonate (MMS), N-acetoxy-2-acetylaminofluorene (AAF), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methylazoxymethanol (MAM) acetate enhanced SA7 transformation of hamster embryo cells regardless of when they were added relative to the time of addition of virus. It was suggested that chemicals which impart reparable damage to cellular DNA enhance viral-induced transformation by increasing the available cellular DNA integration sites for viral DNA during (unscheduled) DNA repair synthesis; alternatively, chemicals which do not induce cellular DNA repair enhance viral-induced transformation through integration of viral DNA into chemically-induced unrepaired discontinuities in cellular DNA during semiconservative DNA synthesis.

Recently, Casto et al. have also developed a focus assay for chemically-induced transformation using diploid hamster embryo cells in the absence of exogenously added virus.[57] Foci of irregular morphology induced after a 6-day treatment with N-acetoxy-2-AAF, aflatoxin B_1, BaP, β-propiolactone, dibenz(a,h)anthracene (DBA), ethyl methanesulfonate (EMS), 3-MC, MMS, or MNNG appeared on a background of untransformed cells within 3 weeks. Cell populations derived from such foci exhibited the phenotypic characteristics typically associated with the transformed state and were tumorigenic in newborn and weanling hamsters. Untransformed cells from chemically-treated dishes, or cells from solvent-treated control dishes did not display phenotypic transformation and degenerated after three subcultivations.

b. Established Aneuploid Rodent Cell Line Systems.

Established rodent cell lines have been used mainly in focus transformation assays, although a mouse BALB/3T3 cell line established by DiPaolo and coworkers has also served as a target cell for a morphological transformation colony assay.[28] A focus assay utilizing an aneuploid line of adult C3H mouse ventral prostate cells on an X-irradiated feeder layer of C3H mouse embryo fibroblasts was developed in the laboratory of Chen and Heidelberger.[19,20] The prostate target cells exposed to carcinogenic hydrocarbons for 6 days formed multilayered transformed foci which were highly tumorigenic in C3H mice (forming fibrosarcomas upon inoculation of only 10^1 - 10^3 cells). A quantitative correlation was found between the carcinogenic potency of the chemical agents tested and their ability to produce foci which were malignant in vivo.

Resnikoff et al. have since developed another focus transformation assay utilizing the hypertetraploid C3H 10T½ cell line derived from C3H mouse embryo cells.[36,37] The 10T½ cells are sensitive to postconfluence inhibition of cell division; upon chemical transformation they form foci of high cell density and/or aberrant cell morphology on a confluent monolayer of contact-inhibited cells after 6 weeks in continuous culture. Three morphologically distinct types of foci were described: a type I focus is a moderately condensed area of cells which are not considered neoplastically transformed and are nontumorigenic in vivo; a type II focus is a highly condensed aggregate of multilayered cells exhibiting some degree of cellular misorientation and cells derived from such foci are tumorigenic in immuno-suppressed (irradiated) 5-7 week-old C3H mice at least 50% of the time; a type III focus consists of a densely packed, deep-straining, multilayered, disorganized arrangement of cells, often corded in appearance and cells derived from such foci yield tumors in immuno-suppressed C3H mice at a frequency of at least 85%. The C3H 10T½ system is quantifiable and has proven most dependable in detecting diverse classes of chemical carcinogens.[37,65-69] Furthermore, the system presents a morphological endpoint with limited subjectivity.

Kakunaga[39] established a quantitative focus transformation assay with a contact-inhibited subclone designated A31-714 derived from clone A31 of the aneuploid BALB/c 3T3 mouse embryo cell line developed by Aaronson and Todaro.[70] Cultures of 1 x 10^4 cells/60 mm dish exposed to a variety of chemical carcinogens (e.g., MNNG, 4-nitroquinoline-1-oxide (4-NQO), 3-MC, and BaP) developed piled-up morphologically aberrant foci of randomly oriented cells on a confluent monolayer of nontransformed cells within 3-4 weeks posttreatment. Morphologically altered foci were shown to grow to high saturation densities, form colonies in soft agar and elicit tumors in vivo. Control cells, both from untreated cultures and from unaltered areas in treated cultures were not tumorigenic. Parameters affecting the frequency of transformation were concentration of chemical carcinogen, duration of treatment, and cell density at the time of treatment. Recently Kakunaga has derived a new subclone of BALB/c 3T3 Clone A31, designated A31-1 as well as daughter subclones of A31-1, one of which was designated A31-1-13 which has properties identical with those of subclone A31-1 and can be scored in a manner similar to that described for the C3H 10T½ cell system (Kakunaga, personal communication).[73,74,96] These subclones have also been in use in the laboratory of Sivak[71] and our laboratory and have proven to be highly

reliable as a nonambiguous system for detecting different classes of chemical carcinogens.[72-74,96] We have also shown it to be amenable for use with an exogenous source of supplementary metabolizing activity (see below) and suitable for the simultaneous assessment of the mutagenic potential of chemical agents.[73,74]

The Fischer rat embryo cell lines employed by Freeman et al.,[47,75,76] Rhim et al.,[33] and Price et al. constitute yet another focus assay system.[77] This system has been used either preinfected with rat-adapted murine leukemia virus or uninfected as target cells for chemical carcinogens. However, in contrast to the mouse systems described above, this rat system requires repeated serial subcultivation of mass cultures previously treated with the chemical in question for the development of phenotypically transformed foci. In this respect, only a qualitative indication of the carcinogenic potential of test chemicals can be obtained. Nevertheless, the reliability of this system has been demonstrated through use in a blind assay of chemical carcinogens and noncarcinogens.[47]

c. Epithelial Cell Systems.

The majority of tumors found in the human population are carcinomas, which are epithelial in origin.[78,79] However, most of the in vitro model systems developed to study carcinogenesis use fibroblastic cells as the target system, which upon transformation, yield sarcomas as an in vivo endpoint. A considerable amount of effort has therefore been invested in the development of in vitro transformation assay systems which employ epithelial target cells. The major difficulties that have plagued such efforts have been generation and maintenance of epithelial cell cultures that remain as such over extended periods in vitro, and establishment of nonambiguous in vitro endpoints of neoplastic transformation similar to those defined for fibroblastic systems. The consequence has been an inability to develop an in vitro epithelial cell bioassay amenable for short-term screening of chemical carcinogens. These restrictions reflect the present "state of the art" and technical limitations of present day tissue culture methods. Fibroblasts are relatively easy to maintain in vitro and multiply more readily in culture than do epithelial cells. Results of Katsuta et al.,[30-32] and Weinstein et al. have indicated that in vitro chemical transformation of epithelial cells is difficult to recognize on the basis of morphological alterations.[80] The in vitro morphological criteria established for fibroblast transformation systems appear not to be applicable to epithelial cells.[80] Thus far, the only unequivocal assays for epithelial cell transformation in vitro are growth in soft agar and neoplastic induction in vivo. Both of these phenotypic characteristics take a considerable period of time to be expressed; however, there appears to be a high degree of correlation between the manifestation of the two events.[81] Nevertheless, the search for earlier anticipatory indices of epithelial cell neoplastic transformation is still ongoing.[82-85] (See 82 for review.)

d. Human Cell Systems.

Of course, the ultimate goal which all these efforts have in common is the development of short-term bioassays for the purpose of identifying agents which are potentially biohazardous to the human population and to develop an

understanding of the molecular mechanisms involved in human carcinogenesis. To this end, a number of investigators have invested major efforts in the development of *in vitro* human cell transformation systems. Unfortunately, inroads into this area have been limited to only a few laboratories. Progress has been reported using two different approaches thus far: (1) the use of human cell lines derived from neoplastic tissues or from patients with a genetic predisposition to cancer, and (2) the use of normal diploid human cell strains derived from newborn foreskin tissues, whole embryos, or skin biopsies. Igel and associates reported on the morphological transformation of human cells (predominantly fibroblastic) derived from neurofibrosarcomas of patients having Von Recklinghausens disease.[86] These cells were chemically transformed by urethane (1.1×10^{-2}M) which induced morphological alterations after repeated subcultivation of the mass primary cultures originally treated three times for 5-day intervals over a 6-week period. Solvent-treated and untreated control cultures did not exhibit the transformed phenotype. Only the transformed cell populations were tumorigenic upon subcutaneous inoculation of 10^7 cells into non-immunosuppressed newborn NIH Swiss mice, although the tumor nodules regressed after 4-6 weeks. Rhim et al. described 7, 12-DMBA-induced neoplastic transformation of a human osteosarcoma clonal cell line after serial subcultivation of cells exposed previously for 7 days.[87] These cells were tumorigenic in NIH nude mice whereas solvent-treated control cells were neither phenotypically transformed nor neoplastic *in vivo*.

Kakunaga has demonstrated the morphological transformation of normal diploid human fibroblastic cells.[58] The cell strain, designated KD, was generated from a skin biopsy of a patient exhibiting no genetic defects. Cultures treated 30 minutes with 4-NQO (0.3μg/ml) or MNNG (01.-1μg/ml) exhibited dense zones (foci) of morphologically altered and disoriented cells on a confluent monolayer of unaltered cells. The average number of cell generations required to express the transformed phenotype was estimated to be ≥ 13. The loss of density-dependent inhibition of cell growth was heritable; the transformed cells exhibited an increased saturation density, an unlimited *in vitro* life span, aneuploid karyotypes, grew in semisolid agar and formed progressively growing tumors upon subcutaneous inoculation of 2×10^5 - 2×10^6 cells into five 16-week-old female nude athymic mice. Solvent-treated control cultures failed to exhibit any of the phenotypic characteristics associated with chemically-transformed cells.

Milo recently reported the transportation of human foreskin fibroblast mass cultures by such agents as β-propiolactone, aflatoxin B_1, MNNG, 4-NQO, EMS, and propanesultone.[59] Transformation was determined by growth of chemically-treated cells in soft agar after extensive serial subcultivation. Control cultures failed to colonize in soft agar and degenerated. Representative chemically-treated cell lines initiated mesenchymal tumors *in vivo*.

2. Role of Metabolic Activation.

A common problem associated with most every target cell system employed in *in vitro* carcinogenesis (and mutagenesis) studies is the inherent lack of, low level of, or rapidly dissipating level of carcinogen/mutagen metabolizing activity. The problem is further compounded by the fact that most chemical carcinogens and mutagens require metabolic activation in order to exhibit

biological activity.[88] TABLE IV shows the relative inherent enzyme activities of several rodent cell lines used in transformation and mutation studies, in terms of their hydrocarbon metabolizing capacities (total amount of organic soluble BaP converted to water soluble forms per minute per mg protein). It is obvious that the cell lines differ widely in their mixed-function oxidase activities and that these differences could account, in part, for the differences in their sensitivity to different chemical carcinogens (and mutagens). In order to render such target cells more universally sensitive to a wide variety of chemicals, to low concentrations of chemicals, and/or to chemicals of weak biological activity, exogenous supplementary metabolizing activity has been supplied either in the form of intact "feeder" cells or subcellular enzyme fractions (e.g., 9000xg postmitochondrial supernatant (S-9) fraction or microsomal fraction, usually derived from hepatic tissues). Each of these approaches offers its own advantages and disadvantages (see following section). This subject has been reviewed more extensively elsewhere.[88,89,134]

One of the main considerations which need be recognized when dealing with exogenous matabolic activation systems is that there is likely no single ideal source of metabolizing activity which is capable of metabolically activating all promutagens and procarcinogens to their bioactive forms. Thus, the choice of the appropriate activation system is necessarily a function of the specific problem being addressed. It may be that in order to identify the myriad of potentially biohazardous agents, a whole battery of activation systems may be required in order to limit the possibility of false negative results attributable to a lack of target cell sensitivity to the particular chemical supplied.

3. Cell Culture Assays for Chemically-Induced Mutagenesis.

A variety of mammalian cell target systems have been employed to examine chemical induction of mutations in vitro. TABLE V lists some of the commonly used mammalian cell systems and some of the genetic loci which are commonly monitored. The majority of these cell systems have been derived from rodents; these include the V-79 Chinese hamster line,[90] Chinese hamster ovary cell lines,[91,92] mouse lymphoma systems,[93-95] subclones of the BALB/c 3T3 Clone A31 line.[96] Various human cell systems, including normal diploid fibroblasts as well as cells derived from patients with a genetic predisposition toward cancer,[97,98] such as xeroderma pigmentosum have also been employed.[99] Discussions of the comparative mutability of different genetic loci have been published.[100-105]

Some of the methods that have been used for in vitro selection of mutant mammalian cells among a treated population include (a) mass selection, (b) lethal growth, and (c) thymineless death. In the mass selection technique, cells previously exposed to a mutagen are subsequently subjected to selective growth conditions in which mutant cells survive and nonmutated (parental) cells are killed. The selective media may contain a toxic drug or antimetabolite or may lack an essential ingredient, or selection may be imposed through a differential temperature sensitivity (TS) between mutated and nonmutated cells.

The lethal growth method relies on the differential growth rates of mutagenized and wild-type cells in a treated population. One such approach makes use of nutritionally deficient medium containing 5-bromodeoxyuridine (BUdR) in which prototrophic cells continue to grow, incorporate BUdR and are killed upon exposure to visible light. The auxotrophic mutant cells, which are nongrowing in the deficient medium, fail to incorporate BUdR, survive exposure to visible light and can be rescued by simply changing the medium to one which will allow these mutant cells to grow into larger populations.

The thymineless death model is one in which thymidine starvation is employed. Through inclusion of a folic acid antagonist (e.g., aminopterin, amithopterin), wild-type cells continue to grow and self-destruct; auxotrophic mutants which have not grown under these adverse conditions, but which survived, are rescued by growth in normal medium. Further details of each of these techniques as well as others have been reviewed by Chu,[106] and Clements.[107]

Since it is not the intention of this manuscript to present a comprehensive review of the extensive amount of work performed in the field of mammalian cell mutagenesis, the remainder of the discussion will be devoted to highlighting some of the more recent developments as they relate to the "state of the art." Comprehensive reports on mutagenicity techniques and screening procedures, as well as detailed literature reviews, can be found elsewhere.[89,106-112,134-135]

At present, two of the important issues in the field of mammalian cell mutagenesis are (a) the limited capacity of most target cells to metabolically activate promutagens (and procarcinogens) to bioreactive intermediates, and (b) the relative ability of chemicals to mutate cells and to neoplastically transform cells in culture. Attempts to circumvent the low level of mutagen/carcinogen metabolizing activity have been directed along two lines. One approach has been to supply specific reactive metabolites of certain chemicals to the target cells,[18,113-119] which may limit or avoid the need for mutagen/carcinogen metabolizing activity on the part of the target cell. This approach has helped to demonstrate the utility of the *in vitro* bioassay systems. However, such an approach has limited applicability in a wide scale screening program and may be useful only in studying particular chemicals and specific metabolites.

The other major approach has been to supplement the target cell-associated metabolizing activity with exogenously supplied metabolic activity. This exogenous activity can be supplied (1) *in vivo* by "coincubation" of the target cells with the test chemical in the intraperitoneal cavity of the host animal during the exposure period, (2) in the form of metabolically active feeder cells (see previous section), or (3) as partially purified enzyme (microsomal and S-9) preparations. The host-mediated assay, originally developed for use with microbial target cells,[120,121] has since been employed with mammalian target cells.[122,123] The cell-mediated assay makes use of lethally irradiated (or nonirradiated nonmitotic) metabolically active "feeder" cells which are cocultivated with the target cells bearing specific mutable genetic markers in the presence of test chemical.[34,124] The activated metabolites of the chemical in question are thus supplied to the target

cells which themselves may have a limited capacity to metabolize chemically nonreactive mutagens/carcinogens to bioactive forms. Both the host-mediated and the cell-mediated assays offer a means by which reactive intermediates can be generated from otherwise inert promutagens and procarcinogens and analyzed for their biohazardous potential without the need to isolate and/or identify those active metabolites. Similar advantages are available by co-incubation of target cells and test agent in the presence of subcellular enzyme fractions (e.g., S-9 homogenates). TABLE VI presents a comparison of some of the advantages and disadvantages of the S-9-mediated activation system and two cell-mediated activation systems (Syrian hamster embryo (SHE) cells and primary hepatocytes). Future studies which center on the identification and comparison of metabolites generated in each of these systems should help to ascertain the relative utility of each of these approaches.

The inclusion of exogenous mammalian enzyme fractions for supplying metabolic activity *in vitro* has been found useful with bacterial mutagenesis tests,[125-128] mammalian cell mutagenesis tests,[73,74,96,129-132] and mammalian cell transformation tests.[73,74,96] Using this approach, our laboratory has developed an assay employing a single target cell system for the simultaneous assessment of the mutagenic and carcinogenic potential of chemicals requiring metabolic activation.[73,74,96] The assay employs a subclone of the BALB/3T3 Cl.A31 line.[70] The 3T3 cells are treated with the test chemical in either of two ways: (a) in suspension for 2 hours at 37°C in the presence of a reaction mixture containing an Aroclor-1254-induced rat hepatic S-9 and an NADPH generating system, according to methods previously described for bacterial systems,[133] and mammalian cells in culture,[129] and modified for use with BALB/3T3 cells;[73,96] (b) for 24 hours at 37°C under conditions in which cells are attached to the petri dish surface in the presence of the same reaction mixture, by procedures[74] modified from those of Kakunaga.[39] Flow charts depicting each of these approaches are presented in CHARTS 1 and 2 respectively. This assay has successfully detected the mutagenic and phenotypic transforming effects of different classes of chemicals including polycyclic aromatic hydrocarbons, aromatic amines, alkylating agents, and various complex biological mixtures such as pesticides, dyes, and environmental pollutants. Furthermore, the assay technique permits the examination of the molecular relationship between carcinogenesis and mutagenesis.

CLOSING REMARKS

From the foregoing discussion, it is apparent that the fields of mammalian cell mutagenesis and mammalian cell neoplastic transformation have made considerable progress in little more than a decade. The basic *in vitro* systems have been expanded and improved upon such that they can detect diverse classes of chemical carcinogens and mutagens--both direct-acting and those requiring metabolic activation. The various methods of supplying exogenous metabolizing activity to supplement that of the target cell system have contributed much toward expanding the sensitivity of these short-term *in vitro* bioassays. In addition, some of these systems have been found useful in the simultaneous assessment of the mutagenic and carcinogenic potential of chemical agents. With the availability of such systems, details of the relationship between mutagenesis and carcinogenesis should be close at hand. Such mammalian cell bioassays have filled the need for rapid, reliable, reproducible, and relatively inexpensive screening tests for the numerous newly

developed chemicals continually being introduced into the environment, as well as for the tremendous backlog of agents to which man has already been exposed. It is felt that such tests help fill the gap between short-term microbial screening tests and the longer term more costly in vivo bioassays, and at the same time maintain a level of relevancy perhaps lacking in the microbial systems. Since a single test can neither clear nor indict a compound in question, such in vitro mammalian bioassays offer an additional level of testing among the battery of available tests for the assessment of the biological activity of environmental agents.

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Chart 1

STANDARD SUSPENSION ASSAY FOR CHEMICALLY-INDUCED MUTATION AND TRANSFORMATION OF BALB/3T3 CLONE A31 CELLS IN VITRO

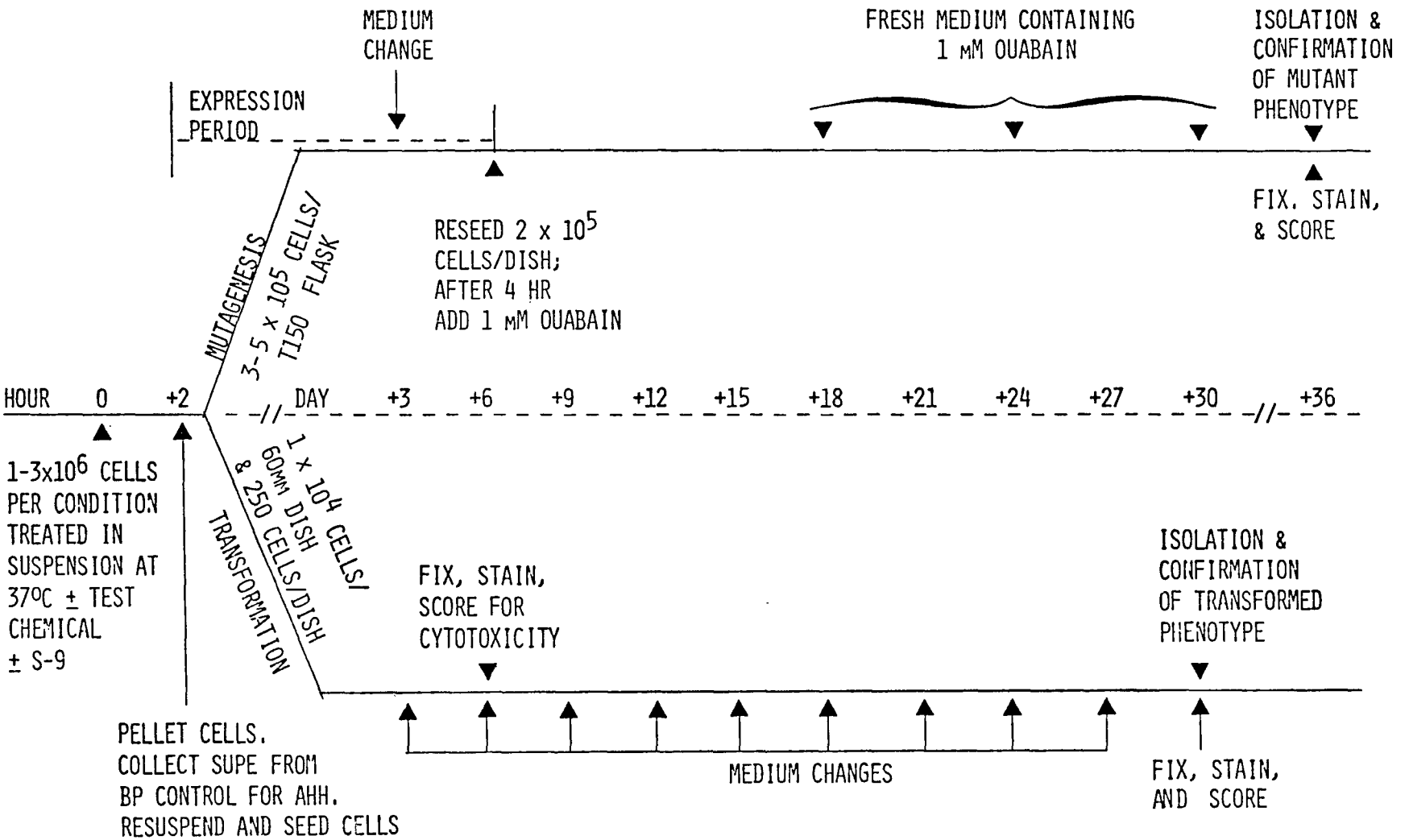

Chart 2

STANDARD PLATE ASSAY FOR CHEMICALLY-INDUCED MUTATION AND TRANSFORMATION OF BALB/3T3 CLONE A31 CELLS IN VITRO

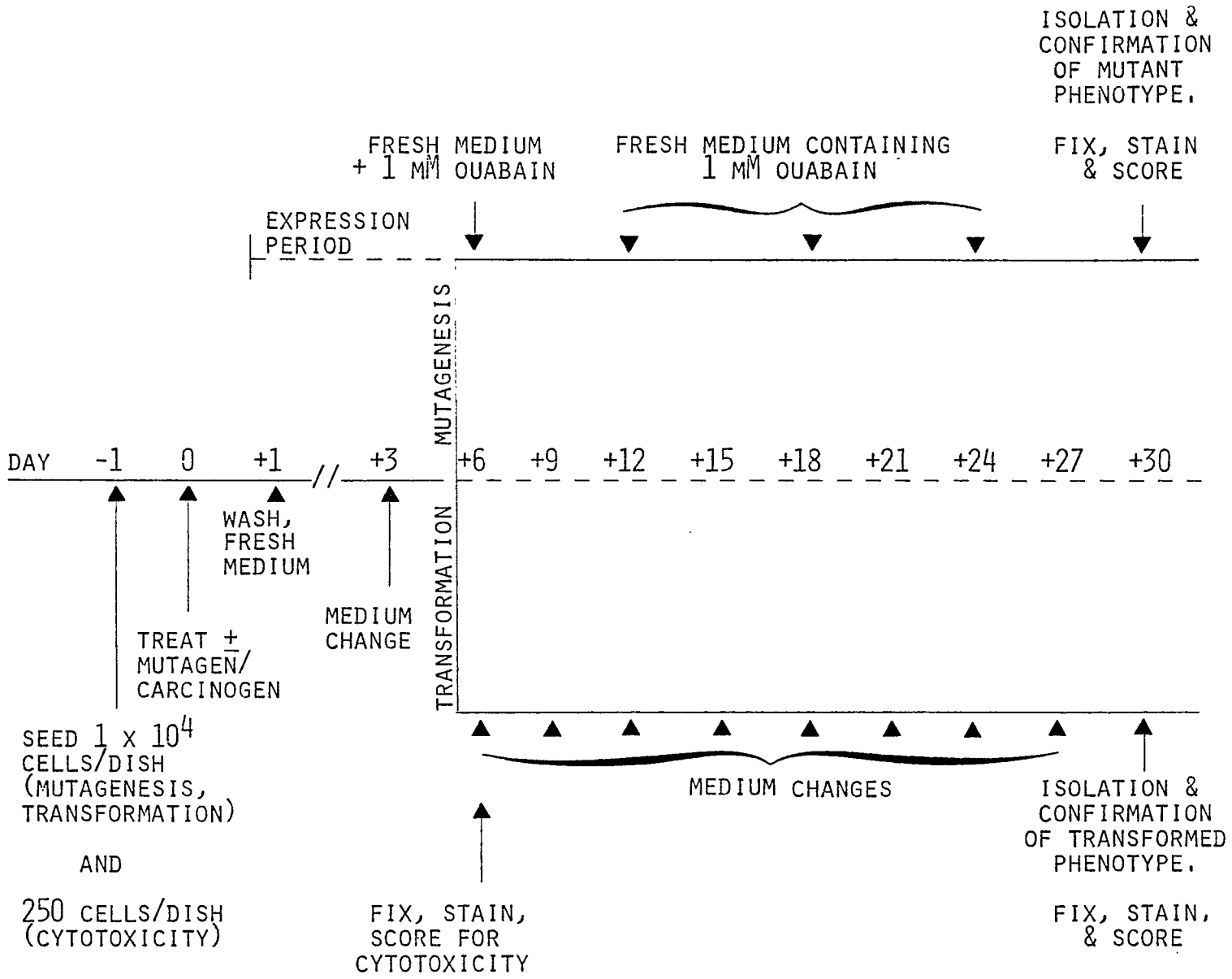

TABLE I

NEOPLASTIC TRANSFORMATION OF CELLS IN CULTURE INDUCED BY CHEMICALS

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
Syrian hamster embryo (SHE) cells	Morphological	Hydrocarbons (BaP, 3-MC, DMBA, etc.)	For BaP (1-10 µg) 8 days 17-10%	Tumor formation in adult hamsters or mice after injection of 5×10^6 transformed cells	1, 2
SWR mouse embryo cells	Morphological	Hydrocarbons (BaP, 3-MC, DMBA, etc.)	10 µg, 3 hour exposure, 10%, etc.	Cloning with feeder layers 1-2 days required after BaP addition to show transformed state	
SHE cells	Morphological Agar Suspension assay Growth saturation level 10-20 times greater than normal cells	BaP, 3-MC	With 10^4-10^6 cells ~9.43% transformed	Large variation in chromosome pattern Tumor formation caused by transformed cells in irradiated adult hamsters	9
SHE cells	Morphological	Hydrocarbons (BaP, 3-MC, DMBA, etc.)	BaP(10 µg) - 6% 1 day exposure	Rat cells as feeder layer	10

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE cells - primary, secondary or tertiary cells	Morphological	BaP, 1 day exposure	% transformation/ cells plated (0.2-0.79%) Linear with log conc. of BaP (0.25-20 µg) % transformation 12 ± 5%	Quantitative evaluation of techniques and effect of feeder layer Always low cloning efficiency	11
SHE cells	Morphological	N-2-FAA N-hydroxy-N-2-FAA N-acetoxy-N-2-FAA 4 hr exposure	N-acetoxy-FAA (0.5-4 µg) gives 0.7-5% N-hydroxy-FAA (5-15 µg) gives 0.3-2% FAA not effective	Also forward somatic mutation (resistance to 8-azaguanine) with Chinese hamster cell lines has been measured.	12
SHE cells	Morphological	BaP or 3-MC (24 hrs) 5,6-benzoflavone (1) α-napthoflavone (2) BA (3)	Pretreatment enhanced % transformation from 1.5 to 4 fold	Pretreatment with (1) did not reduce cytotoxicity of BaP or 3-MC but treatment with (2) or (3) did, indicating a dissociation between cytotoxicity and transformation.	13

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE cells	Morphological	BaP; pretreatment with X-ray, 150-1000 R	Pre-irradiation at 250-500 R 48 hrs prior to BaP increased % T by 8-fold. X-ray 72 hrs after BaP had little effect.	X-ray treatment alone produces no transformation	14
SHE cells	Morphological	Treatment with BaP, 3-MC, etc. together with oncogenic adenovirus SA 7	Pretreatment with hydrocarbons 18 hrs prior to virus increased % T up to 20-fold. Post-treatment with hydrocarbon 5 hrs after viral infection decreases % T by 10-fold.	System applicable to the study of herpes virus. Colonies transformed by virus and hydrocarbons can be distinguished.	15
SHE cells	Morphological with continuation by tumorigenesis in animals	Aflatoxin B_1	7%	Toxicity of MNNG and aflatoxin B_1 clearly indicated	16
		N-acetoxy-FAA	15%		
		MNNG	7%	Cells from embryos were transformed and produced tumors in animals--transformation by chemical that required activation of the pregnant mother.	
		MAM	8%		
		FAA, urethan, DEN	~0%		
		DEN, urethan added to pregnant mother 2 days before removal of embryos (transplacental)	~15%		

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE cells	Morphological (Cytotoxicity measurement)	DBA + K-region epoxide	~0.2-2%	Rat cells in feeder layer; control (0.5% acetone) ≈ 0.5% T	17
		BA + epoxide	~0.2-2%		
		3-MC + epoxide	3-10%	Chrysene epoxide and phenanthrene epoxide also cause transformation	
SHE cells	Morphological	Similar to Ref. 17		Phenol derivatives are toxic but have less effect on transformation	18
Ventral prostate cells of C3H mouse	Morphological	MNNG	~2-10%		
		3-MC	~0.2%		
		3-MC epoxide	2.5-10%		
		BA	0		
		BA epoxide	2.5-5%		
Adult C3H	Morphological with confirmation by tumorigenesis in animals	3-MC (6 days)		Irradiated C3H mouse embryonic fibroblasts as feeder layer	19
		BaP	~2-7%		
		DMBA, etc.			
Mouse ventral prostate cells				Quantitative assay technique	20

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
Single cells--aneuploid cell lines derived from C3H mouse ventral prostate cells	Morphological with confirmation by tumorigenesis in animals	3-MC (6 days)	0 concentration: 6% (could be due to horse serum) 25 µg/ml: 100% T 50-60 days for transformation	Single-cell cloning efficiency about 72% Carcinogen may not select for pre-existing malignant cells.	21
Cell line from Fischer rat embryo cells	Morphological; Increased growth rate, Tumorigenesis in newborn rats	DMBA (2 to 8 days) BaP 3-MC		Qualitative assay	22
Normal human epidermis	Cytotoxicity observed together with early appearance of giant cells and disorderly pattern	3-MC and BaP (1 µg/ml) 4 days	No evidence of malignant transformation can yet be established		23
Pseudodiploid male Chinese hamster line	Morphological chromosome analyses, in vivo tumorigenicity	3-MC BaP DMBA	Semi-quantitative analyses of morphological transformation, chromosomal abnormalities and tumorigenesis	Compared with SV 40 transformation Cellular changes that accompany ability to induce tumors noted	24

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
Primary cultures of lung cells from Syrian hamsters 2-3 days after birth	Biological	MNNG 6×10^{-5} to 5×10^{-6} M for 24 hrs	50% immediate cell death; cell pattern abnormal; chromosomal count no higher; tumor formation after transplantation	No quantitative data on morphological transformation at low cell density culture	25
Epithelial-like cells from rat liver	Biological	Aflatoxin B_1 DMN MNU N-hydroxy-FAA DMBA	Morphological changes (no general pattern); injection of $10\text{-}20 \times 10^6$ cells in irradiated rats produced tumors after months of latency	No quantitative data	26
Mouse embryo cell lines	Morphological; growth rates and tumorigenesis	MNU (2 mM, repeatedly)	1 to few percent; semi-quantitative	Low density cell cultures were prepared without feeder layer Results indicate time lag needed to produce 1) increased plating efficiency, 2) morphologically transformed colonies and 3) transplantability	27

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
BALB/3T3 mouse cloned cell lines	Morphological; growth rate, with confirmation by tumorigenesis	DMBA (10 µg, 48 hr)	10%	Quantitative estimation, about 20-50% cloning efficiency in low cell density culture without feeder layer; not with DEN.	28
		BaP (10 µg, 48 hr)	10%		
		MNNG (1 µg)	2%		
		Aflatoxin B_1	1.2%		
		N-acetoxy-FAA (1 µg)	1-2%		
SHE cells	Tumorigenesis, morphological, growth rate and saturation level	ARA-C, 10^{-3} M, 24 hrs	Qualitative results on transformation and tumorigenesis	Comments made that FUdR (10^{-6} M) and hydroxy urea (10^{-2} M) also cause transformation of cells--indicated by tumorigenesis. Some correlation between inhibition of DNA synthesis and transformation.	29
Diploid strain of rat liver parenchymal cells	Tumorigenesis, chromosomal analyses, morphological and agglutination with Con A	4-NQO 3×10^{-6} M, 30 min, (1-7 times)	Tumor formation, little change in morphology	Control cells can also produce tumors after prolonged culture	30-32

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
Rat-embryo cell line infected or uninfected with Rauscher leukemia virus Mouse cell line infected or uninfected with AKR wild-type C-type RNA virus Uninfected Syrian hamster embryo line	Morphological at low cell density culture; tumorigenesis	BaP, 1-5 µg/ml 7 days	Infected cells were transformed; Uninfected rat and mouse cells not transformed; uninfected hamster embryo cells were transformed	RNA-virus-infected mouse cultures are most sensitive to BaP transformation Suggestion as to mechanism of transformation	33
G23 clone of fibroblast cells derived from C3H mouse ventral prostate co-cultured with irradiated rat or mouse embryo feeder cells	Morphological	3-MC DMBA K-region epoxide	Transformation by 3-MC but not DMBA was enhanced by feeder layer; addition of inducer or suppressor of microsomal mixed-function oxidase in G23 cells increases or decreases transformation	Suggest that the enzymes that metabolize hydrocarbons may in fact activate them to carcinogens Discussion of activation vs. detoxication--delicately-balanced enzyme system	34

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
M2 clone of fibroblast cells derived from C3H mouse ventral prostate	Morphological, focus formation	MNNG (0.2 µg/ml) DMBA (0.1-10 µg/ml) 7-hydroxymethyl BA (0.1-10 µg/ml) K-region epoxide (0.05-5.0 µg/ml) 24 hr exposure	1.7 1.3 - 11 0.5, 1.0, 0.1 0.4 - 2.8	K-region epoxides induced transformation but less active than parent compounds. Epoxide was more toxic; ring hydroxylated derivatives were inactive.	35
C3H 10T1/2 clone 8 embryonic fibroblast cells derived from C3H mouse	Morphological, focus formation; confirmed by tumor formation	3-MC (10 µg/ml) DMBA (1.5 µg/ml) etc.	1% 7%	Aneuploidy. Plating efficiency 12 - 30%. MNNG is toxic but does not cause transformation in an unsynchronized cell population.	36, 37
M2 clone from C3H mouse ventral prostate, synchronized by a double thymidine block	Morphological, focus formation	DMBA-5, 6 oxide MNNG	No transformation when treated at G_2 and M, but transformed when treated at G_1 or S	-	38

TABLE I (Cont'd)

Cell Types	Criteria of Transformation	Agents	% Transformation (T)	Comments	Reference
BALB/3T3	Morphological, focus formation; confirmed by growth characteristics and tumor formation	MNNG (0.3 µg/ml) 4NQO (0.03 µg/ml) 3-MC (10 µg/ml) BaP (1-10 µg/ml)	3.5 foci/plate 1 focus/plate .2, 8.3 foci/plate .2 foci/plate	Transformation frequency reduced at high seeding density.	39
BHK_{21}	Plating in soft agar	DMN	-	No quantitative measurement. The frequency is about 10^{-5}.	40
Wistar rat embryo fibroblasts	Morphological on feeder layer, agar suspension, tumorigenicity	BaP for 1 or 0 passages ± phorbol ester in succeeding passages	Increased morphological transformation frequency using initiator + promoter after 21 passages. Dense colony formation in soft agar by cells treated with both BaP + phorbol ester. Cells treated with phorbol ester alone gave a few colonies in soft agar after 29 passages; not tumorigenic.	Suggestion of two-stage induction of malignant transformation <u>in vitro</u>.	41

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
Mouse embryo cells and cell lines, SHE cells and cell lines, Chinese hamster embryo cells and cell lines	Tumorigenicity, exfoliate cytology	4NQO, 10, 1, 0.1 μM, once or repeatedly; MNU, 1 mM, repeatedly; 3-MC, 1, 3, 9 μg/ml, repeatedly	Tumor production in vivo with varying predictability based upon morphological criteria	No single morphological event could be categorically related to neoplastic transformation, but combinations of morphological characteristics did correlate	42
SHE	Colony morphology: criss-cross orientation of cells on SHE feeder layers	MMS EMS X-ray BaP 3-MC DMBA MNNG N-acetoxy-AAF	Pretreatment with MMS, EMS, or X-ray yielded a 9-fold enhancement of transformation induced by BaP, 3-MC, DMBA, N-acetoxy-AAF	Maximum enhancement at approximately 48 hrs after pretreatment with X-ray or alkylating agents	43
Mouse fibroblast M2 line	Transformed foci	DMBA, 10 μg/ml ± cysteamine-HCl, 1 μg/ml at various times	4-fold reduction in transformed foci without affecting toxicity (plating efficiency)	Oncogenesis and toxicity examined in vivo as well (Sprague-Dawley rats)	44
SHE	Colony morphology: criss-cross pattern of cells on SHE feeder layers	BaP N-acetoxy-AAF MNNG ± caffeine	Caffeine enhances the transformation frequency and toxicity by these carcinogens	Enhancement depended upon caffeine concentration, time of addition, exposure period, and carcinogen used. Caffeine alone, at concentrations used, was nontransforming and nonlethal.	45

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE	Morphological, upon reseeding cells previously treated at confluency	BaP, 10 µg/ml Pyrene, 10 µg/ml	Maximal (7%), with cells treated at confluency for 8 hrs with BaP	BaP also induced DNA replication in confluent SHE cultures; pyrene could not induce DNA replication. BaP also induced unscheduled DNA synthesis.	46
WI-38		BaP, 10 µg/ml		BaP could not induce nonreplicating WI-38 cells (unable to metabolize BaP) to enter DNA synthesis	
Fischer rat embryo cell line F1706, ± Rauscher leukemia virus	Focus formation of disoriented non-contact inhibited cells. Tumorigenicity	Over 30 polycyclic hydrocarbons, azo dyes, aromatic amines, and miscellaneous chemicals	Frequency of focus formation was a function of dose and correlated with tumorigenicity	Carcinogenic activity can be related to the optimal dose of a given chemical carcinogen	47
SHE cells pretreated with test agent and then infected with adenovirus SA7	Focus formation, cytomorphology	UV irradiation, BUdR, IUdR, BCdR, DBA	Enhancement of adenovirus SA7 transformation of SHE cells by UV, DNA base analogs, and DBA	Enhancement was a function of dose of test agent.	48

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE cells treated with chemical carcinogens before or after adenovirus SA7	Focus formation	MNNG, MMS, N-acetoxy-AAF AAF MAM acetate DMN DEN	MNNG, MMS, N-acetoxy-AAF, and MAM acetate enhanced adenovirus SA7 transformation of SHE cells; AAR, DMN, and DEN did not.	Correlation between induction of DNA lesions by chemicals and enhancement of SHE cell transformation by adenovirus SA7; time relationship between chemical treatment and virus treatment.	49
Chinese hamster embryo cells	Colony formation in soft agar	4NQO, 0 or 0.4 µg/ml, 2 hrs before SV40 infection	Transformation of Chinese hamster embryo cells by SV40 was enhanced by a factor of 2-8 by pretreatment with 4NQO	Positive correlation between enhancement of SV40 transformation of Chinese hamster embryo cells and enhancement of integration of SV40 DNA into host cell genome as a function of 4NQO pretreatment.	50
Mouse 3T3 fibroblast line, Polyoma-transformed 3T3 cells, SV40-transformed 3T3 cells, mouse embryo cells (Swiss, HA/ICR), SHE cells	Morphology, karyotyping, tumorigenicity, extended in vitro lifespan	DMBA, BaP, Croton oil, Tobacco leaf extract, cigarette smoke condensate, pyrene	DMBA and BaP transformed SHE cells after 7-9 generations in culture. BaP-transformed SHE cells produced fibrosarcomas after 34 generations in culture. BaP produced morphological alterations in mouse embryo cells (after 15 generations) and in mouse neonatal cells (after 54 generations), but no tumors could be produced.	Toxicity effects of these agents also described.	51

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE cells on rat feeder layers: -mixed embryo SHE cells -embryo SHE cell clones	Morphological, cytotoxicity	BaP at varying concentrations	0.3-23%, depending on dose; 0-61%, depending upon cloned cell population	Transformation frequency was directly related to BaP dose up to 10 µg/ml, using mixed embryo SHE cells. Morphology of the normal cell type that is transformed influences morphology of transformed colony.	52
Guinea pig embryo fibroblasts	Morphological, soft agar, tumorigenicity	Hydrocarbons, MNNG, etc.	-	Morphological transformation may require $\geq$ 4 months; long phenotypic lag for *in vivo* tumorigenicity	53
SHE + simian adenovirus SA7	Morphological, tumorigenicity	Hydrocarbons, alkylating agents, chemotherapeutic agents, etc.	Related to sequence of viral and chemical treatment	Certain chemicals positive only when chemical treatment preceded virus infection; others when chemical followed virus; others, sequence had no effect	15, 48, 49, 54-5

TABLE I (Cont'd)

Cell Types	Criteria for Transformation	Agents	% Transformation (T)	Comments	Reference
SHE	Morphological, focus formation	Hydrocarbons, alkylating agents, etc.	4-7 foci per 10^5 surviving cells	Focus assay (3 weeks) requiring 6-day treatment	57
Human KD diploid fibroblasts (skin)	Morphological, tumorigenicity	4-NQO, MNNG	-	Extended phenotypic lag for expression of transformed state	58
Human foreskin fibroblasts (diploid)	Soft agar, tumorigenicity	aflatoxin B_1, MNNG, 4-NQO, etc.	-	Long phenotypic lag in expression of transformed state.	59

TABLE II

METHODS FOR ASSAY OF CHEMICAL CARCINOGENS IN VITRO*

Established In Vitro Systems

1. Syrian hamster embryo cells (colony assay and mass culture assay)
2. Mouse prostate cell line (focus assay)
3. Mouse embryo C3H 10T½ cell line (focus assay)
4. Mouse BALB/3T3 cell line (focus and colony assay)
5. Guinea pig embryo cells (mass culture assay)
6. Rat embryo cells (F111 and F1706; focus and mass culture assay)

In Vitro Systems Being Developed

1. Syrian hamster embryo cells (focus assay)
2. BHK21 hamster cell lines
3. Epithelial cells
    a. Mouse and rat epidermis
    b. Rat fetal lung
    c. Epithelial-like cells from organs and tissues (liver, bladder, trachea, salivary gland, submandibular gland)
4. Human tumor cells
    a. Osteosarcoma (mass culture assay)
    b. Neurofibrosarcoma (mass culture assay)
5. Human diploid cells
    a. KD skin (lip) fibroblasts (focus assay)
    b. Foreskin fibroblasts (soft agar assay)

In Vivo - In Vitro Systems

1. Hamster embryo fibroblasts
2. Rat trachea
3. Rat kidney
4. Mouse BALB/3T3 cell line

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*Adapted from: Casto, B. C. and G. G. Hatch. 1978. Developments in neoplastic transformation. In: Short-Term In Vitro Testing for Carcinogenesis, Mutagenesis and Toxicity. J. Berky and P. C. Sherrod, eds. Franklin Institute Press, Philadelphia, Pa., pp. 192-214.

TABLE III

PARAMETERS ASSOCIATED WITH TRANSFORMATION OF CELL IN VITRO

1. Altered cell morphology
2. Random patterns of cell growth
3. Loss of density-dependent inhibition
4. Growth at low serum concentrations
5. Production of tumor angiogenesis factor
6. Dexamethasone resistance
7. Loss of surface proteins
8. Acquisition of fibrinolytic activity
9. Agglutination by Concanavallin A and wheat germ lipase
10. Resistance to various polysaccharides
11. Sensitivity to peritoneal exudate cells and lysates
12. Loss of anchorage dependence
13. Cloning in soft agar
14. Tumor formation in susceptible hosts

SOURCE: Reference #4

TABLE IV

BaP-metabolizing activity of various rodent cells *in vitro*

Cell line[a]	Nanomoles BaP metabolized[b]	Relative activity[c]
FU 5-5	25.0	1
BALB/3T3 Cl. A31-714	6.4	0.26
BALB/3T3 Cl. A31-1	19.8	0.79
C3H 10T½ Cl. 8	20.5	0.82
V-79 Cl. 8	<1.4	<0.06
SHE-12f	67.1	2.68

[a]FU 5-5 is an established rat hepatoma line that has maintained its mixed-function oxidase activity after repeated subcultion *in vitro*. V-79 Cl. 8 is an established Chinese hamster line (92) that has virtually lost its mixed-function oxidase activity. The 3T3, 10T½, and SHE lines are described in Table 1.

[b]Nanomoles converted to water-soluble forms/mg protein/24 hr.

[c]Relative BaP-metabolizing activity.

TABLE V

Some commonly used mammalian cell mutagenesis systems

Target cell	Source	Phenotypes monitored[a]
V-79	Chinese hamster lung	8-AG^R, 6-TG^R OUA^R, TS
CHO	Chinese hamster ovary	Cyclohexamide^R, 8-AG^R, 6-TG^R, OUA^R, TS
L5178Y, S49, P388	Mouse lymphoma	OUA^R, 6-TG^R, excess TdR^R, dibutyryl c-AMP^R, BUdR^R, IUdR^R
Xexoderma pigmentosum	Human	8-AG^R
Fibroblasts (2N)	Human (e.g., lung)	8-AG^R
3T3	BALB/c mouse	OUA^R, TS

[a] 8-AG^R, resistance to 8-azaguanine
6-TG^R, resistance to 6-thioguanine
OUA^R, resistance to ouabain
TS, temperature sensitivity
TdR^R, resistance to thymidine
BUdR^R, resistance to bromodeoxyuridine
IUdR^R, resistance to iododeoxyuridine

TABLE VI

COMPARISON OF S-9 AND CELL-MEDIATED SYSTEMS FOR BIOACTIVATION OF CHEMICAL CARCINOGENS

ADVANTAGES

S-9	SHE Cells	Hepatocytes
1. Relatively easy to prepare and stable at -70°C.	1. Relatively easy to prepare.	1. High MFO activity.
2. Level of MFO activity can be controlled by varying species or strain of animals, tissue of origin, or kind of inducer.	2. High MFO activity which is unaffected by lethal (5000 R) X-irradiation.	2. Cells attach to dishes and are mitotically arrested without the need for X-irradiation.
3. Reaction time can be strictly controlled.	3. Both cells and MFO activity are stable at -120°C for indefinite periods.	3. Cells and MFO activity are stable at -120°C.
4. Permits some degree of control in amounts and kinds of metabolites generated.	4. Metabolites are continually generated for long periods of time (~24 hr) so that long-term exposures are possible.	4. Used as source of metabolic activity in mutagenicity tests and is presently being used for transformation assays.
5. Can be made sterile by filtration through 0.45 μ filters.	5. Has been used in mammalian mutagenesis tests with high success, but in transformation tests with only limited success.	5. Metabolic profile of whole cells may be more analogous to <u>in vivo</u> condition.
6. Has been used in conjunction with bacterial and mammalian mutagenesis systems with great success.		
7. Versatile: i.e. can be used with bacterial and mammalian cell mutagenesis and mammalian cell transformation assay systems.		

DISADVANTAGES

S-9	SHE Cells	Hepatocytes
1. High inherent toxicity to test cells; therefore, level of MFO added is determined by this toxicity.	1. Inability to strictly control level of MFO activity.	1. Time-consuming and relatively difficult preparation.
2. MFO activity is not linear with time and only relatively short incubation times (<4 hr) are feasible.	2. Limited control over time of co-incubation.	2. Inability to strictly control level of MFO activity.
3. Requires an exogenous NADPH-generating system; cost of assay is relatively high.	3. Metabolism is mostly that of fibroblasts, and these cells do not activate certain carcinogens (e.g. 2-AAF).	3. MFO activity very labile (half-life of only ~4 hr).
	4. Level of MFO is limited by number of SHE cells per unit area culture vessel.	4. Some enhanced stability to these enzymes if other cells are present, but the mechanism of this action is not understood.
		5. Level of MFO is limited by the number of hepatocytes attaching to the culture flask.

IN VITRO TERATOGENICITY TESTS

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In recent years, there has been increasing concern over the effects of occupational exposure on human reproductive failure. Many agents encountered in the workplace and environment have been implicated in the causation of adult sterility, miscarriage, birth defects, and childhood diseases.[1] Growing awareness of reproductive failure as a potential outcome of environmental exposure has culminated in the Toxic Substances Control Act, which requires the testing of environmental chemicals for carcinogenic, mutagenic, as well as teratogenic activity.

The influence of environmental agents on reproductive capacity is currently tested in laboratory animals. FDA recommendations for reproductive testing include three phases of tests, with exposures (a) before mating to weaning, (b) during the organogenesis period, and (c) during the perinatal period. Outcomes monitored include breeding, fertility, implantation, parturition, lactation, embryotoxicity, teratogenicity, and neonatal effects.[2]

While laboratory animal testing is comprehensive in scope, it is also expensive and time-consuming. New chemical agents are produced and released into the environment at a rate which makes laboratory animal testing prohibitive in terms of expense and time. The Chemical Abstract Service of the American Chemical Society reported 4,039,907 distinct chemical compounds as of November 1977, with an average growth rate of 6,000 entries per week.[3] It has been estimated that a total of 1,930 compounds have been tested for teratogenicity as of 1976.[4]

The approach taken by Federal agencies responsible for enforcing the Toxic Substances Control Act has been to develop a tier-testing approach, where rapid, inexpensive screening tests are first used to determine the necessity for whole animal testing.[5] This approach is useful for studies of carcinogenesis and mutagenesis because *in vitro* bioassays have been established that are reasonably predictive of *in vivo* toxicity. No comparable *in vitro* tests exist for detection of compounds with teratogenic activity.

The overall purpose of *in vitro* bioassay is to accurately identify those compounds requiring further whole animal testing. Test systems yielding a minimum of false negatives that react to a broad spectrum of agents are required to achieve this goal. False positive results are of less concern if their occurrence is infrequent enough to reduce the number of compounds required for whole animal testing. The main consideration in *in vitro* bioassays is whether a toxic effect occurs when the agent is delivered directly to the target tissue. The same agent that tests positive *in vitro* may be nontoxic in the whole animal because it does not reach the target tissue through detoxification/excretion mechanisms. Thus, a certain level of false positive results are to be expected from *in vitro* tests, while false negative results are less acceptable.

Selection of a representative target tissue is important in the development of an in vitro assay. The same molecule, cell time, or organ that is the target for in vivo toxicity should be incorporated into the bioassay. The fundamental event in mutagenesis is heritable alteration in DNA; the target molecule in microbial test systems is bacterial DNA. In carcinogenesis, the underlying event is cell transformation leading to excessive proliferation. Several mammalian cell lines have been employed as target cells to measure this event (see reference 6 for review). The situation is somewhat more complicated in reproductive testing. The target tissue continually changes, and thus has a wide spectrum of different susceptibilities. Although the basic mechanisms of teratogenesis are not fully understood, events such as interference with mitosis, insufficient subtrates for biosynthesis, reduced energy sources, alteration of membrane permeability, water-osmolite imbalance, chromosome nondisjunction, enzyme inhibition, altered nucleic acid function, and mutagenesis play a role.[9]

Two distinct types of target tissues function in reproduction that should be included in in vitro bioassays: germs cells and the developing embryo. Mutagens are capable of damaging germ cells in adult males and females, leading to sterility, pregnancy wastage, and birth defects. According to Wilson, microbial mutagenesis tests could be used to predict potential damage to germ cells.[7] Given the diversity of basic mechanisms in embryogenesis, however, mutagenesis assays alone would not be predictive of embryotoxicity and teratogenicity. Many compounds that are not mutagenic can exert a teratogenic effect. Conversely, mutagenic damage to the embryo does not always lead to birth defects because of the high repair capacity of embryonic tissue.

The ideal target tissue for teratogenesis screening would be the intact postimplantation mammalian embryo. Unfortunately, culture methods are not available for long-term maintenance of postimplantation embryos. Rat embryos have been maintained in vitro from day 9.5 to day 11.5 of gestation at rates of growth and differentiation indistinguishable from in vivo conditions.[10] Teratogenic agents have been administered in whole embryo culture, and depression of ^{3}H-thymidine uptake measured as an index of teratogenicity.[11] While this approach is ideal for administering teratogenic agents during the organogenesis period, it has limited potential for predicting teratogenic outcome. Fetuses have to be maintained up to days 16-18 of gestation for assessment of structural and functional integrity. DNA synthesis can be appreciably inhibited in day 12 rat embryos without teratogenic outcome; thus, depression in ^{3}H-thymidine uptake is a potentially repairable event, and not predictive of teratogenic outcome.[12] A novel approach to the long-term maintenance problem has been developed by Agnish and Kochhar who exposed intact day 11 mouse embryos in culture to bromodeoxyuridine, and then organ-cultured the forelimbs for an additional 9 days.[13] Combining whole embryo and organ culture satisfies the conditions for exposure during the critical period and assessment of differentiated structure/function, but cannot be considered a rapid and inexpensive approach relative to whole animal testing.

Culturing intact amphibian, fish, and chick embryos has been proposed for the bioassay of teratogens.[7] These systems can be discounted, based on hypersensitivity to nonspecific irritants (chick), and low permeability to agents in aqueous media (fish and amphibian). The yolk sacs and jelly coats

of aquatic embryos severely limit passage of chemicals.[14] The advantages of these organisms are that large numbers of subjects can be studied, and the response of the whole embryo measured.

The potential bioassay system I will address in greatest detail is organ culture of embryonic organs, specifically, limb bud culture. Limb buds can be placed in culture and exposed during the sensitive period, and maintained for 6-9 days while the cartilaginous skeleton forms. Structural malformations can be easily visualized by staining the skeleton with toluidine blue.[15] Methods involved are relatively straightforward; day 11 mouse embryos with 44-48 somite pairs are obtained from time-pregnant mice. Embryos are dissected away from uterine horns, and placental membranes under a dissecting microscope in a laminar flow hood. Limbs are dissected away from the body of the embryo in tissue fragments containing flank tissue with associated somite and nervous tissue.[16] Fragments are transferred to millipore filters supported at the surface of the media by glass rings. BGJ_b media are supplemented with ascorbate and glutamine, antibiotics, and 25% calf serum. Limbs are incubated at 38°C in 5% CO_2, and transferred to fresh media every 3 days of a 6-day culture period.

Experiments performed in several laboratories[11,13,17-21] have demonstrated that mouse embryonic limbs developing in culture are capable of the same pathologic responses to approximately 15 teratogenic agents as those developing in vivo (TABLE I). The approach taken was to expose pregnant mice i.p. on day 11 of gestation to teratogenic agents, and analyze term fetuses for limb malformations. Day 11 limb buds were then placed in culture where they were exposed to teratogens for various lengths of time, and allowed to develop for 6 days. Morphologic and biochemical abnormalities obtained by the two approaches were compared. As can be seen from FIGURE 1, limb abnormalities resulting from in vivo exposure to the teratogen cytosine arabinoside are similar to those resulting from in vitro exposure.

The compounds listed in TABLE I are not representative of environmental agents for two reasons; they are all potent drugs that are direct-acting teratogens. As such, the parent compound is the toxic agent, and drug metabolism is not necessary for toxicity to be expressed. Many environmental agents, such as polycyclic aromatic hydrocarbons, halogenated hydrocarbons, aminoazo dyes, and some insecticides, require mtabolic activation before their toxicity can be expressed. (See reference 22 for review.) In order to detect the teratogenicity of compounds with unknown activity in an in vitro bioassay, it is necessary to combine the target tissue with a metabolizing system that transforms compounds to their active state.

The major activation (and detoxification) system for xenobiotics is a multicomponent, membrane-bound complex of enzymes called "mixed function oxygenases" (MFO). (See reference 23 for review.) Drug metabolism occurs when a complex of substrate, cytochrome P-450, and molecular oxygen forms; with NADPH as an energy source, an oxidized form of the compound is produced. These oxidized intermediates have several fates including nonenzymatic rearrangement, conjugation, further enzymatic degradation, and binding to cellular macromolecules.

We have been performing experiments in my laboratory to add a drug-metabolizing system to limb bud culture. The teratogen cyclophosphamide has been used as a model compound in these studies. Cyclophosphamide is an antineoplastic agent that is teratogenic in many mammalian species,[24,25] including humans.[26] The parent compound is inactive, while metabolites generated by the hepatic P-450 system possess alkylating actively and are the teratogenic moieties. Hill et al.[27] elucidated the metabolism of cyclophosphamide in adult mouse liver; four metabolites, possessing various degrees of alkylating activity, have been identified (FIGURE 2).

When pregnant mice were exposed i.p. to cyclophosphamide, preaxial ectrodactyly and hemimelia of limbs were produced. When cyclophosphamide was added to limb bud culture, no adverse effect on limb development occurred. In vitro exposure to the metabolite 4-ketocyclophosphamide, however, resulted in formation of limbs with a "hemimelic" appearance and distal limb reduction (FIGURE 3). Thus, in the absence of a metabolizing system the alkylating metabolite possessed teratogenic activity, while the parent compound was inactive.

Further experiments were performed in which an activating system and cyclophosphamide were added to limb bud culture, and limb abnormalities induced. Several sources of MFO enzymes were tested; the 9000 x g supernatant (S9) and purified microsomal fraction from adult mouse liver, and co-culture of limb buds with intact cells capable of MFO metabolism.[18] The liver fractions were capable of converting cyclophosphamide to five identifiable metabolites with alkylating activity. The cytotoxicity of these preparations, however, limit their use in limb bud culture. Use of hamster embryo cells as sources of MFO metabolism appeared promising; damage to limb buds co-cultured with HEC did not occur, and HEC were able to continue metabolism for 3 days, in contrast to the 2 hours for liver preparations.

The products of cyclophosphamide metabolism by HEC were capable of inducing abnormal limb development in vitro. Exposure of limb buds to HEC and cyclophosphamide resulted in the same malformation syndrome observed in vivo (FIGURE 3). Delivery of parent compound and metabolites to limb buds in the presence and absence of an activating system was measured with ^{14}C-cyclophosphamide. When ^{14}C-cyclophosphamide was added in the presence of HEC, radioactivity accumulated in limb buds for 72 hours (TABLE II). In the absence of HEC, little radioactivity was found in the target tissue, indicating that the parent compound does not bind to cellular macromolecules.

The presence of a drug-metabolizing system in any in vitro bioassay for teratogens is necessary because many chemical agents require activation before their toxicity can be expressed, and embryonic cells often lack the specific enzymes to metabolize these chemicals. Now that methods for metabolism of the model compound, cyclophosphamide, have been elucidated, studies are underway in my laboratory to assess the teratogenicity of representative environmental agents in vitro.

CONCLUSIONS

Two types of *in vitro* bioassays for teratogens have been discussed; whole embryo and organ culture. It is recognized that the intact, post-implantation mammalian embryo constitutes the ideal target tissue for *in vitro* bioassay of teratogens. Unfortunately, culture methods have not been developed to the point where long-term maintenance of the intact embryo is possible.

Intact fish, amphibian, and chick embryos have been employed in teratology studies for many years; chick embryos have proven to be hypersensitive to nonspecific agents.[14] Amphibian and fish embryos are relatively impermeable to agents in aqueous media.

Organ culture of mammalian embryonic limb buds has been discussed as a potential bioassay for teratogens. The advantage of this approach is that limb buds can be exposed during the organogenesis period and maintained for assessment of differentiated structure and function *in vitro*. Limb buds exposed to 15 known teratogenic agents *in vitro* have developed the same structural malformations as those exposed *in vivo*. A drug metabolizing enzyme system has been added to limb bud culture to assess the teratogenicity of agents requiring metabolic activation.

The major disadvantage of this approach is that the response of the intact embryo cannot be assessed. Agents that interfere with the interaction between the central nervous system and peripheral tissues cannot be measured in limb bud culture. This disadvantage may be circumvented by removing large tissue fragments containing somites and nerves along with the limb bud, or by performing transfilter experiments with limb buds on one side of the filter innervated by nervous tissue on the other side.[16] Experiments are underway in my laboratory to test the feasibility of this approach, and the response of limb buds in culture to environmental agents.

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TABLE I

COMPARISON OF DOSES CAUSING IN VIVO AND IN VITRO LIMB MALFORMATIONS

AGENT	REMARKS	TERATOGENIC DOSE PER G BODY WT.	DOSAGE IN VITRO PER ML MEDIA	RATIO
6-AMINONICOTINAMIDE	NICOTINAMIDE ANTAGONIST	25 µg	100 µg	.25
AMINOPROPIONITRILE	COLLAGEN INHIBITOR	NA	200 µg	-
AMINOPTERIN	FOLIC ACID ANTAGONIST	25 µg	25 µg	1
AZASERINE	GLUTAMINE ANALOG	50 µg	10 µg	5
5-BUdR	THYMIDINE ANALOG	1 mg	15 µg	66
CHLORAMBUCIL	ALKYLATING AGENT	20 µg	1 µg	20
CYTOSINE ARABINOSIDE	INHIBITS DNA SYNTHESIS	40 µg	1 µg	40
3,4-DEHYDROPROLINE	PROLINE ANALOG	NA	100 µg	-
DON	GLUTAMINE ANALOG	5 µg	.5 µg	10
HYDROXYUREA	INHIBITOR DNA SYNTHESIS	2 mg	10 µg	200
L-AZETIDINE-2-CARBOX-YLIC ACID	PROLINE ANALOG	NA	50 µg	-
6-MERCAPTOPURINE	PURINE ANTIMETABOLITE	25 µg	10 µg	2.5
URETHAN	CARBAMATE	1 µg	50 µg	.02
VITAMIN A ACETATE	RETINYL ACETATE	1000 IU	5 IU	200

TABLE II

DELIVERY OF ^{14}C-CYCLOPHOSPHAMIDE AND/OR METABOLITES TO LIMB BUDS IN THE PRESENCE AND ABSENCE OF AN HEC ACTIVATING SYSTEM

	Uptake (dpm/µg limb bud DNA)	
Time (hrs.)	+ HEC	- HEC
3	117 ± 24	-
18	3471 ± 549	-
24	3616 ± 284	19 ± 2
48	4237 ± 759	19 ± 2
72	2898 ± 38	20 ± 2

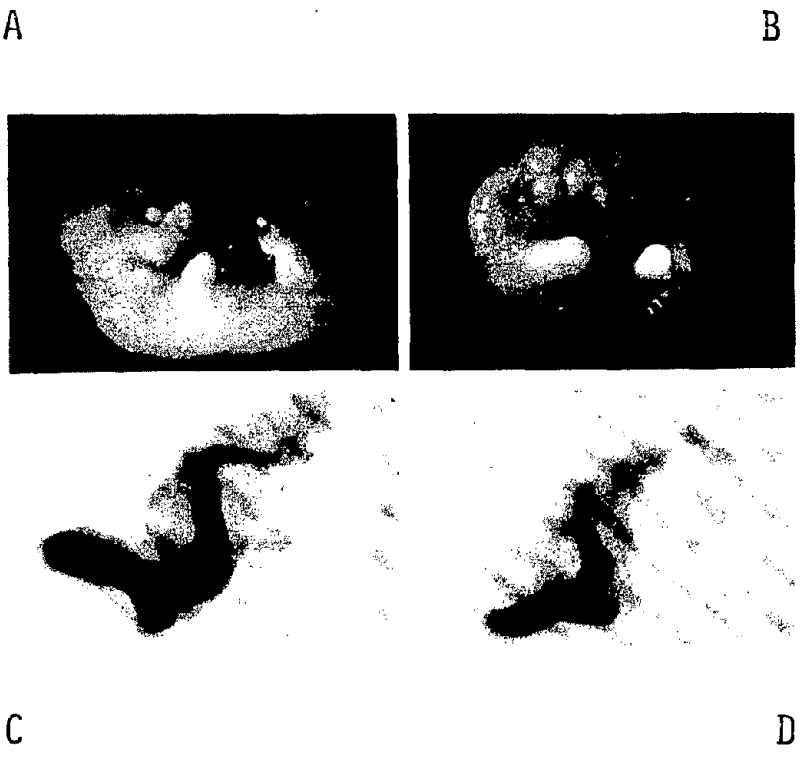

FIGURE 1. Influence of cytosine arabinoside on mouse limb development.

A. Normal mouse fetus on day 11 of gestation.

B. Fetus exposed to 40 mg/kg cytosine arabinoside on day 11 of gestation. Note blistering of hindlimb bud.

C. Day 11 limb bud cultured for 6 days under control conditions.

D. Day 11 limb bud exposed to 1 μg/ml of cytosine arabinoside for 3 days in culture.

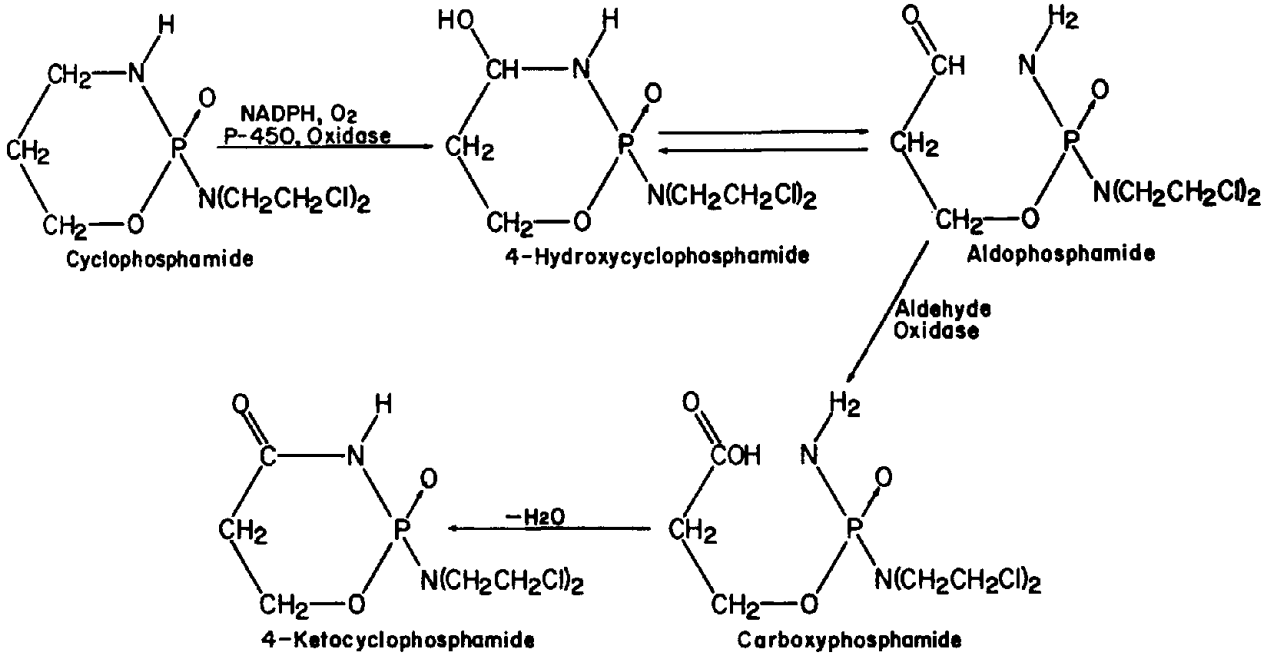

FIGURE 2. Metabolism of cyclophosphamide in adult mouse liver according to Hill (27).

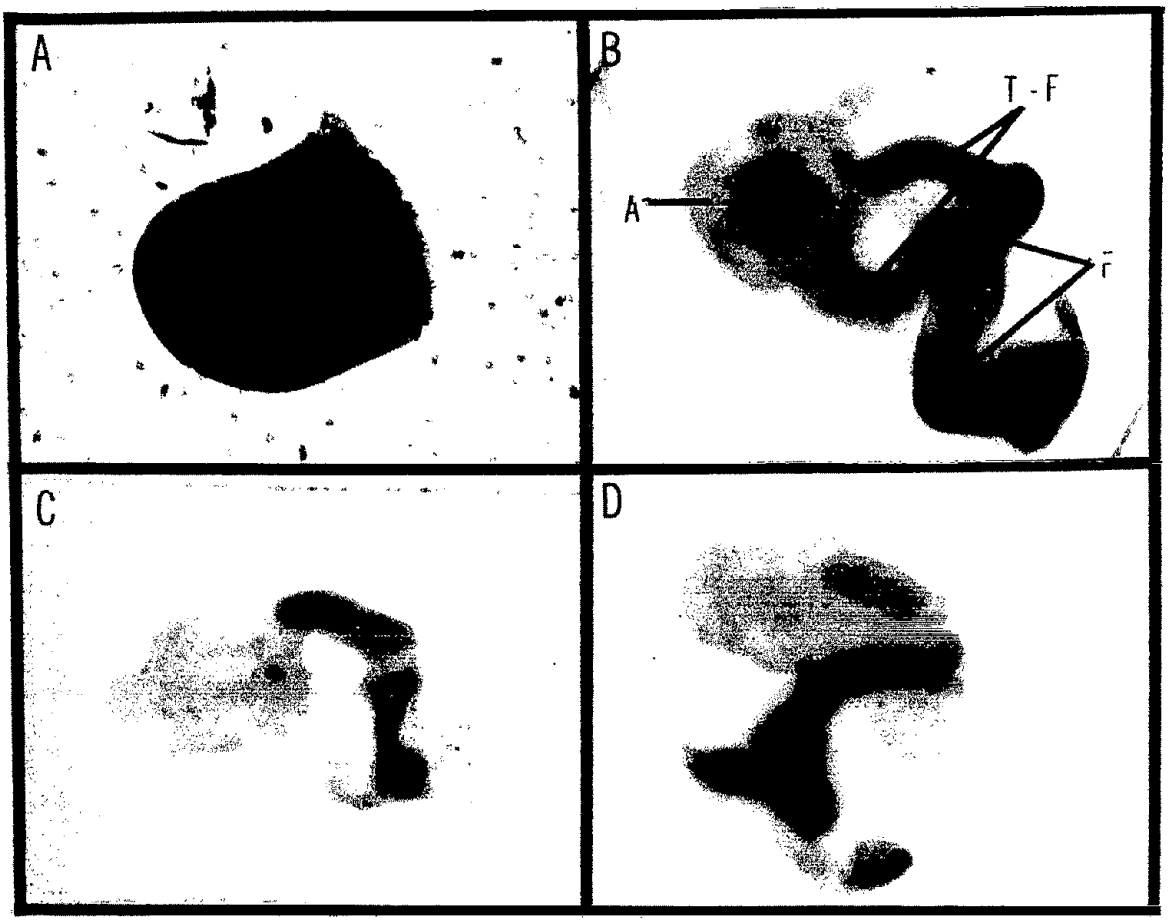

FIGURE 3. Influence of cyclophosphamide and metabolites on limb development in vitro.

A. Appearance of day 11 limb bud at start of culture.

B. Control limb bud after 6 days in culture. Same appearance as limbs treated in vitro to cyclophosphamide.

C. Limb treated with 10 µg/ml of 4-ketocyclophosphamide for 3 days in culture.

D. Limb exposed to cyclophosphamide + HEC activating system for 3 days in culture.

THE DETECTION OF ENVIRONMENTAL MUTAGENS IN DROSOPHILA

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INTRODUCTION

Microbial assay systems such as the Ames' test with Salmonella provide a useful tool for a quick identification of the majority of mutagens and carcinogens in the human environment.[1] It is a widely accepted concept, however, that the assessment of possible genetic hazards to man should be based on data obtained with a battery of different assay systems, among these the Drosophila system.[2]

In this brief survey we will focus attention on four different aspects of mutagenicity screening with Drosophila: First, the possibility of assessing simultaneously a wide spectrum of induced genetic changes; second, the testing procedure available for routine tests; third, the enzymic activation of indirect mutagens in Drosophila; and finally, we will report some recent results obtained with environmental mutagens.

SELECTION OF THE MOST SUITABLE GENETIC ENDPOINT

In contrast to most other assay systems, where only one class of genetic damage can be studied, Drosophila permits the simultaneous assessment of a wide spectrum of genetic changes. The existence of special tester strains provides opportunity for a quantitative comparison of the detection capacity of different genetic endpoints. Such comparative studies have been performed for sex-linked recessive lethal mutations, dominant lethals, sex chromosome loss, and reciprocal translocations.[3]

Recessive lethals are determined in the F_2-generation of treated males. This class of genetic changes includes gene or point mutations, deletions, and, although at a small percentage, also gross structural changes which are occasionally recessive lethal.[4] Dominant lethality in Drosophila is usually determined as the relative percentage of egg hatchability. This quick and easy test provides a measure of gross chromosomal damage, mainly unrepaired chromosome breaks. The major drawback of this test is that non-genetic damage may contribute also to what is generally called "dominant lethality" which makes it of very little value for routine testing.[5]

Total or partial losses of sex chromosomes are an indication of unrepaired chromosome breaks, but such effects may arise also from disturbances in chromosome distribution.[4] Reciprocal translocations are the result of of misrepair of chromosome breaks. In other systems translocations are usually detected by cytological means. In Drosophila, however, a translocation bearing gamete can be recognized <u>in vivo</u> by the absence of two out

of four possible phenotypes two generations after treatment with the mutagen.[4,6]

In most Drosophila tests a distinct set of two or four mutations coding for eye color, body color, or eye shape are used to identify the chromosomes being tested. Thus recognition in F_1 and F_2 progeny of flies carrying exposed chromosomes is easy, and a high degree of skill is not essential. As an example of a comparison of different genetic endpoints, studies by Vogel and Leigh, and Vogel (unpublished), on the monofunctional alkylating agent methyl methanesulfonate (MMS), and on the tetrafunctional alkylating agent tetraethylene-imino benzoquinone (TEB) may be quoted.[3] For the presentation of the results in summarized form, a comparison of the different genetic endpoints is given as the ratio between the lowest effective concentration (LEC) and the LD_{50} (TABLE I). LEC is defined as the lowest concentration of a mutagen which causes a significant increase of the mutation frequency over the control. MMS, for example, gives an LED:LD50 ratio for recessive lethals of 1 to 100. But a 10-fold higher concentration (ratio 1 to 10) is required for dominant lethals, chromosome loss, or translocations. A similar discrepancy between different genetic endpoints is found for TEB. Here 5-fold or even higher concentrations are needed for the induction of chromosome breakage events.

The main conclusion from these comparative studies has been the existence of two effective levels of exposure. In other words, the concentration of a mutagen required to increase the amount of point mutations is far lower than that needed for chromosome breakage effects, even for potent chromosome breakers such as MMS and TEB.

The phenomenon of two effective levels has been observed for all mutagens examined extensively until now. These are about 25 mutagens of different mode of action and chemical structure. A mutagen which exerts extreme specificity is diethylnitrosamine.[3] DEN produces, in Drosophila, no chromosome breakage effects, unless high levels of exposure (LD_{60} - LD_{90}) are applied. In contrast, point mutations induced by DEN are detectable at 180-fold lower concentrations (Vogel unpublished, personal communication). Consequently, in situations where a rate limiting factor restricts the amount of active test substance in the target cells, chromosome breakage may not be observed at all, whereas mutations can be detected to a noticeable extent. Such rate limiting factors can be high toxicity of the substance under test, low stability or water solubility, or from limits in the bioactivation of indirectly acting mutagens.

Indeed, such a situation was observed for vinyl chloride, cyclophosphamide, trophosphamide, iphosphamide, and pyridyl dimethyl triazene for which induction of recessive lethals did not follow any concentration-effect relationship, being between 1% and 3%.[7] All these substances failed to produce chromosome breakage effects. This is consistent with the expectations, because low concentrations of MMS or TEB, resulting in only 1% or 2% lethals, also failed to produce chromosome aberrations. This clearly demonstrates that tests based on chromosome breakage effects can generate false negatives in quite a number of cases. Therefore, a crucial point in screening programs with Drosophila is the selection of the most suitable genetic endpoint, which obviously is the recessive lethal test.

TESTING PROCEDURE

Adult Drosophila males are exposed to the test substance either by feeding, by injection, or by inhalation.[6] When using the feeding technique, tester males are put into glass filter cups and fed with a sugar solution containing the test substance. Flies can withstand such a treatment for up to 9 days.

Another application technique is the injection of adult males with the test substance in saline. To do so, narcotized flies are injected with an extremely fine glass needle to apply the potential mutagen into the haemocoel surrounding the testes. A well-trained technician can inject over 100 flies per hour.

In inhalation experiments, a very simple technique can be used. Males are put into blood plasma bottles, which contain a sufficient amount of standard Drosophila food. Then a known quantity of a gaseous test compound is injected via the rubber part of the screw cap.[8] In order to detect sex-linked recessive lethals among the progeny of exposed males, these males are individually mated to virgin females according to a scheme which is widely known as the Muller-5 test or the Basc technique.[4,6] In this scheme, the two sex chromosomes of P-females are marked with the recessive eye-color mutation white-apricot (w^a), and with the semidominant eye-shape mutation Bar, whereas the X-chromosome of treated males is wild type (that is with alleles for red, round eyes). F_1-females have red kidney-shaped eyes, because these are heterozygous for w^a, and for the semidominant marker Bar.

Among the F_2-progeny the treated X-chromosome is present in two out of four phenotypes, namely in heterozygous condition in one class of females, and in hemizygous condition in wild type males. If the treated X-chromosome in question carries a recessive lethal mutation, this class of wild type males is absent, whereas the other three classes are present. For confirmation, each lethal bearing culture can be retested in another generation using heterozygous F_2-females.

Recessive lethals can be caused by quite different events at the DNA-level, such as basepair substitutions, frameshift mutations, deletions, and gross structural changes. A large part of the Drosophila genome (20% of the genome, that is about 700-800 loci can give rise to a recessive lethal) is covered by the sex-linked recessive lethal test, and this is another reason for the high discriminating power of the test.

BIOACTIVATION OF INDIRECT MUTAGENS

It has become evident that the majority of environmental mutagens require metabolic activation. Therefore, it is important to know the extent to which any detection system is capable of activating indirect mutagens. Insect microsomes can metabolize a wide variety of foreign compounds, and the enzymic reactions are similar or even the same as those in mammalian liver.[9] The first experimental evidence supporting this view came from work with the housefly Musca domestica, but in recent studies carried out in Leiden, existence of cytochrome P-450 and considerable aryl hydrocarbon

hydroxylase activity have also been demonstrated in Drosophila.[10] Genetic evidence that Drosophila can execute the essential activation steps which convert precarcinogens to highly reactive species has accumulated over 15 years. In a review article,[11] Vogel gives an overview of literature data on the mutagenic response of Drosophila to several classes of precarcinogens (TABLE II). Among these are model substrates like triazenes, cytostatics such as oxazaphosphorines, naturally occurring carcinogens such as aflatoxin and the pyrrolizidine alkaloids which were already detected in 1959 by Clark, and suspect environmental mutagens such as vinyl chloride, chloroprene, and polycyclic hydrocarbons.[12] The induction of recessive lethals by this wide variety of indirectly acting compounds provides a pertinent proof of the metabolic versatility, and substrate nonspecificity, of the Drosophila enzyme system. One problem, however, which has yet to be solved concerns the detection of aromatics. Most aromatic amines and polycyclic hydrocarbons induced no recessive lethals, but were active in the production of another type of genetic damage, bobbed-deficiencies.

There is ample evidence supporting the view that--apart from metabolic activity in the gut, the fat bodies, and the Malpighian tubuli--metabolism also takes place in certain parts of the gonads. There, enzymic transformation is only executed in metabolically active germ cells, such as spermatids and spermatocytes, but not in mature sperm which are less sensitive to indirect mutagens. Therefore, routine testing with Drosophila should always include an analysis of progeny from treated spermatids and spermatocytes.

ACTIVITY OF ENVIRONMENTAL MUTAGENS

TABLE III presents examples of recently tested environmental mutagens by means of the recessive lethal test. Activity is expressed as an n-fold increase over the spontaneous mutation frequency observed in the cell stage most sensitive to the mutagen in question (indicated in the last column). It will be seen that beta-propiolactone, as a direct mutagen, is most active in sperm, whereas most other compounds have their maximum activity in metabolically active spermatids.[13]

1,2-dibromoethane and 1,2-dibromopropane--the latter is structurally related to the nematocide DBCP--are mutagenic, whereas no mutagenic activity has been found for 1,1-dibromoethane and 1,3-dibromopropane. Apparently the 1,2-configuration is of critical importance for mutagenic action of these agents.[14] Chloroprene (2-chlorobutadiene-1,3) and three structurally related compounds are also clearly mutagenic.[15] Vinyl chloride, when administered by inhalation, is already mutagenic at concentrations of 30 ppm.[8] Vinyl bromide is also mutagenic via inhalation.[16] In the recessive lethal test, p-phenylene diamine, and three related hair dyes, show weak, but consistent, mutagenic activity.[17] Estragole and hydroxyestragole, in contrast to safrole and hydroxysafrole, display mutagenic effects in Drosophila.[15] The latter two may be considered "false negatives." Of three nitrofurans tested, AF-2 and furazolidone are mutagenic, whereas no decision can be made for H 193.[18]

Epichlorohydrin is mutagenic when injected, but hardly so when fed.[19] Then, four carcinogenic cyclic nitrosamines and methyl-phenyl-nitrosamine are mutagens in Drosophila.[15] The precarcinogen methyl-phenyl-nitrosamine

seems of particular interest. Until now, this compound has been found to be mutagenic only when assayed *in vivo*: that is, cytogenetic effects in plants,[20] micronuclei in mice,[21] and recessive lethals in Drosophila.[15] But so far the compound is negative *in vitro* with and without microsomal activation (V-79,[22] CHO-cells,[21] E. coli,[23*] and Salmonella[24]).

CONCLUSIONS

The two generations test on recessive lethals in Drosophila offers a reliable tool for discrimination between mutagens and nonmutagens. The test is relatively easy to conduct, and the reproducibility of experiments is good. Since false positives in the test are hardly known, a positive response in this system has a high predictive value for possible mutagenicity and carcinogenicity, including such compounds that require metabolic activation. Current attention of our group in Leiden is focusing on further increasing the sensitivity of the test in order to get a better response to the group of aromatic amines and hydrocarbons. One approach is the use of repair-deficient strains, which have already enabled the detection of mutagenic activity of 4-dimethyl-amino-transstilbene. The second approach tries to modify the enzymatic machinery of Drosophila by means of new mutations, and treatment with enzyme inducers and inhibitors. These studies are linked with biochemical characterization of the enzymes involved in the bioactivation of indirect mutagens.

ACKNOWLEDGMENTS

I wish to thank Drs. P. G. N. Kramers, A. D. Tates, F. G. Verburgt, and E. Vogel for their permission to mention some unpublished results. Dr. E. Vogel is also acknowledged for critical reading of the manuscript.

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*Note added: After the workshop, mutagenic activity of methylphenylnitrosamine was demonstrated in E. coli by Dr. G. R. Mohn (personal communication).

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TABLE 1.

LEC:LD_{50} RATIO IN DROSOPHILA FOR VARIOUS GENETIC END POINTS

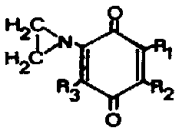
COMPOUND	RECESSIVE LETHALS	DOMINANT LETHALS	CHROMOSOME LOSS	TRANSLOCATIONS
$CH_3SO_2OCH_3$ MMS	1:100	1:10	1:10	1:10
 TEB	1:6700	1:100	1:200	1:1000

TABLE II

MUTAGENIC RESPONSE OF DROSOPHILA TO INDIRECTLY ACTING CARCINOGENS AND RELATED COMPOUNDS [a]

Group	Chemical Class	Number tested	Activation required by	Mutagenicity in Drosophila	
				Recessive lethals	Deletions[b]
I	Arylalkyltriazenes	17	12	+	
II	Nitrosamines (carcinogenic)	6	6	+	+ (DMN tested)
	Nitrosamines (non-carcinogenic)	2	-	0	
III	Hydrazo-, Azoxyalkanes	3	3	+	
IV	Oxazaphosphorines	3	3	+	
V	DDT, DDA, HEMPA	3	3	+	
VI	Aflatoxin B_1	1	1	+	
VII	Pyrrolizidine Alkaloids	11	11	+	
VIII	Vinyl chloride, chloroprene and related compounds	5	4 ?	+	
IX	4-Nitroquinoline-N-oxide	1	1	+	
X	Aromatic Amines and polycyclic Hydrocarbons				
Xa	4-Dimethylamino-trans-stilbene	1	1	+	
Xb	Others	10	10	0 or (+)	+
		63	55		

[a] For literature, see the review article of Vogel and Sobels (5)

[b] Induction of bb's and Minutes; for literature, see the review by Vogel (11)

TABLE III

ACTIVITY OF ENVIRONMENTAL MUTAGENS IN DROSOPHILA

COMPOUND	PEAK ACTIVITY [a] (recessive lethals)	CELL STAGE
beta-propiolactone	90 x	sperm (sp)
1,2- dibromoethane	8 x	spermatids (st)
1,2- dibromopropane	6 x	st
2-chlorobutadiene-1,3	10 x	st
1-chlorobutadiene-1,3	8 x	st
1,4-dichlorobutene-2	6 x	st
1,4-dichlorobutene-2,3-epoxide	8 x	st
vinyl chloride	20 x	st
vinyl bromide	35 x	st
p- phenylene diamine	3 x	st
2,4- diaminoanisole sulfate	4 x	st
2,4- diaminotoluene	4 x	st
4- nitro - o - phenylene- diamine	4 x	st
estragole	4 x	st
1' -hydroxyestragole	6 x	st
safrole	0	
1' -hydroxysafrole	0	
AF-2	4 x	st
H 193	(2 x) ?	
furazolidone	5 x	st
epichlorohydrin	7 x	sp
methyl-phenyl-nitrosamine	30 x	
N-nitroso-N'-methylpiperazine	20 x	st
N-nitroso-morpholine	80 x	st
N-nitroso-piperidine	20 x	st
N-nitroso-pyrrolidine	5 x	st

a, increase over spontaneous mutation frequency

THE DOMINANT LETHAL AND HERITABLE TRANSLOCATION TESTS

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The dominant lethal test has been used, for several years, as a means of predicting or detecting the mutagenicity of certain types of chemicals and irradiation.

The genetic basis for the test lies in the production of chromosomal aberrations in sperm to the extent that when the sperm fertilizes an egg, the egg does not survive beyond the stage of implantation, which is roughly around day 5, and at that point the zygote invokes an implantation reaction on the uterus, that results in a plaque, decidua, or resorption. Any of those terms would suffice, and all have appeared in the literature describing the implantation reaction.

The test can be conducted in the mouse or the rat with relative ease. The procedures are relatively simple, but, as with most of the test methods in the area of mutagenicity, the evaluation and interpretation is the most difficult part of the test.

In the dominant lethal test, two parameters are usually assessed. The first parameter is called preimplantation loss. The preimplantation loss is defined as the difference between the number of corpora lutea on the ovary, and the number of embryos on the uterus. If the number of corpora lutea is much greater than the number of implantations on the uterus, this indicates that there were more eggs ovulated than implanted. The assumption is that those eggs failed to implant simply due to chromosomal aberrations produced in sperm which, when fertilizing the egg, did not allow the egg to even survive to the implantation stage. That, in essence, is the preimplantation index.

The other parameter is that of postimplantation loss. This index is determined by counting the total number of implantations on the uterus (live and dead). The term postimplantation, however, refers to the loss of eggs or the death of the egg after it has implanted on the uterus.

In terms of actual performance, at least two (and preferably three) dosages should be employed, the highest of which should be the maximally tolerated. (This produces some evidence of toxicity, e.g., weight loss or death.) Substances should be administered over a 5-day period, or continuously for 8 weeks in the case of the mouse or 10 weeks in the case of the rat. If one is employing the 5-day dosage regimen, one should make two females per male for each of 8 or 10 weeks. If the males are treated over the entire spermatogenic cycle, one need only mate for 2 weeks following cessation of treatment.

There is one deficiency with use of the latter approach, and that is that information with respect to the specific stage affected is not provided. That, however, is of academic importance only. The basic question that one

is asking in this test is whether or not a chemical can cause chromosomal aberrations in sperm which can result in dominant lethality. This procedure answers that question. If one is concerned about the stage that is affected, one can then return to the technique of administering five daily injections and mate over 8 or 10 weeks, depending upon the species, and then determine at which point in spermatogenesis that agent produces dominant lethality. For a more detailed description of the approach, employing extended treatment, the reader is referred to Green et al., 1977.[1]

With respect to the information obtained, there are numerous ways of tabulating dominant lethal data. The following is suggested but all indexes need not be employed.

1. Fertility index: This index is calculated as number of pregnant females per number of mated females, and the chi-square test is used to compare the values for each treatment group with the control value.

2. Total number of implantations: The "t" test is utilized to determine significant differences between average number of implantations per pregnant female for each treatment, compared with the control value.

3. Total number of corpora lutea: The "t" test is also utilized to determine significant differences between average number of corpora lutea per pregnant female for each treatment, compared with the control value.

4. Preimplantation losses as determined by counting the number of corpora lutea: The preimplantation losses for each female are transformed by the Freeman-Tukey square root, and the "t" test is then used to compare values for each treatment with the control value.[2]

5. Dead implantations: Statistical treatment is the same as that used for preimplantation losses.

6. Proportion of females with one or more dead implantations: Values for each treatment are compared with the control value by the chi-square test.

7. Proportion of females with two or more dead implantations: Statistical treatment is the same as that for proportion of females with one or more dead implantations.

8. Dead implantations per total implantations: This index, computed for each female and the Freeman-Tukey arc-sine transformation, is performed on the data for each female.[2] The "t" test is then utilized to compare each treatment value with the control value.

THE HERITABLE TRANSLOCATION TEST

The heritable translocation test detects reciprocal translocations between nonhomologous chromosomes. Reciprocal translocations cause little problems in cells that divide by mitosis, but the chromosomes of cells that divide meiotically must achieve close pairing of homologous parts. This

leads to a cross-like configuration at pachytene with the eventual formation at meiotic synapse of a ring, tetravalent, trivalent plus a univalent, etc.[3]

Reciprocal translocations alter the linkage association for genes and result in sterility and semi-sterility for that particular animal.

The test is performed by treating parental males, mating, and obtaining the F_1 males. The test is usually performed in the mouse, for the simple reason that large numbers of animals are required, and the karyotype of the mouse facilitates cytogenetic confirmation of translocation heterozygosity. Three hundred to five hundred F_1 animals per dosage are required. At least two dosages should be used.

One then mates those F_1 males, in a ratio of one male to three virgin females, and then selects presumptive translocation heterozygotes. One determines whether or not a male is presumptive, based upon the total number of implantations and the number of dead implantations in mated females.

Animals which carry reciprocal translocations are usually sterile or semi-sterile. Therefore, in those females which are mated to those animals, one usually finds a reduction in the number of live implantations. One can use a 50% reduction as a cutoff point. That is, any animal which has 50% fewer implants than control is a suspect translocation heterozygote. But one is still faced with demonstrating that the animal actually carries a translocation.

In order to confirm the presence of translocations, a cytogenetic biopsy of one of the testes of the animal is performed. One usually finds rings, chains of four, or other types of cytogenetic damage, which proves that the animal carries a heritable translocation.

The statistical analysis of the data is in the evolutionary state. The test has not been routinely used. The advantage to this test is that one actually detects mutation in live progeny. Therefore, one does not have a problem in answering the query: of course, this chemical produces dominant lethality, but those animals do not survive, so why should we really be concerned about it in terms of danger or harm to future generations? The heritable translocation test answers that question, for the F_1 sons obtained their translocations through exposure of the father.

For a more detailed discussion of the methodology involved, the reader is referred to Sheu et al.[4]

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STANDARD IN VIVO TESTS FOR THE IDENTIFICATION OF MUTAGENS: MITOTIC AND MEIOTIC EFFECTS

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INTRODUCTION

In recent years it has been demonstrated that various environmental agents have a mutagenic potency which, in humans, leads to mutation problems. This stems from a variety of observations:

1. There are more than 1,500 inherited diseases with a simple mode of Mendelian inheritance, primarily due to point mutations.

2. Among clinically recognizable spontaneous abortions, there are more than 35% with chromosome aberrations which are supposed to be the cause of miscarriage.

3. Among 10,000 newborns there are--on the average--50 babies with a chromosome anomaly, the chromosome anomaly of 37 of these babies being due to a new chromosome or genome mutation.

4. On the basis of the frequency of chromosome aberrations among abortions and newborns, we can conclude that at least 7% of all human zygotes contain a chromosome aberration. Especially chromosome and genome mutations are a great danger. Usually they manifest themselves in the first generation, and their consequences are severe.

5. Besides ionizing radiation, a high number of chemicals have been characterized as mutagens, and a growing number of them have been proved to be mutagenic in mammals, and some even in man.

For all these reasons, we cannot but evaluate and, if necessary, test the chemicals of our environment for possible mutagenic activity im man.

Already in 1966, the Genetics Study Section of the National Institute of Health made the following recommendations:

1. A register of mutagenic chemicals should be kept.

2. Mutagenicity testing should be made of compounds used in pharmaceutical drugs, foods, or to which large numbers of persons may be exposed.

Our investigations were supported by the "Bundesministerium für Forschung und Technologie," F.R.G.

3. Mutagenicity tests which are cheaper, more sensitive and relevant to man should be developed.

4. The genetic monitoring of the human population should be explored.

In Germany, the "Bundesministerium für Forschung und Technologie," the "Umweltbundesamt," the "Bundesgesundheitsamt," the "Deutsche Forschungsgemeinschaft," and other government agencies also support these recommendations and investigations.

Today, in this workshop, we will examine which of these recommendations are fulfilled. Therefore, we will present and evaluate practicable cytogenetic in vivo tests for the identification of mutagens.

NECESSARY ATTRIBUTIONS OF CYTOGENETIC MUTAGENICITY TEST SYSTEMS

Before presenting the standard test systems, we should mention some desirable attributions of those assays. Especially for later value this knowledge is necessary. Some of the important criteria are:

1. The assay should be of a high relevance to man. Mechanisms of mutations are often deduced from studies in microorganisms or from cell culture. These investigations sometimes ignore that the test compound may not reach the DNA of somatic or germ cells of higher organisms in the same reactive form. For instance, a specific compound could be activated or inactivated by metabolic processes before reaching the DNA in certain mammalian tissues. For these reasons, tests in mammals in vivo have the greatest relevance to man.

2. As in other fields of toxicology, thresholds may exist for mutagens. Therefore, the dose effect relationship should be carefully examined. We know that the extrapolation of dose effect dependency is problematic from one mammal to another species--especially from mammals to man--but it is more easily performed than from cell cultures or from microorganisms. Also, for this reason, tests should be carried out with mammals, as in other fields of toxicology.

3. A wide spectrum of chromosome aberrations should be detectable with the test system. This implies that structural aberrations (e.g., gaps, breaks, fragments, deletions, exchange figures, pulverizations), as well as aneuploidies, should be recognizable.

4. The test should be simple to handle.

5. The test should not be expensive.

6. It should be a quick test system.

Some of these recommendations are accomplished by the following standard in vivo tests:

TEST SYSTEMS

A. Analysis of Somatic Cells

1. The Micronucleus Test.

Some years ago Schmid and coworkers developed a test to investigate mutagenic effects in bone marrow cells of mammals.[1,2,3] The principle of this test system is based on the following data: Chromatid breaks in mitotic dividing cells led to disturbances in the anaphase. In general, the chromosome fragments lag in the anaphase, and after telophase such displaced chromosome pieces are not included in the nuclei of the daughter cells. Instead of this, they form one or multiple so-called "micronuclei." These are detectable in a variety of different cell types of the bone marrow, in myeloblasts, myeloclasts, as well as in erythrocytes. Of main interest for this test system are the polychromatic erythrocytes. This cell type expels its nuclei a few hours after the last mitosis. The micronuclei, however, remain behind, and are easily detectable in this nuclei-free cell type (FIGURE 1). A second advantage to restrict scoring to this cell type is the following: Young erythrocytes, e.g., polychromatic ones, less than 24 hours old, stain bluish instead of red, like the older normochromatic erythrocytes. Therefore, all observed micronuclei in the polychromatic erythrocytes are known to have arisen during the last 24 hours, that means some hours after treating animals with the test compound.

Using this test system,[4] we investigated the effects of our standard mutagen, the cytostatic drug cyclophosphamide (CP), the mutagenic activity which was first detected by Röhrborn in 1966.[5] In our experiments, Chinese hamsters (5♀♀, 5♂♂ per dose and interval) were given two intraperitoneal injections of CP, 24 hours apart. The single doses were 13.3, 40, and 120 mg per kg body weight. The preparations were made 12 or 24 hours after the second application. In untreated hamsters, the yield of polychromatic erythrocytes with micronuclei was 5 per 1000 analyzed. In the experimental series (FIGURE 2), clear-cut dose effect relationships are to be seen. In addition to these investigations, we scored the ratio of normochromatic to polychromatic erythrocytes. This ratio dropped from 1,000:671 after two applications of 40 mg/kg, to only 1,000:144 after injection of 2 x 120 mg/kg, referred to a bone marrow depression. This demonstrates that besides mutagenic effects, cytotoxic effects are also detectable with this test system.

2. Bone Marrow Chromosomes.

The previously described micronucleus test is appropriate for screening in the primary safe evaluation of compounds. However, for the differentiation of cytological effects induced by meaningful chemical compounds, for example, drugs, metaphase studies are indispensable. Only these investigations give information of the severity of genetic damage (aneuploidies, exchanges, breaks, gaps). The following remarks concern metaphase studies in bone marrow cells of mammals, using the method by Boller and Schmid.[6] Usually, the animals were treated twice with the test compound, 24 hours apart. Two hours prior to preparation, the animals received a metaphase blocking agent, e.g., colcemid. The femora are dissected, the marrow rinsed out. The cells are treated in a hypotonic solution, fixed, spread on a

slide, and stained. For these investigations, we prefer Chinese hamsters as test animals because of the appropriate karyotype. They have only 22 well distinguishable chromosomes; 8 great metacentric, 6 acrocentric, 6 small metacentric, and the sex-chromosomes. The various types of chromosome aberrations, such as gaps, breaks, exchange figures--including deletions--(FIGURE 3), are well analyzable. Furthermore, the spontaneous rate of aberrations--below 1% gaps excluded--is extremely low.

The suitability of this test system is shown in our next experiments. In addition to micronucleus tests, and chromosome aberration tests, the dose effect relationship of CP in bone marrow of Chinese hamsters was investigated.[4] The same experimental setup was chosen. Gaps, breaks, and exchange figures can be seen (FIGURE 4). With higher doses, even completely fragmented and pulverized metaphases occur. Twelve hours after the last application of CP, the dose effect curve is very distinct. The best correlation exists between the rate of exchange figures and applied doses. A similar result was found from preparations after 24 hours; however, a drastic decrease of the portion of aberrant metaphases was found after the application of the highest dose (2 x 120 mg CP per kg), which might be caused by cytotoxic effects.

3. Sister Chromatid Exchanges (SCE) In Vivo.

Taylor et al. were the first who described sister chromatid exchanges in plant chromosomes treated with ^{3}H-thymidine in the S-phase of the cell cycle, and analyzed at the second mitosis, following the incorporation of the tracer.[7] New methods demonstrate that SCE are based on the substitution of thymidine by bromodeoxyuridine (BUDR) during two S-phases of the cell cycle, resulting in a substitution of thymidine in both strands of the DNA in one chromatid, and one strand in the other. The BUDR substituted chromatids can be differentially stained with Giemsa after pretreating with Hoechst 33258 and UV-light. The differential staining of sister chromatids was so far restricted to cultured cells. Recent methods were developed to demonstrate these effects also in vivo, which means in spermatogonia[8] or in bone marrow of treated animals.[9] In this assay, mice or hamsters were injected with BUDR, inhibiting the production of thymidine. Three hours later, they received six times BUDR over 6 hours. Chromosome preparations were made in the conventional way. The chromosomes were stained as described above. Thus it was possible to demonstrate differential chromatid staining by in vivo treatment. Compared to in vitro experiments, the spontaneous rate of SCE is extemely low (3.84). The mechanism for the SCE formation is not yet understood.[10] Perhaps it is initiated by a process similar to postreplicational repair. Nevertheless, good correlations between the potency of mutagens to induce chromosome aberrations and SCE are recognizable. One might even suppose that the SCE test is much more sensitive than the other cytogenetic assays. Mutagenic compounds elevate the rate of SCE in concentrations that are not yet active in inducing chromosome aberrations.[11] This is also shown in our laboratory in testing caffeine. In plants and mammalian cells, high doses of caffeine induce chromosome aberrations. But the effects of caffeine, which caused chromosome breakage in human cells, are restricted to cell cultures. Up to now there was no evidence for mutagenic effects in mammals in vivo. Only if applied in combination with mutagens, a synergistic effect resulting in a higher frequency of chromosome aberrations, can be

stated in vivo.[10] In our experiments, two applications of 200 mg caffeine, given orally, do not increase significantly the aberration rate. On the other hand, a dose dependence of induced SCE in bone marrow cells could be shown (TABLE I, FIGURE 5). A significant increase induced by caffeine only in mammals could be recognized in vivo. Concluding, we can state that perhaps this method opens a new field in mutagenicity testing.

B. Analysis in Germ Cells

It has frequently been alleged that somatic cells are as sensitive to the induction of mutations as germ cells. This leads to the conclusion that findings in treated somatic cells have the same importance, as far as the damage to germ cells is concerned, as findings in treated germ cells themselves. Up to the present, however, there is no evidence for this statement. On the contrary, the possibility of a difference in sensitivity of germ and somatic cells must be taken into consideration. One argument for this assumption is based on the fact that even the various germ cell stages show considerable differences in sensitivity to most of the chemical mutagens. For these reasons, the germ cells of mammals are of great interest and importance for mutation research.

1. Male Germ Cells.

Spermatogenesis was recognized to be an especially suitable subject for testing mutagens. Its regularity permits the testing of various stages of development. Two contributions have enabled this. The exact spermatogenesis timetable was first described by Leblond and Clermont.[12,13] The second point was the development of an air-drying method for the preparation of meiotic chromosomes by Evans et al.[14] Using this method, it is possible to investigate either chromosomes of spermatogonia, or the first meiotic division in spermatocytes. The analysis of spermatocytes, however, seems not to be very sensitive for detecting mutagens; chromosome aberrations induced in spermatogonia are no longer detectable in metaphase I, since they were already eliminated during spermatogonial cleavage. Thus, for demonstrating induced chromosome aberrations in male germ cells, the investigation of spermatogonia might be the more sensitive method. As demonstrated in experiments with cyclophosphamide,[4] spermatogonia are not as sensitive to the induction of chromosome aberrations as bone marrow cells (FIGURE 6). Whereas five injections of 40 mg/kg increased the yield of aberrations in bone marrow cells from 1% (gaps excluded) in the control to 21.1%; the yield in spermatogonia was elevated only from 0.5% to 0.6%.

2. Female Germ Cells.

With all the present methods it is only possible to analyze induced structural chromosome aberrations. In humans, however, aneuploidies are of great importance. They lead to abortions or to severe malformations in the next generation, like mongolism or Klinefelter's syndrome. Nondisjunction has to be considered the basic mechanism. The factors causing nondisjunction in mammals and man are practically unknown. Therefore, the experimental investigations of this problem seem to be especially important. Russell and Saylors developed a method to analyze the occurrence of induced nondisjunction

in mice with the help of X-chromosome markers.[15] This Tabby-X-method, however, applies only to nondisjunctions of the gonosomes and neglects all the autosomes. Furthermore, this method is not very sensitive because of the high proportion of chromosomally aberrant embryos which die during pregnancy.[16] Meiotic chromosomes in female mammals were studied shortly after the application of the test compound, by a method first described by Röhrborn and Hansmann.[17] The principle of this test method,[17,18] the analysis of unfertilized oocytes, is based on the following data: In the dictyotene stage, the oocytes rest from about the time of birth until shortly before ovulation. After a very short period with leptotene-like and diplotene-like structures, the meiotic diakinesis and metaphase I follow. After anaphase I, a short telophase I, and interkinesis, the metaphase II follows. In this stage, the results of mitotic nondisjunction in oogonia and nondisjunction induced during the first meiotic division can be analyzed directly by counting the number of chromosomes of the single oocytes. To perform this test, adult female mice were pretreated with hormones to synchronize the oestrus. Three hours after the injection of human chorionic gonadotropin, the oocytes are in the stage of metaphase I; 16 hours after the injection all oocytes are in the stage of metaphase II. At this time, the females were killed, the oocytes flushed out of the oviduct, treated hypotonically, and the single oocytes fixed under microscopic control.

Using this test system, we analyzed the frequencies of spontaneous nondisjunction in metaphase II oocytes of mice,[19] and found that the preovulatory phase is very sensitive to the induction of aneuploidies, as well as structural aberrations. This could be shown, for example, by experiments with X-rays,[20] triaziquone,[16,17] cyclophosphamide,[17] and trypaflavin.[21] In all these experiments the yield of aneuploid metaphases with 19 or 21 chromosomes instead of the usual 20 increased.

Furthermore, structural chromosome aberrations were also analyzable with this test system. Breaks, fragments, deletions, and even exchange figures, like rings, were detectable.[20] Because of the uniform shape of mice chromosomes, which are only acrocentric, cytogenetic analysis might, at times, be difficult. It is hardly possible to detect isochromatid deletions. Moreover, a classification of chromosomes is not possible. These problems can be avoided using Syrian hamsters (Mesocricetus auratus), which have easily distinguishable chromosomes (2 n = 44): great metacentrics, small metacentrics as well as acrocentrics. For these reasons, we recently developed a method to analyze chromosomes of Syrian hamster oocytes.[22] The preparation of metaphase II chromosomes (FIGURE 7) is similar to the method in mice. Also, in this species, the induction of chromosome aberrations and its analysis is possible (FIGURE 8).

CONCLUDING REMARKS

After comparing the standard in vivo tests for the identification of mutagens, the following conclusions can be drawn:

The micronucleus test, which indirectly measures the occurrence of chromosome aberrations, is one of the most useful methods. It is quick, simple, and easily learned by those not trained in cytogenetics.

Metaphase studies are indispensable for the differentiation of cytological effects induced by meaningful compounds. Bone marrow cells or spermatogonia must be analyzed to determine the severity of genetic damage.

The SCE test is much more sensitive than all other cytogenetic test systems. Mutagenic compounds elevate the rate of SCE in concentrations that are not yet active enough to induce chromosome aberrations. Despite using the SCE test, the mechanisms of induction are still unknown, and we do not know if SCE are mutations. Before this method opens a new field in mutagenicity testing, more experience is needed.

With all these methods it was only possible to investigate structural aberrations. The analysis of meiotic stages in oogonia is the only method to analyze nondisjunction processes. Use of this method for screening does not appear advisable because of the relatively time-consuming preparation involved. Therefore, this method which has the highest relevance for man should be used only if special problems arise which have to be clarified. As demonstrated, there is no test fulfilling all requirements. Various test systems have to be used to clarify all the problems in mutagenicity testing.

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TABLE I

In vivo induced sister chromatid exchanges (SCE) in Chinese hamster bone marrow cells through caffeine

Agent	SCE per metaphase mean ± SEM	Number of metaphases	Number of hamsters	t-test (compared to the controls)
control: 2 x 0.5 ml drinking water; given per stomach tube	3.84 ± 0.50	600	12	
2 x 20 mg caffeine per kg b.w.; per os	4.35 ± 1.30	400	8	$p > 0.2$
2 x 100 mg caffeine per kg b.w.; per os	5.43 ± 1.30	400	8	$p < 0.01$
2 x 200 mg caffeine per kg b.w.; per os	6.20 ± 1.72	400	8	$p < 0.01$
2 x 400 mg caffeine per kg b.w.; per os	5.00 ± 0.47	200	4	$p < 0.01$

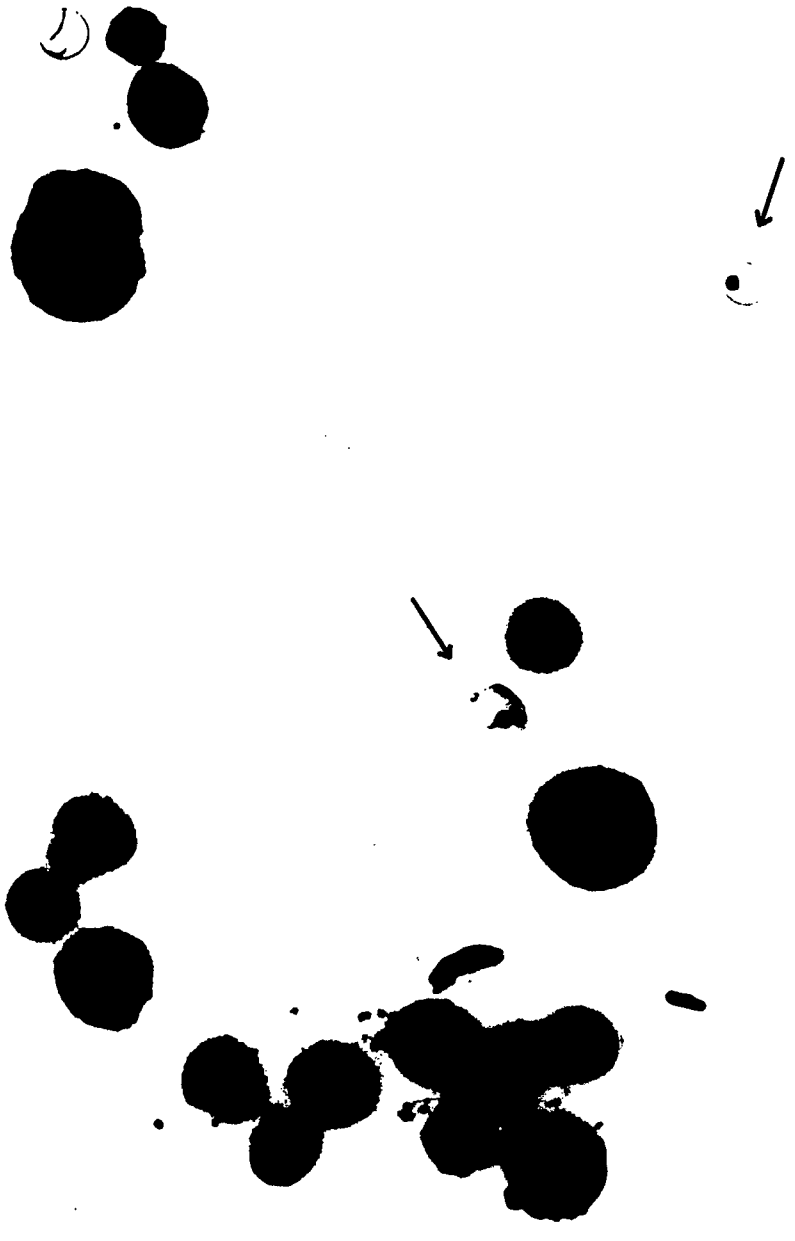

FIGURE 1. Erythrocytes with micronuclei.

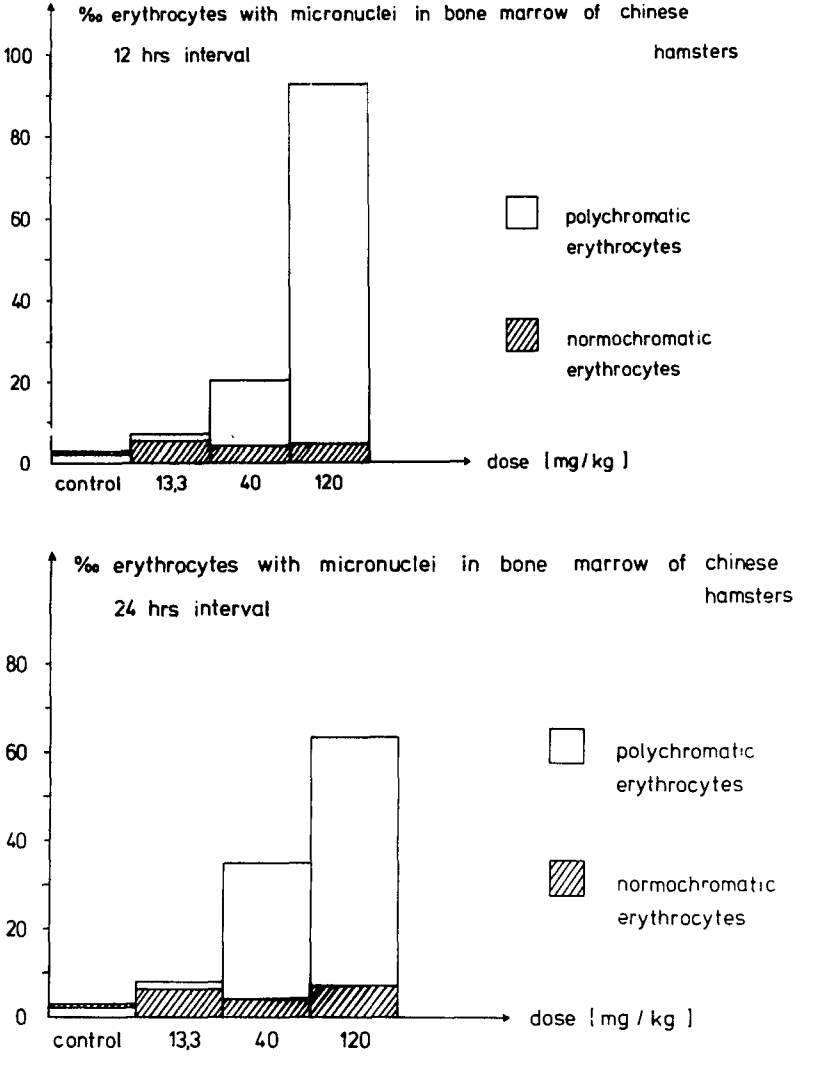

FIGURE 2. Erythrocytes with micronuclei 12 and 24 hours after the second cyclophosphamide injection.

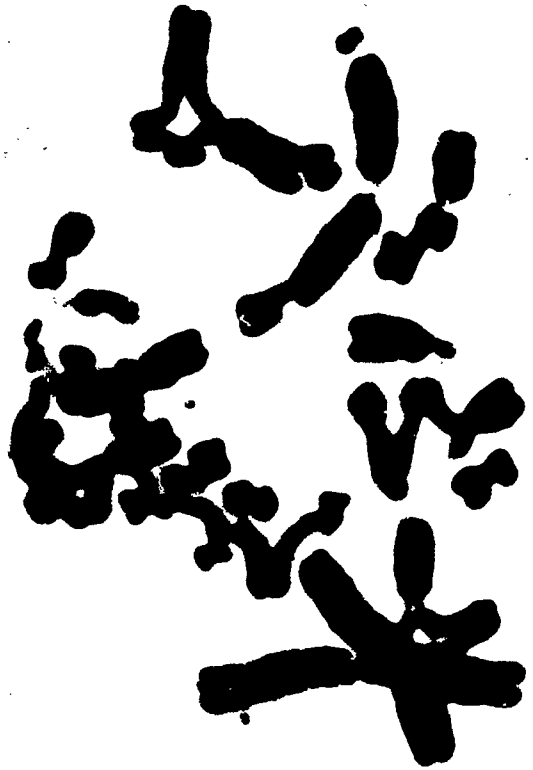

FIGURE 3. Multiple chromosome aberrations in a Chinese hamster bone marrow cell.

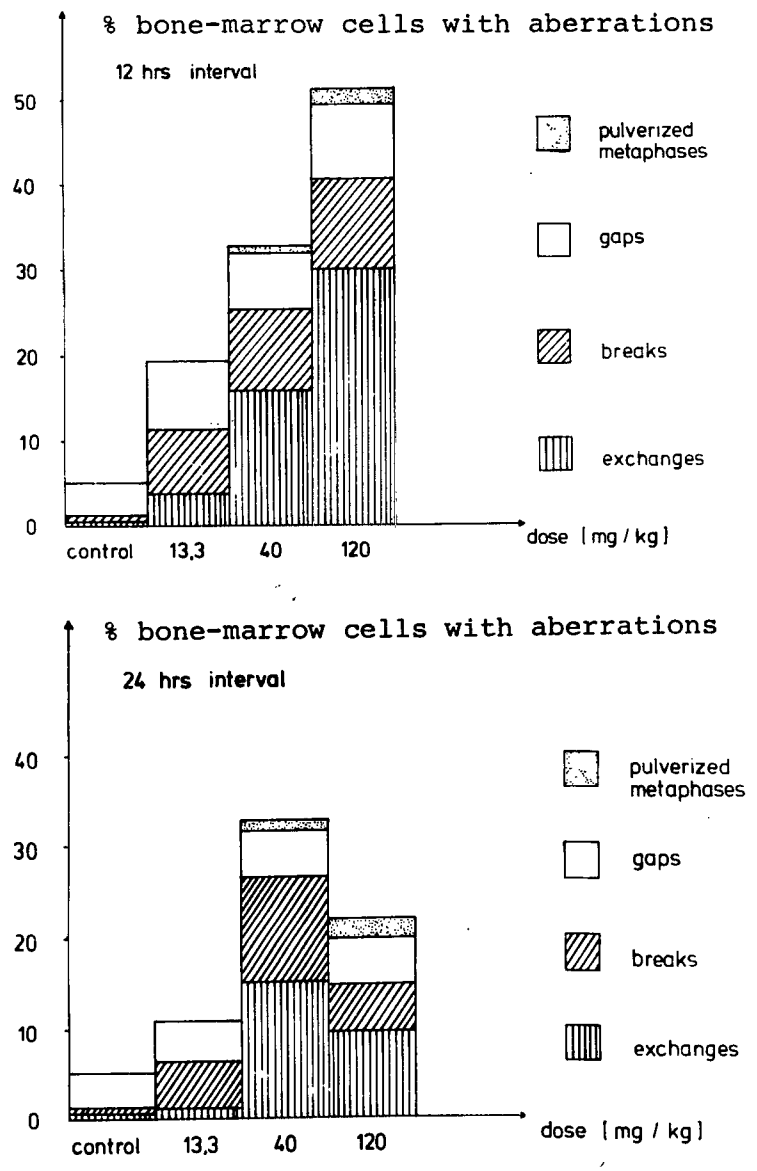

FIGURE 4. Chromosomal aberrations of Chinese hamster bone marrow cells 12 and 24 hours after the second cyclophosphamide injection.

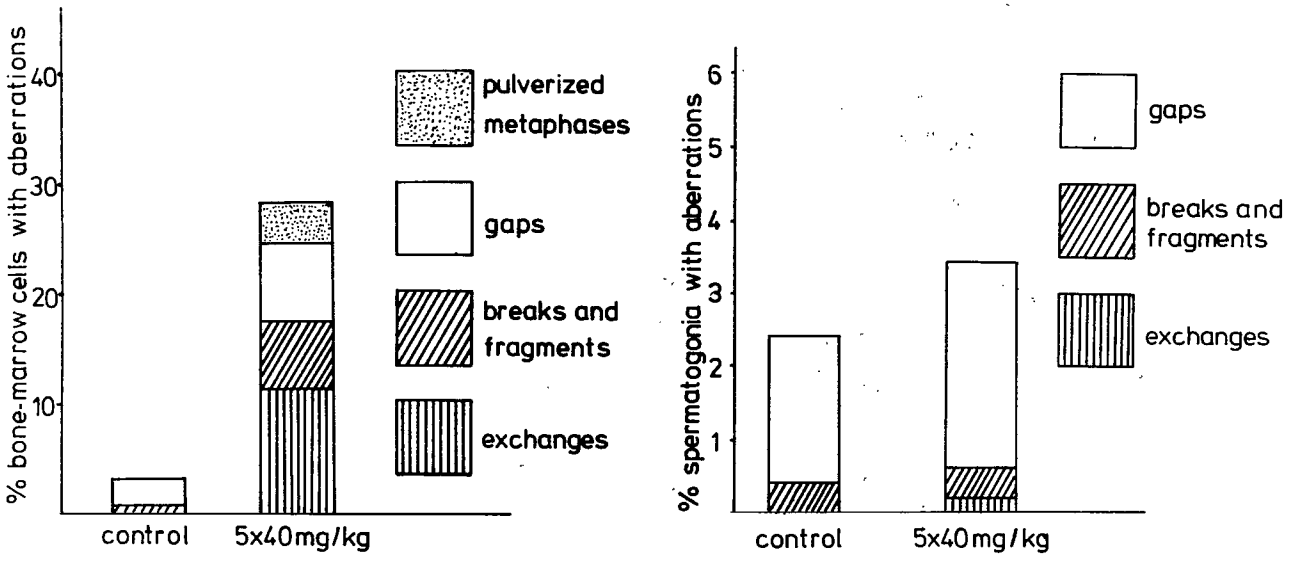

FIGURE 6. Chromosomal aberrations of Chinese hamster bone marrow cells and spermatogonia 24 hours after the last of five cyclophosphamide injections.

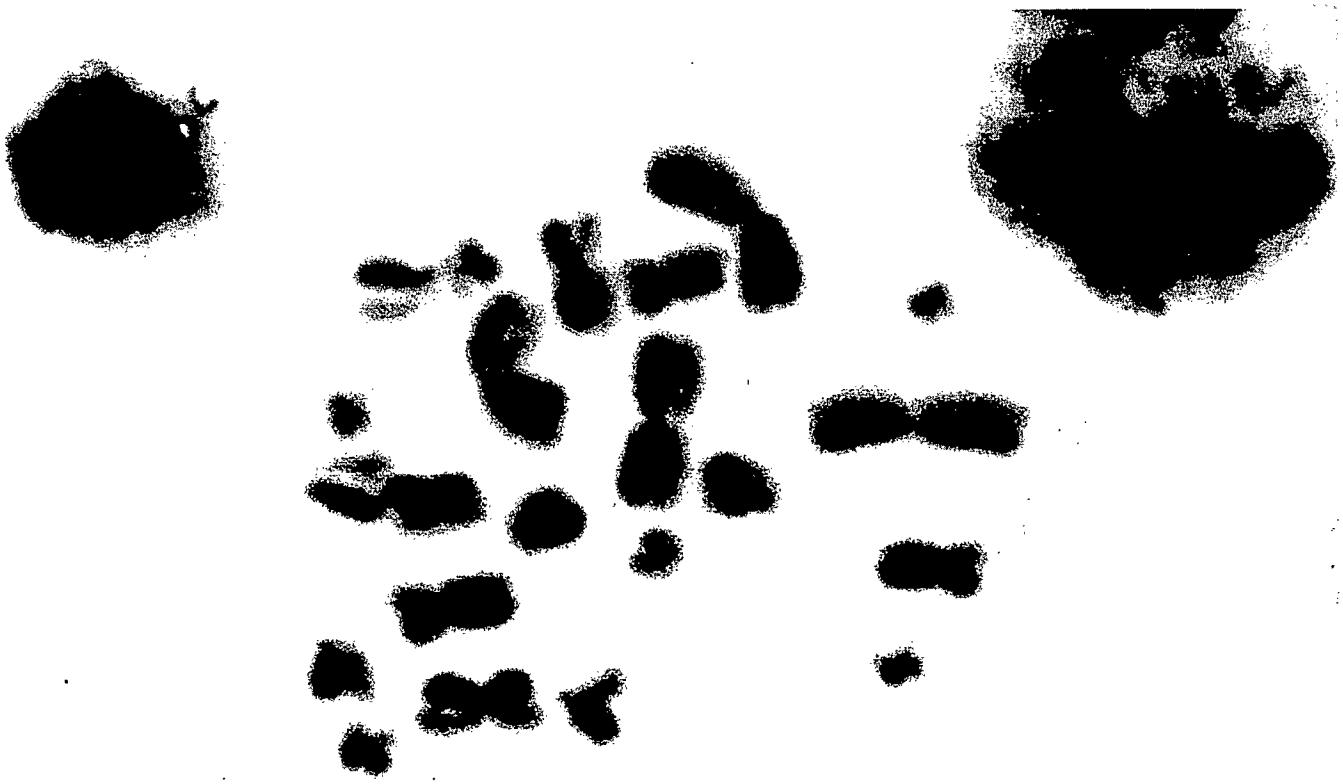

FIGURE 5. In vivo induced sister chromatid exchanges in Chinese hamster bone marrow cells through caffeine.

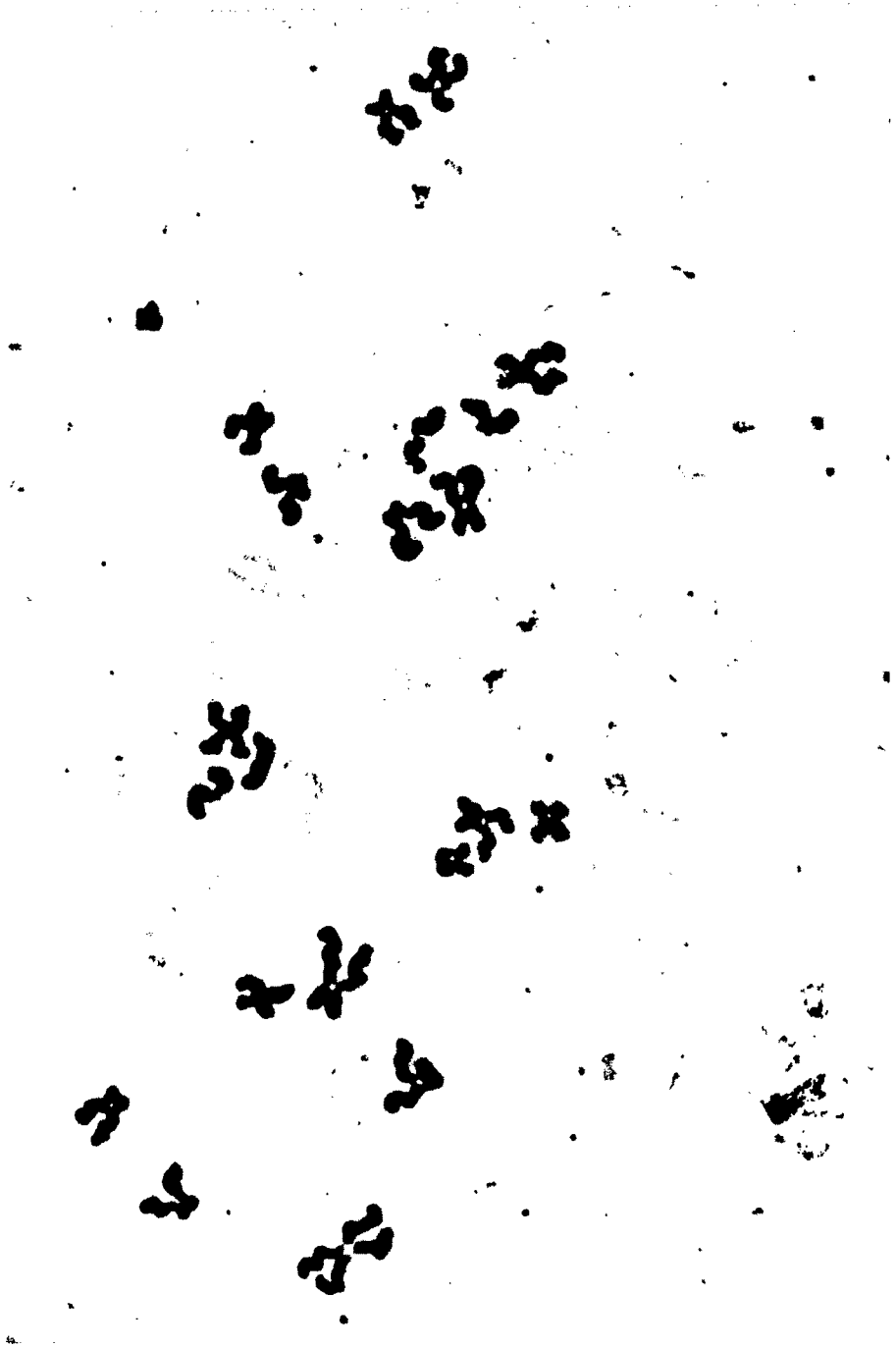

FIGURE 7. Metaphase II of Syrian hamster (Mesocricetus auratus) oocytes.

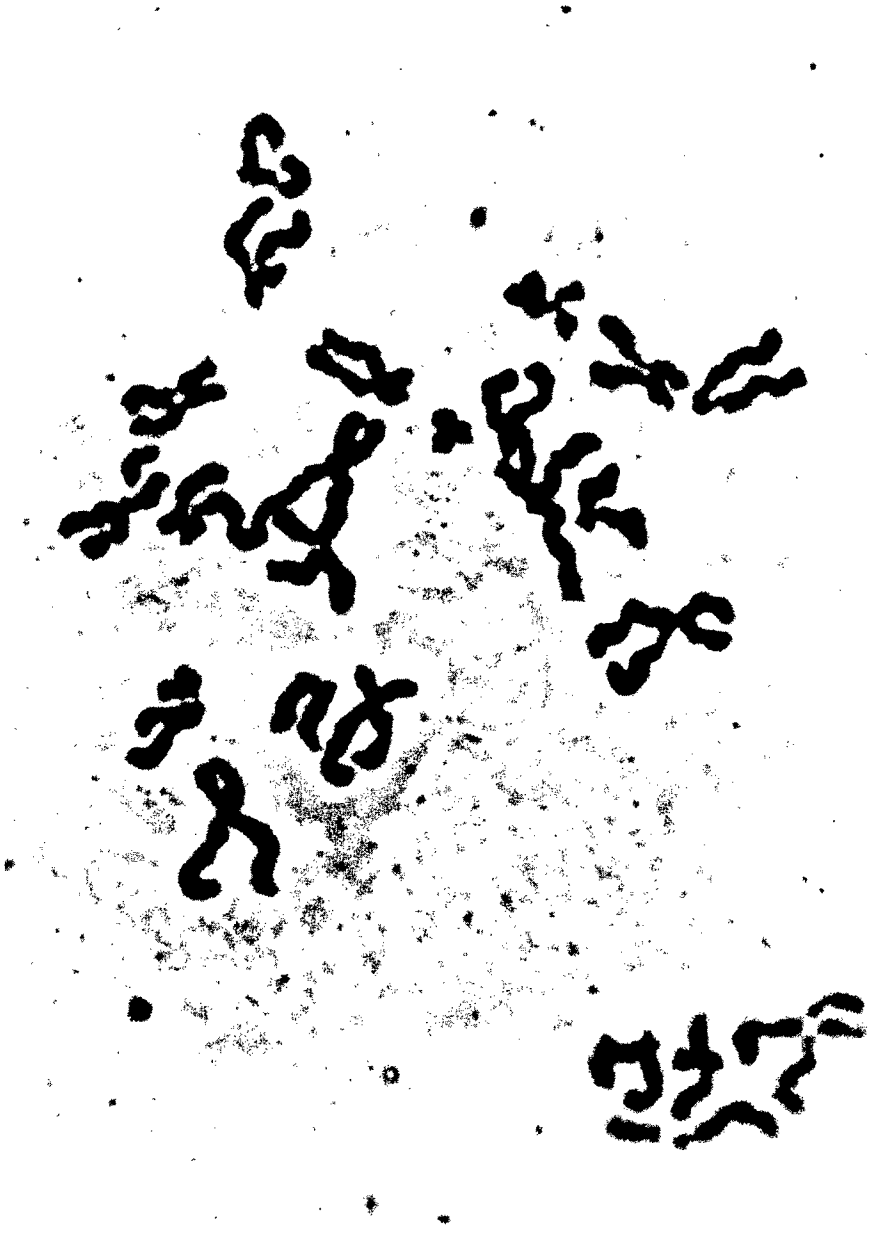

FIGURE 8. Metaphase II with multiple chromosome aberrations.

IN VIVO TERATOGENICITY TESTS

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Prompted by the thalidomide incident of the early 1960's, regulatory agencies initiated requirements for animal testing of therapeutic drugs that hopefully would detect teratogenic potential prior to their widespread distribution. The experience of two subsequent decades of extensive testing has failed to demonstrate the reliability of animal studies in the prediction of human teratogenic potential. There are so many examples of inconsistency between results of animal teratologic studies and the human experience that a credibility gap has developed. Recently published epidemiologic studies have shown that numerous drugs had insignificant human teratogenic potential,[1] and yet these drugs have produced positive results in multiple animal species.[2] This paper will outline the current procedures for in vivo teratogenicity testing in animals, discuss probable reasons for deficiencies, and provide a preliminary critical evaluation of the degree of predictability.

PROCEDURES

Teratogenicity means the capacity to induce congenital monsters. Historically, the focus has been on major overt morphological abnormalities. There is, however, a growing tendency to broaden the definition of teratogenicity beyond major dysmorphogenesis, by including minor and latent (covert) structural abnormalities. Furthermore, it is recognized that functional and behavioral abnormalities should also be considered, particularly in mature offspring. Unfortunately, the majority of teratologic testing has focused only on the state of anatomic development at the end of pregnancy. Pregnant animals are treated during the period of embryonic development (organogenesis), and fetuses are removed from killed mothers a few days prior to parturition. It is argued that mothers will cannibalize abnormal or dead offspring if allowed to deliver spontaneously. The usual periods of treatment (in days of gestation, with day zero being the day of conception) are: mice and rats --6 to 15; rabbits--6 to 18. Treatments are avoided before and after these periods to minimize the chance that the teratogenic potential of a chemical might be obscured by its lethal effect on the conceptus. In addition, there is some evidence that initiating treatment substantially before the critical period of gestation will provide an opportunity for induction of detoxification enzyme activity, resulting in reduced fetal exposure to the agent. Potential embryolethality is evaluated by decreased litter size, or disparity between ovarian corpora lutea and implantations. It is common practice to initially evaluate gross abnormalities, and then to group living fetuses for examination for internal visceral anomalies or skeletal anomalies. Results are separately tabulated as: litter size (number of implantations), lethal effect (number of resorbed embryos and dead fetuses), teratogenic effect (number of malformed live fetuses), and fetal growth retardation (reduced

body weight of live fetuses). This practice of separate categorization of offspring hampers statistical analysis for dose-related effects, since an embryopathic chemical often causes deaths primarily at high dose levels resulting in decreased apparent malformation rates. In studies of thalidomide in rabbits, Schumacher et al. demonstrated a linear relationship between the log of dose and total "abnormalities," when dead or malformed fetuses were considered to be abnormal.[3] Such a categorization seems reasonable unless fetal death and fetal malformation can be shown to be mutually exclusive events. A linear dose-response curve provides the opportunity for statistical determination of the 50% embryopathic effect level which can be compared to the maternal LD_{50} for evaluation of direct embryotoxicity. Using this approach to evaluate animal teratogenicity data from the literature, Jusko has demonstrated that drugs can be classified into two categories: those that have a dose threshold for teratogenic effect and those that do not.[4] The former group (including aspirin) has 50% embryopathic doses that are close to the maternal lethal doses, whereas the latter group (including thalidomide) has greater direct embryopathic potential. Presumably, there is greater teratogenic risk with a compound that has no threshold to its effect, and causes malformations at a dose level considerably below that causing maternal toxicity. Although such dose-effect analyses are common in other branches of toxicology, it is rare to find them in the teratologic literature.

Multigenerational reproductive studies have been advocated by regulatory officials,[5] although an advisory panel has questioned their value beyond the second filial generation.[6] It is generally accepted that a complete evaluation of reproductive toxicity should include study of the reproductive performance in the F(1) animals which have been exposed continuously to the test substances from the time of conception, and during the periods of embryogenesis, infancy, puberty, and reproductive maturity. This assessment requires observation of the growth and development of the F(2) generation through weaning.

POSSIBLE REASONS FOR DEFICIENCIES

Dosage. As previously discussed, meaningful interpretation of teratologic studies requires consideration of the relationship between the teratogenic dose range and the maternal toxic dose range. Since abnormal fetal development is likely to result if the mothers are "sick," it is generally recommended that the highest dose level produce minimal, but measurable, maternal toxicity. Indirect pseudoteratogenicity can also occur if the treatment causes excessive depression of maternal eating or drinking.[7,8]

Pharmacokinetics. The fraction of administered dose ultimately reaching sites of teratogenic action in the conceptus or placenta is governed by multiple kinetic factors, including rates of absorption, biotransformation, placental passage, and excretion. Inconsistent teratologic results between experiments, laboratories, species and strains of animals can often be explained by variations in these factors. Keller and Blake demonstrated a 400% difference in plasma levels of thalidomide in rabbits depending on the oral dosage formulation.[8] The widely investigated strain-dependent susceptibility of mice to cortisone-induced anomalies correlates with slower maternal elimination of the drug in the more susceptible strain.[9] Although transport of chemicals across the placenta late in gestation has received a great deal

of experimental attention, there is much less information on maternal-fetal exchange early in gestation when morphologic teratogenesis is induced. Moreover, there is a complete void in our knowledge of the amount of maternally administered drug reaching the human embryo at known levels of exposure. Thus, it is impossible to rationally devise dosing regimes in animal experiments that would be relevant to the human situation.

Until recently, it was thought that the fetus lacked the enzyme activity responsible for biotransformation of xenobiotics. Through improved analytic methodology, it is now known that fetal liver, particularly in primates, possesses many of the metabolic capabilities of maternal liver. Some of these metabolic transformations result in the formation of reactive metabolites that can bind to cellular macromolecules, and thereby cause cancer, mutations, and cell death. Emerging evidence suggests that metabolic intoxification may also be a mechanism of embryopathy. Because the enzymatic activity responsible for these reactions is related to genotype and multiple environmental factors, it may also provide an explanation for species and other variations in teratologic results. The anticonvulsant phenytoin (Dilantin) is metabolized to a dihydrodiol metabolite via a reactive arene oxide intermediate. The arene oxide intermediate presumably binds covalently to fetal macromolecules, and may be the cause of the well documented teratogenic effect of the drug in mice.[10] Studies in our laboratory have shown a species correlation between susceptibility to phenytoin-induced teratogenesis and formation of the dihydrodiol metabolite in fetal liver.[11] There is also a strain-dependent embryopathic sensitivity to certain polycyclic aromatic hydrocarbons (PAHs) in mice, which is related to the genotype determining inducibility of aryl hydrocarbon hydroxylase (AHH).[12] AHH converts PAHs to reactive toxic metabolites. We have recently found that fetal livers from four strains of mice activate benzo(a)pyrene to mutagenic metabolites at an efficiency that linearly correlates with their induced levels of AHH.[13] These findings provide a basis for the widely discussed genetic-environmental interactions that presumably subserve multifactorial inheritance of susceptibility to birth defects.

PREDICTABILITY OF TERATOGENIC POTENTIAL

The ultimate utility of any animal toxicologic testing procedure depends on the degree of extrapolatability to human beings. Thalidomide is the only chemical known to have a profound teratogenic effect in humans, and the failure to detect positive results with standard teratologic tests in mice and rats is well known. It was determined retrospectively that thalidomide was teratogenic in rabbits and monkeys, but there is no assurance that these species would be better predictors for other human teratogens. Since there are only a few drugs with known human teratogenic potential, it is difficult to evaluate predictability against known positives.[1]

In contrast, there are numerous therapeutic drugs now known to have little or no human teratogenic risk.[1] This information is derived from a review of the data from the Perinatal Collaborative Project, a prospective and concurrent epidemiologic study of more than 50,000 pregnancies. The exact determination of drug exposure in the first 4 months of pregnancy, and uniformity of categorization of major structural anomalies, are unparalled by any other study to date. For many popular drugs, there were sufficient

numbers of exposed cases to permit statistical confidence of the lack of teratogenic effect, at least under prevailing conditions of use. The results obtained for 16 drugs are listed in the table; values are given for the number of exposed cases (at least 100 for each drug selected), and the relative risk ratio, after standardization, for race, and survival. A ratio of 1.0 indicates an identical frequency of congenital anomalies between exposed and nonexposed cases. The only drug on this list with a ratio significantly greater than unity is insulin. Also shown in the table are the results of animal teratologic tests in various species taken from the reference text of Shepard.[2] A degree of subjective judgment was required to translate Shepard's comments into + or - categories, and no attempt was made to review individual reports.

It can be seen that for 8 of 16 drugs (aspirin, salicylamide, sulfisoxazole, phenytoin, phenobarbital, meclizine, prochlorperazine, and d-amphetamine) there was disagreement between animal tests and the human experience; the animal test results were positive (for at least one species), and the human experience negative. For 6 drugs there was agreement; positive animal results correlating with a positive human result for 1 drug (insulin), and negative results with both animals and humans for 5 others (propoxyphene, penicillins, doxylamine, diphenhydramine, and norethynodrel). No decision could be reached for 2 drugs (tetracycline and caffeine) because of disagreement between studies with the same animal species. It should be realized that the reference source used for animal teratologic results is incomplete, and other studies exist in the literature which would alter the comparability for a few of the drugs, particularly those with negative findings. In addition, the relative merits of individual animal studies could be argued extensively. These considerations aside, the obvious conclusion is that in vivo teratologic tests have poor predictability of human teratogenic potential. Generally stated, it would appear that most drugs can be found to be teratogenic in some animal models, while very few drugs have a clinically significant teratogenic potential.

Hopefully, through improvements in design and interpretation of animal tests, and through more appropriate selection of species based on comparability of metabolism, etc., it may be possible to improve on what currently must be regarded as an unreliable toxicologic test procedure.

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Predictability of Teratogenic Potential

Drugs	Human (1)		Animal (2)	
	No. Exposed	Relative Risk	Species	Result
Analgesics				
Aspirin	14,864	1.04	Rat	+
			Mouse	+
			Monkey	+
			Rabbit	-
Salicylamide	744	0.95	Hamster	+
Propoxyphene	686	0.99	Rat	-
			Mouse	-
			Rabbit	-
Antibacterials				
Sulfisoxazole	796	1.04	Mouse	+
			Rat	+
Penicillins	3,546	1.07	Mouse	-
			Rat	-
Tetracycline	341	1.14	Mouse	+/-
			Rat	+/-
Anticonvulsants				
Phenytoin	132	1.56	Mouse	++
			Rat	+/-
			Monkey	+/-
Phenobarbital	1,415	1.03	Mouse	+
			Rabbit	+
Antinauseants				
Meclizine	1,014	1.21	Rat	+
			Ferret	+
			Mouse	+
Doxylamine	1,169	1.06	Rabbit	-
Prochlorperazine	877	1.18	Mouse	+
			Rat	+
Diphenhydramine	595	1.33	Rat	-
			Rabbit	-
			Mouse	+ (vitro)

Drugs	Human (1)		Animal (2)	
	No. Exposed	Relative Risk	Species	Result
Hormones				
Insulin	121	2.09 ($p < 0.01$)	Rat	-
			Mouse	+
			Rabbit	+
Norethynodrel	154	1.10	Rat	-
Stimulants				
d-Amphetamine	367	1.11	Mouse	+
			Rabbit	+
Caffeine	5,378	0.98	Mouse	+/-
			Rat	+/-
			Rabbit	+/-

1. From Heinonen et al. (1977).

2. From Shepard (1976).

THE ROLE OF BEHAVIORAL ASSESSMENT IN STANDARD IN VIVO TERATOGENICITY TESTING

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INTRODUCTION

Werhoff and Gottlieb brought to our attention the possible manifestation of teratogenic effects as behavioral changes in developing offspring.[1] They also recognized the inherent difficulties in establishing cause-effect relationships from human clinical data. The problem is two-fold: (1) the range of normal human behavior is extremely broad, and (2) intervening extraneous variables can intrude during the interval between exposure and evaluation to confound the relationships between cause and effect.

As a result of this problem, many researchers have argued for increased reliance upon laboratory investigation in order to detect the potentially hazardous agent before it is introduced into the environment.[2] Not surprisingly, there has been a rapid increase in research directed toward the process of behavioral teratogenesis. Several review articles have recently appeared which further attest to the increasing reliance on postnatal functional evaluation.[3-6]

This brief review will address the following issues:

1. What do we hope to gain through the inclusion of a behavioral evaluation program in the assessment of reproductive hazards?

2. What testing strategies are currently in use or have been proposed?

3. Which protocols will provide the maximum amount of relevant information at the least additional cost to our testing programs?

In addition, our preliminary findings relating to the effects of prenatal methylene chloride exposure on postnatal behavioral function will be presented.

BENEFITS OF BEHAVIORAL EVALUATION

As mentioned in the introduction, the detection of deficits in behavioral function will probably never occur outside the laboratory. It thus becomes mandatory to include such capabilities in our current screening procedures if we deem the detection of such effects to be a desirable goal. In order to reach such a decision, we must first deal with the relevance of behavioral testing.

Like other animal researchers, behavioral teratologists must deal with the issue of extrapolation of animal data to humans. Unlike most other disciplines, there appears to be some reluctance on the part of researchers

outside the field of behavioral investigation to accept the validity of any level of extrapolation of behavioral data from other animals to man. Perhaps this is because their attention is too narrowly focused on the specific detail of an individual behavioral task, e.g., a rat pressing a lever at some prescribed rate in order to gain access to food. Such a behavior is then usually compared with some aspect of human behavior, e.g., the abstract reasoning required for the mathematical solution of an equation, and then followed by the challenge, "What's the relevance?"

Rather than focus attention on the technology of behavioral evaluations of animals, attention should be focused on the observable behavior that occurs when an organism interacts with its environment. The environment can be simple or complex, artificial or natural, relatively unchanging or rapidly changing. The behaviors under observation may be innate, acquired, sex-dependent, species dependent, etc. In a toxicological screening situation, one is interested in assessing how a well-defined class of organisms interacts with a well-defined environment, and what changes occur in the nature of this interaction during, or following, exposure to a toxin. A difference in the observed behavior of treated and control animals indicates that the treatment has altered the organism's response to its environment. It is unlikely that the structural or developmental change responsible for the change in behavior will be immediately clear. However, one thing will be self-evident: Treatment has altered the manner in which the organism interacts with its environment. In order to determine the site of toxic action, whether it be the nervous system, endocrine system, sensory or motor system, additional specialized tests will be required.

Behavioral endpoints are apical, thereby representing the culmination of the interaction between the function of numerous anatomical subsystems, developmental processes, and the environment, and are well suited for inclusion in screening programs. (cf. footnote reference 2) Butcher has argued that the detection of behavioral effects in the absence of morphological alteration is evidence that behavioral endpoints provide a more sensitive endpoint.[2] This argument has been discounted by those in the pharmaceutical industry, who feel that there is little need for such an increase in sensitivity, since an agent which produces obvious skeletal, or soft tissue, malformation, will automatically be withheld from the market. Such an argument assumes that the mechanism underlying the morphological effects is the same as that responsible for the behavioral effects, which remains to be demonstrated. In the case of some materials, it may be possible, impractical, and/or unnecessary to ban their use, if we can identify the population at risk and the exposure conditions at which this risk becomes manifest. Thus, a continuing search for procedures which have high sensitivity is warranted.

In addition to their apical nature and high sensitivity, behavioral endpoints have an inherent functional relevance. Low-level teratogenicity may often appear as an increase in the frequency of occurrence of skeletal or soft-tissue variations. The biological relevance of these variations to the survival of the individual is questionable, and to the survival of the species probably irrelevant. This is not the case for behavioral changes brought about by perinatal toxin exposure. Changes in such things as decisionmaking and reaction time can have readily demonstrable effects on the survival of the individual and the species. Beyond that, behavioral changes such as learning

disabilities, mental retardation, and psychological disturbances alter the quality of life for the individual and those around him.

TESTING STRATEGIES

If we agree that detection of behavioral changes is desirable, and that we wish inclusion of such capabilities in our screening procedures, what testing strategies are available? Currently, considerable debate exists among those in the field as to the best manner in which to proceed. The most frequently mentioned approach is the use of a comprehensive behavioral screening battery. These batteries often include every distinct class of behavior for which we have the technological assessment capability. Testing at every distinct stage of development is also recommended. This approach is obviously the most conservative. While it minimizes the chance of a behavioral teratogen going undetected, such approaches are very costly in terms of time, equipment, and personnel.

Weiss has recognized this problem, and attempted to scale the level of testing to the outcome at each level of testing and the need to obtain further information.[8] The strategy utilizes a decision tree with three basic tiers, or levels, of analysis. The first tier is similar to that used in the pharmaceutical industry to screen for CNS activity. It includes measures of gross motor and ambulatory function, reflex changes, elicited responses to gross stimulation, and growth rate. The second tier includes selected sensitive behavioral assays such as spontaneous motor activity and avoidance learning. The final tier includes the utilization of specific function tests designed to assess sensory and/or motor processes, and complex cognitive function. The decision to continue with the next level of testing depends on the cost/benefit ratio associated with the compound under investigation.

The second strategy under consideration might be called the apical task approach[2] which limits the initial screen to one or two specific tasks. Essentially, this approach eliminates tier one of Weiss' decision tree and begins with the second tier.[3,8] Both locomotor activity and learning tasks have been suggested as appropriate for this level of screening. Arguments in favor of eliminating the gross tier one screen include its general insensitivity, high cost in terms of personnel, and its vulnerability to subjective bias. Thus, while positive results call attention to the need for further testing, negative results are open to questions which can only be resolved by further testing. Therefore, one might be best advised to start with apical testing. In fact, a review of the literature indicates that the vast majority of behavioral teratology investigations have used either some form of general activity measure, or a "learning" measure.[4] Because of the current central role of these two measures in behavioral evaluation, a more detailed description and discussion of these procedures may be useful.

Activity measures are probably the most frequently used measure in behavioral pharmacology and behavioral toxicology. This is no doubt due to the apparent ease with which the data can be collected. However, as has been most recently pointed out by Norton and Reiter, the ease is more apparent than real.[11,12] The main drawback is the extreme sensitivity of the method to extraneous influences at the time of testing. Fortunately, with awareness and reasonable effort on the part of the investigator, this problem can be minimized.

Activity is not a unitary concept. Rather, it includes behaviors as diverse as simple motor reflexes, tremor, stereotypic movement, species specific movement, ambulation, self-directed grooming, and social interactions. Quantification of these behaviors can be greatly influenced by the recording apparatus, the environmental and social setting, the past experience and maturational level of the subject, and the current hormonal, motivational, and arousal level of the subject (cf. 12).

Of the previously mentioned behaviors, ambulation or locomotion is most frequently studied (cf. 11). Although numerous combinations of testing procedures and apparatus have been used, most can be classified on the basis of the duration of the measure into acute and chronic tests.

Acute measures range in duration from several minutes to several hours and attempt to evaluate such processes as initial reactivity, exploratory behavior, and habituation to novel environments. Equipment used to acquire such measures usually includes jiggle platforms, photocell cages, open fields, or their equivalent.

Chronic measures of activity range in duration from several days to several weeks. Such measurement usually takes place in a home cage, residential maze, or running wheel. These procedures measure the stable level of activity of animals in familiar environments with minimal associated stress.

The acute, short-term measures of activity may prove quite useful as a screening procedure. The behavior observed in such situations can be described as apical (cf. 2) since the behavior observed is the culmination of the interaction between motivational processes, sensory and motor capabilities, the information processing ability, and learning and memory processes of the animal. The novelty of the test situation and the stress reaction elicited by it are a crucial element in this type of behavioral assessment. This stress may unmask a deficit which is hidden under less stressful conditions. In addition to its apical nature, several other features make it ideally suited for inclusion in any toxicity screening system. First, the short duration of the testing interval permits the testing of large numbers of animals. Second, since exploratory behavior can be assessed in animals of almost any age, it permits longitudinal evaluation of behavioral development. Finally, the cost in man-hours and equipment is minimal in comparison to some of the more elaborate operant procedures.

Many of the arguments presented above for utilizing short-term activity testing could also be made in support of "learning" tasks. The major problems have been the length of time required to test animals, stress associated with food deprivation, which is commonly used to motivate the animals, and the difficulty in testing animals before they become adult.

Two years ago, Dr. Tina Levine and I became interested in developing an automated learning paradigm which would correct some of these problems.[18] Since many of our fellow researchers were using mice in investigations of carcinogenicity and teratogenicity, we felt that a procedure applicable to mice might facilitate future collaboration. Several other benefits soon became apparent. In particular, mice offer economy in collection in the correlative biochemical and pathological data essential in any toxicological investigation. They are also more active than rats which facilitates training.

We have successfully trained mice as early as 21 days of age. The technique to be described offers several advantages over other methodologies. First of all, it is not necessary to subject the animals to the stress of a high level of deprivation in order to maintain adequate motivation and performance (18 to 20 hours of water deprivation is sufficient). Such stress might interact with the direct effects of the agent in question. Second, acquisition of the operant, a nose-poke, is rapid and requires no shaping, thereby decreasing the experimenter-subject interaction which could bias the results of behavioral studies. Third, the nose-poke response can be studied in very young animals allowing the assessment of developmental variables. Fourth, the level of nose-poke behavior seen in the animals before training can be used to assess exploratory activity. Fifth, the task difficulty may be manipulated readily by selecting: (1) the level of proficiency required, (2) the time interval between problems, (3) the stimulus intensity or type of pattern, (4) the problem type (visual discrimination vs. position habit; simultaneous vs. successive discrimination; acquisition vs. reversal). Finally, the speed with which the evaluation can be performed permits the screening of large numbers of animals: about 20 per day.

The basic experimental task is a discrete trial, simultaneous two-choice, visual discrimination. The number of trials required to attain a predetermined criterion of accuracy and mean latency of responding, two commonly used measures of learning, can be compared in treated and control animals. The experimental apparatus has been described previously.[13]

Mice are placed on a 2-hour limited access drinking schedule 3 days prior to their first placement in the chamber. For the remainder of the study, the animals are given ½-hour access to water approximately 2 hours after testing. Food is available ad libitum. This deprivation schedule produces about an 8% weight loss in mice and a 5% weight loss in rats.

Prior to the initiation of the discrimination task, an experimentally naive mouse is placed in the test chamber. The chamber is dark and the mouse is permitted to explore for 5 minutes. Exploratory nose-pokes are recorded. The mouse is then simultaneously presented with two visual stimuli (bright light vs. dim light) and required to touch the correct visual stimulus with its nose. The first response usually occurs within 60 seconds. Interruption of a photocell beam is used to monitor the response. If the correct response is made, a dipper containing 0.01 cc condensed milk is activated for 5 seconds to provide a reward. If the animal responds incorrectly, the trial ends and a 15-second intertrial interval begins. After this interval, a new trial begins and the animal is again presented with the two stimuli.

The mice remain in the test chamber until they complete 10 consecutive correct responses. This requires about 80 minutes per subject among control mice. The number of correct responses, the mean number of nonrewarded, extraneous intertrial responses (responses made between consecutive presentation of problems), as well as mean response latency and total number of trials to reach criterion are recorded for each animal (see TABLE I).

One week later all mice are retested on the same problem and again remain in the test chamber until they attain 10 consecutive correct responses. Performance on the retest, which should require fewer trials, provides an index of the animal's ability to retain information acquired during the

previous test session. Normal memory function is inferred on the basis of improvement in performance between the first and second session. Three to five days after the first retest, the mice are again retested with the same learning criterion. However, immediately after the attainment of 10 consecutive correct responses, the positive and negative stimuli are reversed and the mice permitted to remain in the chamber. Mice are thus required to switch their response to the opposite stimulus condition in order to continue receiving rewards. All mice are again run to a criterion of 10 consecutive correct responses.

A typical record of the acquisition history of an individual mouse is shown in FIGURE 1. A summary of the performance of a group of control mice appears in TABLE I. The procedure described above has been useful in detecting changes in the acquisition and reversal-learning ability of mice exposed to lead during early stages of development.[14] Furthermore, the paradigm has recently been adapted for rats and is currently being used to assess the behavioral effects of perinatal cadmium exposure.[15]

Although locomotor reactivity and learning assessments are sufficiently sensitive to permit detection of the effects of toxin exposure, they provide little if any indication of the mechanism underlying the observed effects. The final screening strategy to be discussed addresses this problem. This strategy employs the use of pharmacological agents in conjunction with a measure of locomotion or an operant schedule of reinforcement. The rationale for this approach has been described by Grant.[9] Grant points out that CNS damage may be followed by a decreased or increased sensitivity to drugs. Therefore, an animal's response to CNS drugs may unmask a CNS deficit.

The concept of using a pharmacological probe to obtain a noninvasive evaluation of organ function is not new. Toxicologists have made frequent use of hexabarbital-sleeping time tests to make inferences about the metabolic activity of the liver. This test, which provides a rapid assessment of liver function, is based on the fact that hexabarbital is (a) a CNS active agent, (b) produces an observable behavioral effect, i.e., sleep, and (c) the effects can be easily quantified. Alterations in the liver metabolism of hexabarbital caused by toxin exposure are readily reflected in the duration of sleeping time of injected mice.

Consideration should be given to the utilization of this simple, yet elegant, procedure in our search for functional correlates of in utero toxin exposure. With the selection of four or five appropriate drugs it should be possible to rapidly screen for abnormalities in the functioning of numerous metabolic and physiological systems including liver and brain metabolism, lung or renal clearance, blood-brain barrier function, and the metabolic and physiologic functioning of numerous neuronal systems.

In order to be included in the pharmacological screen, the drugs should meet the following criteria: (a) the metabolic pathways used by the drug should be well known; (b) to the greatest extent possible, each drug in the screening battery should be metabolized by different metabolic pathways (e.g., Type I vs. Type II vs. conjugation, etc.); (c) each should use different routes of entry into the CNS (e.g., passive diffusion vs. active transport via a number of different systems); (d) each drug should act specifically on relatively well-defined neuronal systems (e.g., cholinergic vs.

dopaminergic) or brain region (cortex vs. limbic system vs. brain stem vs. autonomic nervous system, etc.) by relatively well-understood mechanisms of action (transmitter release vs. receptor stimulation); and (e) each should produce effects at the behavioral or physiological level which can be simply and accurately quantified (e.g., induced locomotor activity, tremor, altered heart rate or body temperature).

Several candidates are available: d and/or l-amphetamine, morphine, alcohol, tremorine, numerous drugs which have been developed to act on the autonomic nervous system, commonly employed anesthetics, etc. For example, amphetamine-induced locomotor activation might be used to screen for effects arising from in utero toxin exposure. If no differences were found between treated and control mice, one could infer that Type II microsomal enzymes, amino acid transport systems in the blood brain barrier, and the noradrenergic and dopaminergic neuronal pathways mediating locomotor activity were intact. If, on the other hand, differences in drug response were seen between treated and control animals, then the test could be repeated using radiolabeled amphetamine. Plasma and brain concentrations of the parent compound and its metabolites could be determined. The outcome of this study would direct attention to specific target organs such as the liver, blood-brain barrier transport systems, or specific neuronal systems.

Several advantages are inherent in this approach. First, excluding the unlikely event that a toxin will disrupt two or more processes and the effects cancel, negative results will tell us that numerous specific metabolic and physiological systems are functioning normally in the presence of or following exposure to a toxin. A second advantage derives from the fact that large numbers of animals can be tested without the need for extensive behavioral training or overly elaborate testing facilities. This, of course, requires the appropriate selection of drugs which produce easily quantifiable behaviors. Finally, the use of radiolabeled drugs following the observance of treatment effects should permit identification of specific target organ sites thereby providing direction to future mechanistic studies.

Robbins et al.[16] are successfully utilizing this procedure to examine delayed teratogenic effects of methyl mercury on hepatic cytochrome P-450 system in rats. In this case, fetal rats were exposed to methyl mercury in utero. They are permitted to develop and evidenced no abnormalities in the acquisition or performance of an operant task. However, when challenged with d-amphetamine, the ability of the offspring of treated dams to perform the task was less impaired than controls.[17] Thus the pharmacological probe unmasked a previously undetected effect of early toxin exposure. This observation was followed up by Robbins et al.[16] with an evaluation of the hepatic cytochrome P-450-dependent mono-oxygenase system in immature (3 and 8 weeks old) and adult (26 weeks old) male and female offspring of methyl mercury exposed dams. An effect was found, but only in adult males. Thus a behavioral evaluation coupled with a pharmacological probe was able to detect a delayed, chemically induced teratogenic effect in otherwise apparently normal offspring. Followup studies of brain concentrations of amphetamine and neurotransmitter release may reveal further subtle changes in functions.

In a similar manner, our laboratory has been examining the effects of perinatal lead exposure on subsequent behavior.[18] In the offspring of dams exposed to low levels of lead acetate (0.10% or 0.02%) via drinking water we have seen no effects on the locomotor activity level of mice ranging in age from 21 days to 120 days of age. However, when these same mice are administered amphetamine, they react with an increase in activity which is attenuated relative to that seen in control mice. This effect is marginally present at 21 days of age and is still present in 120-day-old mice. Furthermore, the effect is present across several doses (see FIGURE 2). The mechanism underlying this treatment effect is currently unknown. However, the use of radiolabeled amphetamine may indicate whether the effect is of peripheral or central origin.

MAXIMIZING RETURNS

Regardless of the particular task selected, the costs associated with behavioral evaluation can only increase an already expensive testing procedure. Five years ago, Gehring et al.[20] estimated the average cost of a teratogenicity study and multigenerational reproduction studies to be $10,000 and $35,000 respectively. With this in mind, serious consideration must be given to conducting behavioral analysis with minimal additional cost.

Although some investigators may suggest drawing subjects for behavioral testing from a testing group separate from those used in other phases of teratology testing, this is not economically practical or necessary. It is possible, and in many instances desirable, to incorporate behavioral evaluation into existing in vivo teratology testing. Many testing protocols currently include measures of postnatal development, in which case the offspring are maintained for 3 weeks; or they include measures of reproductive function in the F_1 generation, in which case the offspring are maintained until sexual maturity. Finally, in the case of protocols developed to examine transplacental carcinogenesis, the offspring are maintained for 18 to 24 months. In each case, time is available for certain types of behavioral evaluation.

Incorporation of behavioral assessment into standard Phase I teratogenicity screening protocol (FDA Guidelines for Reproduction Studies) offers economy in terms of the number of animals which must be exposed and maintained. In addition, it permits animal observation of growth effects and behavioral effects prior to sacrifice and teratogenic and carcinogenic effects after sacrifice. Such a procedure permits the collection of valuable correlative information which may ultimately facilitate interpretation of data. For example, considerable debate surrounds the significance attached to the production of an increase in the incidence of skeletal variations. Do those individuals which develop wavy ribs represent a genetically more susceptible segment of the gene pool? Did they receive a higher exposure level in utero? Does the presence of such a variation permit any prediction of future functional deficits had the animal been permitted to live? By utilizing apical tasks in conjunction with pharmacological probes prior to sacrifice of the offspring, and followed by a teratological evaluation, we may be able to answer such questions.

METHYLENE CHLORIDE

The following is a brief description of a study, currently in progress, which exemplifies the manner in which behavioral evaluation can complement standard teratogenicity and carcinogenicity evaluation. The study is being carried out under the direction of Dr. Jeanne Manson, with Dr. Lloyd Hastings and myself as coinvestigators. The investigation deals with the potential teratogenicity and transplacental carcinogenicity of the hydrocarbon solvent methylene chloride.

Pilot studies were carried out to determine the maximum tolerated dose, i.e., that exposure level which did not interfere with mating, normal course of pregnancy as reflected by maternal weight gain, food and water consumption, or produce excessive preimplantation loss or fetotoxicity. It is very important to work in an exposure range which does not produce preimplantation loss or fetotoxicity if one plans to investigate the behavioral development of the offspring since observed differences between treated and control offspring may merely reflect a sampling bias brought about by the selective action of the toxin on a susceptible portion of the fetal population. The pilot study indicated the appropriate exposure level to be 4500 ppm.

The basic experimental protocol is shown in FIGURE 3. Four groups, each consisting of 30 female Long-Evans hooded rats, were exposed either to methylene chloride (Groups A and B), or to filtered air (Groups C and D) for 6 hours/day, 7 days/week for 2 weeks, whereupon breeding was begun. Pregestational exposure continued unchanged until a rat was successfully mated. The day on which sperm was seen in a vaginal smear was considered day 1 of gestation. Groups A and D rats continued to receive solvent or filtered air exposure through day 17 of gestation. On day 1 of gestation for Groups B and C rats, exposure was reversed (e.g., from methylene chloride to filtered air for Group B rats), and the new exposure was continued through day 17.

Ten females from each group were allowed to deliver their litters normally for later studies of functional and behavioral development. Pups were weighed as soon as possible following birth, were examined for externally visible abnormalities (none were seen), and litters were reduced to eight pups each. Surplus pups were preserved for later skeletal or soft tissue examination. The remaining 20 females per groups were killed on day 21 of gestation, and litters were collected for teratologic evaluation, with half of each litter being preserved for skeletal and half for soft tissue examination.

Soft tissue and skeletal examination on day 17 of gestation revealed no treatment related increase in the incidence of anomalies and a very slight increase in the incidence of skeletal variations. A 6% decrease in the fetal weight of Group A offspring was also evident. This decrease was not evident in the offspring of those animals which were permitted to litter.

On days 5 and 10 following parturition, all offspring were placed in groups of four litter mates on Motron Motility Meters for a 5-minute period. The test quantitated the reactivity and associated body movement brought about by the stress of removal from the nest box. The results appear in

FIGURE 4. The Groups A and C offspring habituated to this stress at a significantly slower rate than Group B or Group D control offspring.

The treatment effects were even more pronounced by day 15, when the offspring were fully active and able to remain separated from the dam for a 60-minute evaluation of the exploratory activity and habituation rate of individually tested animals. Significant treatment effects were also demonstrable in a 5-minute open field test conducted at 4 weeks of age.

At approximately 6 weeks of age, the male offspring were housed individually in running wheels for 9 weeks in order to assess long-term activity profiles under constant environmental conditions. No significant treatment effects were evident during the time. Likewise, at about 4 months of age, all offspring were evaluated on their ability to learn a shock elicited one-way avoidance response, and again no treatment effects were seen.

At about 5 months of age, the exploratory-reactivity-habituation process was once again evaluated in all offspring. The test was carried out by transferring the animals from their home cages to individual "jiggle platforms," which monitored all movement during the 90-minute test session. The results appear in FIGURE 5. As was previously seen, as early as day 10, the offspring of Groups A and C exhibited a significantly different pattern of reactivity to the test situation. However, at this time the effect was evident only in the opposite direction from that seen on early tests, i.e., Groups A and C habituated more rapidly than did Groups B and D.

Several general summary statements can be made at this point in the study.

1. Functional behavioral alterations were produced in the offspring of dams exposed to methylene chloride during gestation.

2. These effects were seen in the absence of maternal toxicity, fetotoxicity, and teratogenicity.

3. The behavioral effects were demonstrable shortly after parturition, and persisted in adult animals.

4. No sex differences were evident in the neonatal rats, however sex effects were demonstrable in mature animals.

5. The effects are reproducible, since the entire experiment was carried out in two cohorts of animals separated in time by about 6 weeks. Effects seen in the first cohort were replicated in the second.

6. The effects can not be definitively attributed to a prenatal effect since a cross-fostering design was not employed.

7. The effects cannot be definitively attributed to a direct effect of methylene chloride on the developing pup. It may be related to elevated maternal carboxyhemoglobin or a methylene chloride induced change in maternal-pup interaction.

8. The effects are unlikely to be the result of a general stress effect on the dam since the magnitude and direction of effect changed as a function of the exposure period.

Presently, we are continuing to follow these animals. The animals will be sacrificed at about 18 months of age and examined for malformations and tumors. These data and the data derived from the behavioral studies will then be analyzed for possible correlation between altered behavioral function, skeletal and soft tissue malformations, and variations and tumor incidence.

CONCLUSION

When one evaluates the potential role of behavioral assessment in standard *in vivo* teratogenicity testing, it is important to bear in mind two important points which set behavioral evaluation apart from classic teratogenicity testing. First, the endpoint, behavioral function, is perhaps the most relevant endpoint. It reflects the magnitude of insult remaining after genetic and cellular repair processes have been evoked, after physiological and behavioral compensatory processes have been initiated, and after the individual's functional reserve capacity has been brought to bear on the insult. Second, the use of behavioral function as an endpoint permits the screening of a large number of potentially toxic processes whereby the development of the offspring could be compromised. In addition to the more commonly examined processes of germ cell toxicity and embryotoxicity, analysis of behavioral function detects toxin mediated effects on fetal nervous system, maternal endocrine system, maternal behavior, mammary function, and maternal-infant interactions. Thus, the assessment of behavioral development of the offspring is both very relevant biologically and very powerful methodologically.

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TABLE I

SIMULTANEOUS BRIGHTNESS DISCRIMINATION IN MICE

AGE (Days)	40	47	50-52	
Task	Acquisition	Retest 1	Retest 2	Reversal
$\bar{X}$ trials to criterion	199 ± 16	60 ± 13	21 ± 3	837 ± 54
$\bar{X}$ intertrial responses	3.8 ± 0.7	2.7 ± 0.4	1.1 ± 0.2	3.5 ± 0.4
$\bar{X}$ response lat. (sec)	8.9 ± 1.0	4.4 ± 0.7	3.5 ± 0.7	5.1 ± 1.0
$\bar{X}$ running time (min)	81 ± 6	19 ± 3	6 ± 2	100 - 180

N = 14 CD-1 male mice
criterion = 100% accuracy on 10 consecutive trials

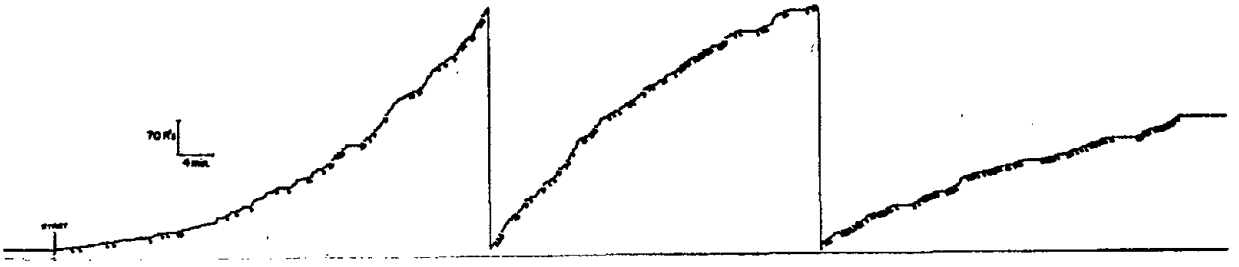

FIGURE 1. Cumulative record of a 40-day old CD-1 mouse during initial acquisition of a simultaneous brightness discrimination response. Each response by the mouse produces an upward deflection of the ink pen. Each correct, rewarded response is indicated by a downward pip. Ten consecutive correct responses occur at about 130 minutes after entering the test chamber.

Subject I.D. No.	68	Totals trials	346
Age (days)	40	Running time (min)	130
Learning criterion		X No. of ITR's	2.6
(consecutive correct)	10	X Response latency	
Total reinforcements	180	(sec)	8.5

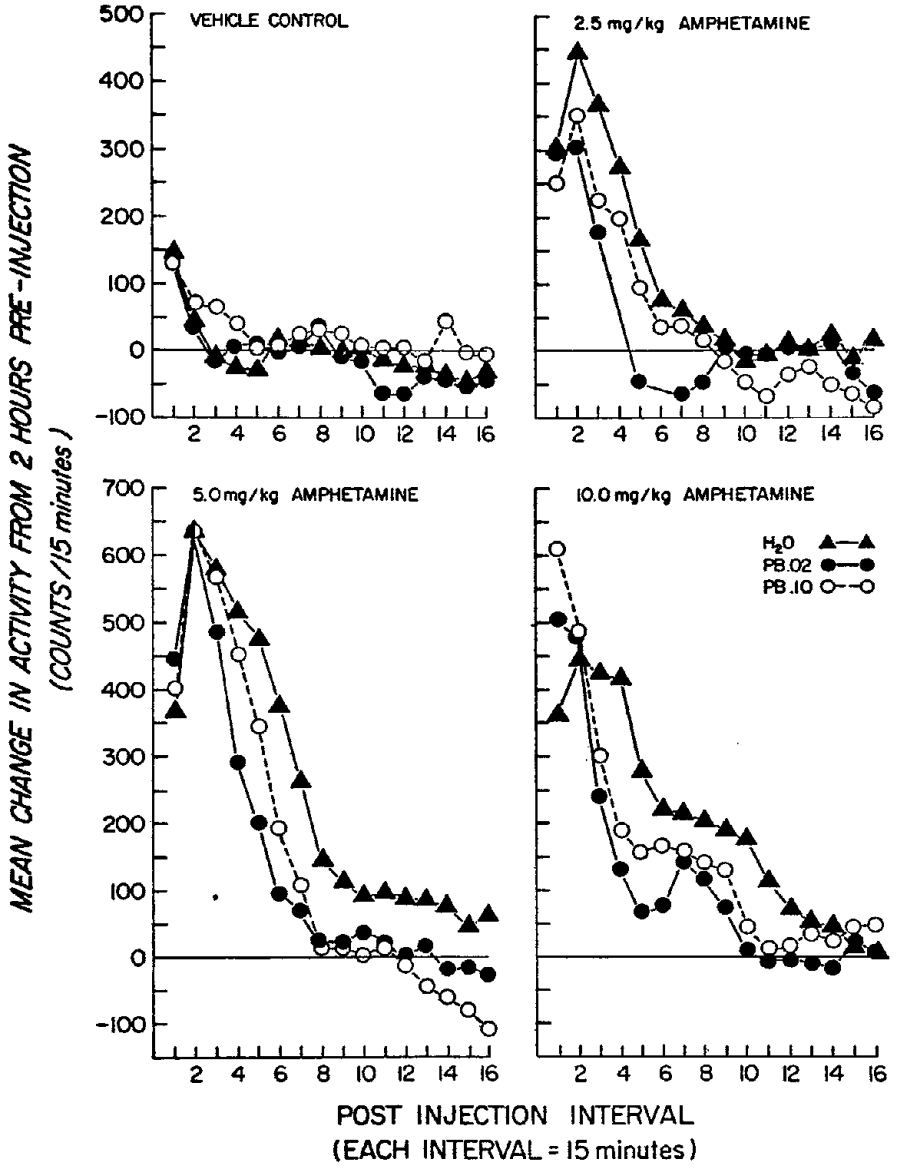

FIGURE 2. Activity level of individually tested 110-130-day old CD-1 male mice following the administration of d-amphetamine (i.p.) N = 10 per treatment group. Mice were exposed to lead only during the first 3 weeks following parturition. Lactating dams were given either water or a 0.02% or 0.10% lead acetate containing water supply during this interval.

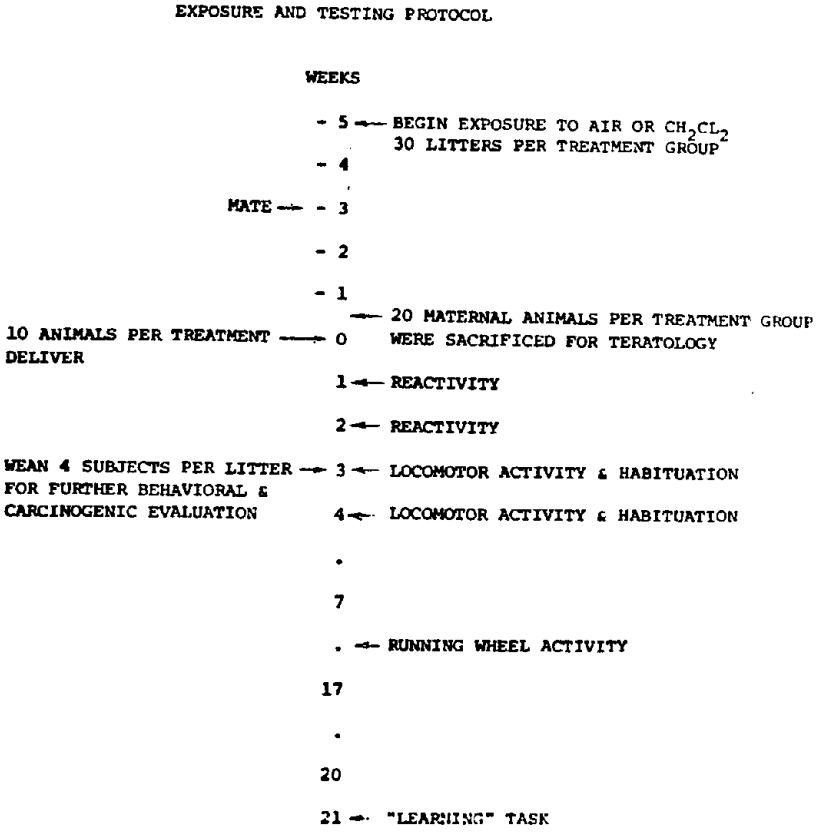

FIGURE 3. Exposure and testing protocol used in an investigation of the effects of <u>in utero</u> exposure to methylene chloride on subsequent behavior of the offspring.

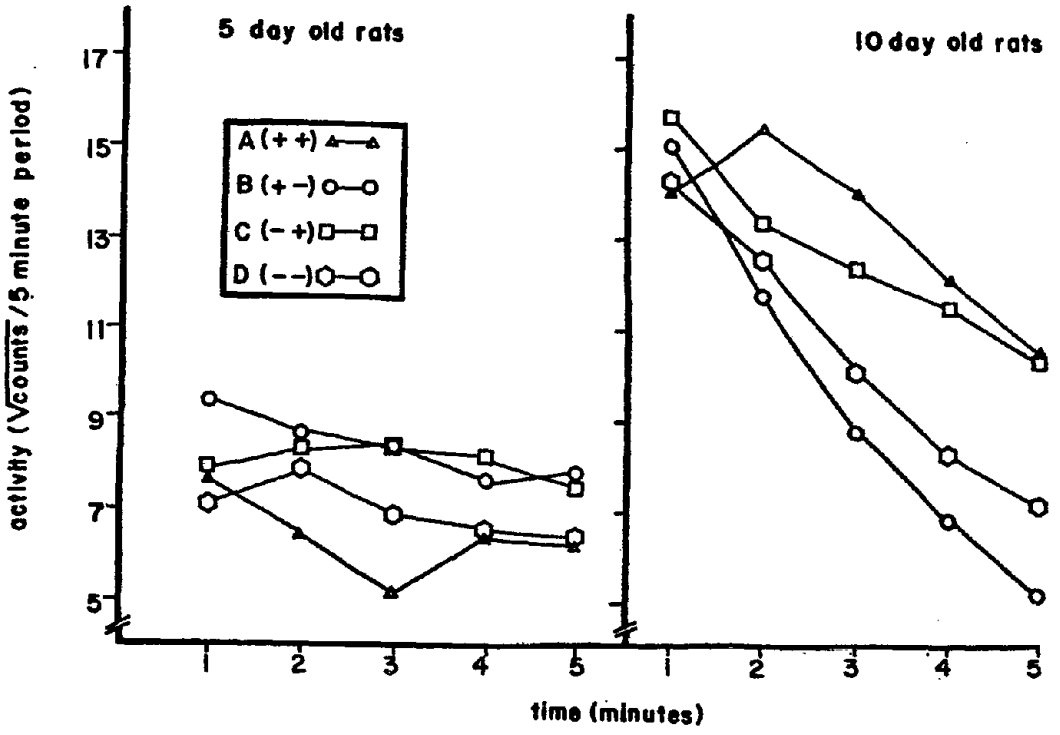

FIGURE 4. Reactivity of 5- and 10-day old rats. A Motron Electronic Motility Meter (photocell sensors) was used N = 80 pups per treatment group. Animals were tested in groups of four. Groups A and C habituated to the test environment at a significantly slower rate than did Groups B and D.

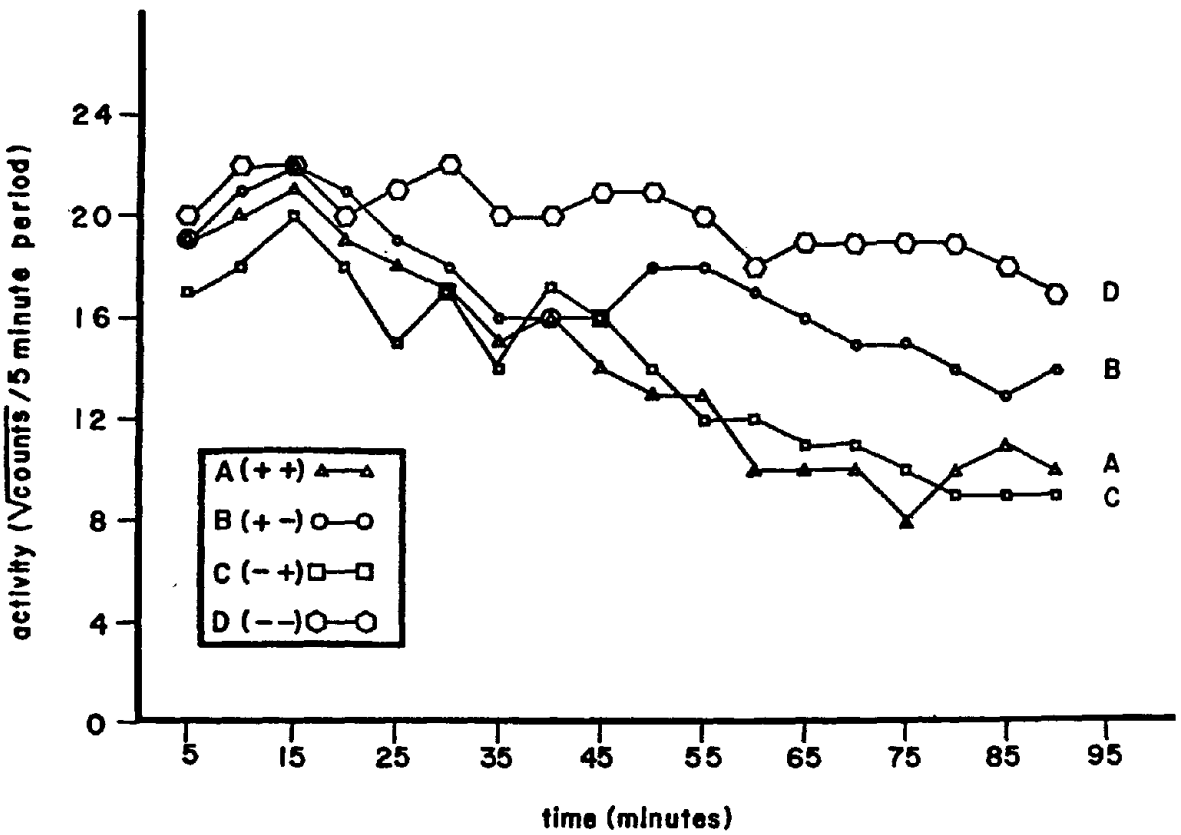

FIGURE 5. Locomotor activity of 180-day-old male rats tested individually on "jiggle platforms" N = 20 male rats per group. Groups A and C habituated to the test environment at a significantly faster rate than did Groups B and D.

INCORPORATION OF PERINATAL EXPOSURE INTO BIOASSAYS FOR CARCINOGENICITY

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INTRODUCTION

The methodology of carcinogenesis bioassays has become increasingly sophisticated during the last decade as the standards established by various government regulatory agencies in many countries have become more rigorous. Factors of special concern in the performance and interpretation of these bioassays have been widely discussed and published,[1,2] and detailed monographs have been entirely devoted to explicit formulation of guidelines for such studies by the U.S. National Cancer Institute[3] and the Canadian Ministry of Health and Welfare.[4] These, in turn, are based on a selection of the most generally applicable procedures from among the many different, and in some cases, highly specialized techniques which have been reviewed in detail by the Weisburgers[5] and by Magee.[6] Conventional testing[3] employs two rodent species, generally rats and mice; involves oral administration of the test substance, beginning shortly after weaning and continuing for the average life span of the species used, generally 18-24 months; includes approximately 50 animals of each sex at each of two or more dosage levels, the highest of which is the maximum tolerated dose; and depends on histopathologic diagnosis of tumors for evaluation. Care is taken to insure that the strains of animals used are known to be susceptible to chemical carcinogens, but do not have so high a spontaneous incidence of tumors that the statistical power of the test to detect increases in tumor incidence is reduced. No other systematic effort has been made to maximize the sensitivity of conventional bioassays.

The experiments of Druckrey and his associates during the 1960's dramatically demonstrated that prenatal exposure to certain chemical carcinogens, notably direct-acting alkylating agents such as ethylnitrosourea, resulted in postnatal tumor development.[7] Furthermore, the quantity of carcinogen necessary to induce tumors in a given proportion of offspring was in some cases two decimal orders of magnitude less than that necessary for a comparable effect in adult animals, at least for certain organ systems. The subsequent finding, that prenatal exposure to diethylstilbestrol (DES) was responsible for the postnatal development of carcinoma of the female genital tract in young women, illustrated the reality of prenatal carcinogenesis for human beings.[8] In consequence of both experimental findings and the DES tragedy, a movement has been developing to include prenatal exposure in bioassays of chemicals for carcinogenic activity.[1,2,4] The Bureau of Foods of the U.S. Food and Drug Administration, for example, now requires carcinogenicity evaluations in two species, with prenatal exposure to be

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The views expressed are those of the author and do not necessarily represent the official position of the National Cancer Institute.

included in the protocol for at least one of these. Prenatal exposure was included in the protocol for the definitive Canadian study on carcinogenicity of saccharin, which resulted in regulatory action against this nonnutritive sweetener in the United States.[9]

The advantages and disadvantages of requiring the inclusion of perinatal exposure in carcinogenesis bioassays, the limitations of this approach, and suggestions for the manner in which combined perinatal-plus-conventional bioassays might be performed are the subject of this paper.

CONVENTIONAL TESTING PROCEDURES

Conventional bioassays for carcinogenic properties of chemicals rely on lifetime studies in young adults, a provision which facilitates the distribution of animals to testing laboratories remote from the breeding facility and simplifies the preliminary determinations of acute and chronic toxicity which must precede selection of dosage levels for long-term administration.[3,4] To incorporate perinatal exposures into bioassay programs, particularly one like that of the National Cancer Institute in which animals are not generally bred at the testing facility, is a very complicated undertaking. From the standpoints both of simple logistics and of toxicology this is difficult, as pregnant animals and their products of conception may differ from young adults in susceptibility to the toxic effects of an agent.[3] Such differences may change as pregnancy progresses, thus complicating dose selection. Accordingly, the possible benefits from such a modification must be carefully considered in the context of both the goals and methodology of conventional testing. Several major aspects of bioassay procedures would be affected by inclusion of perinatal exposures. Among these are the choice of strains of test animals; dose selection; the numbers of animals assigned to each experimental group, and the methods employed for assignment; the duration of the test; and the statistical evaluation of results. For purposes of illustration the NCI testing program can be considered representative.[3]

The various species of rodents, inbred strains and random or outbred lines within a species, have markedly different natural incidences of tumors of all kinds, and the sites and types of these naturally occurring tumors likewise differ according to genetic background. In the mouse especially, naturally occurring tumors include alveolar pulmonary adenomas and adenocarcinomas and hepatocellular adenomas and adenocarcinomas. As these are among the principal index tumors induced by the overwhelming majority of chemical carcinogens, it is a matter of great importance to choose for bioassays animals which will be susceptible to the induction of these tumors by the widest possible variety of chemical agents, yet will not have so high a "background" of naturally occurring tumors that it becomes difficult to detect a marginal carcinogenic effect, i.e., a small but statistically significant difference between treated and control groups of animals. There are arguments in favor of the use of inbred animals or their F_1 hybrids; chief among these are the uniformity of response among individuals in a test group and the relative constancy--and thus predictability--of intercurrent noninfectious diseases, including naturally occurring tumors. For both reasons, tests performed on inbred animals tend to yield more reproducible results than tests on noninbred animals. On the other hand, the smaller gene pool of inbreds might result in a reduction of the range of

substances to which they can respond, either by metabolism of certain classes of chemicals or by other more subtle factors governing the potential for neoplastic expression at the cellular level in various organ systems. An alternative is the use of F_1 hybrids between two different inbred parents, which increases the gene pool and adds "hybrid vigor" without sacrificing uniformity. The NCI program has elected the advantages of uniformity, choosing the inbred Fischer 344 rat and (C57BL/6 x C3Hf)F_1 hybrid mice.

Dose selection is based on estimation of a maximum tolerated dose (MTD), determined separately for each sex of each species, and defined as "the highest dose of the test agent given during the chronic study that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity."[3] In practice, this means the highest dose that causes no more than a 10% weight decrement in comparison with control groups, and results in no mortality, clinical signs of toxicity, or pathologic lesions other than those related to development of neoplasia. (To these criteria some toxicologists would add the provision that the MTD not perturb the normal metabolic processes of the test animals; this is not easily determined, and is not considered in the NCI program.) The MTD is chosen as the highest dosage level for chronic (carcinogenesis) studies; one (or more) lower levels are chosen as simple fractions (e.g., one-half) of the MTD. Determination of the MTD depends on successive studies of acute and subacute toxicity carried out in young adult animals, in which metabolic capacities and responses to toxic agents are postulated not to be rapidly changing with increasing age.

The number of animals in each group crucially affects the power of statistical tests for significance of results. For practical purposes, 50 animals of each sex are generally included in each test group (including untreated and vehicle control groups), and are randomly assigned to various groups in the study from a pool of weanling animals of identical age. (One should note that "random" is not synonymous with "haphazard"; to avoid unconscious bias the distribution of animals should be performed with the aid of a computer-generated table of random numbers.)

It is well known that the period of latency between initial exposure to a test substance and appearance of tumors is often inversely related to effective dose. This aspect of dose-response relationships in chemical carcinogenesis effectively dictates that, for detection of a weak carcinogen, animals should be held for observation for their lifetimes, or a major fraction thereof. The NCI program recommends at least 2 years for both rats and mice, with exposure to the test substance continuing until shortly before termination.

For statistical evaluation of the results, each animal in a conventional test represents an independent entity, as each is independently exposed to the test agent.

Finally, bioassays in laboratory rodents do not generally predict the organ or site in which tumors may be expected to occur in man. Aromatic amines, for example, which in man induce carcinoma of the urinary bladder, induce hepatocellular tumors in mice. A bioassay generally reveals only the capacity to induce tumors, and the observed sites of tumor occurrence in any given test species cannot be extrapolated to a second species.

RESPONSES OF FETAL, NEONATAL, AND INFANT RODENTS TO CHEMICAL CARCINOGENS

Transplacental Carcinogenesis. To many, but by no means all, carcinogens, the fetal rodent is more susceptible than adults of the same genetic background.[10-14] This phenomenon has been demonstrated convincingly in the rat, mouse, Syrian golden hamster, rabbit, and patas monkey. In the rat and mouse, susceptibility of the conceptus generally becomes demonstrable at approximately day 11 of gestation, after definitive organogenesis is complete and the transition from embryo to fetus has begun. Grossly visible tumors in the offspring are rarely present at birth in the rat and mouse, and may become evident at any time during subsequent postnatal life, depending on the species, the organ system affected, and the effective dose of carcinogen. Enhanced prenatal susceptibility is generally evident as a high incidence of tumors at the end of their lifespan in the offspring of treated animals, in comparison with their female parent or with virgin animals given the same treatment after weaning. These differences may be as great as two decimal orders of magnitude, as in the case of rats given ethylnitrosourea (ENU) during the final week of pregnancy.[7] Latency may, in high-dosage groups, be extremely short; we have seen rats die of intracranial neurogenic tumors less than 2 months after birth, following a single transplacental exposure to ENU.

A large number of chemicals have now been shown to induce tumors by transplacental exposure alone in one or more species, and new examples continue to appear in the literature. An incomplete but representative list is given in TABLE I. Without exception, these agents had previously been bound to induce tumors in adults of at least one species. Consequently there is at present no agent known which is exclusively a transplacental carcinogen. This includes DES which, although best known for its transplacental effects in humans, was previously shown to be a renal carcinogen in adult Syrian hamsters.

The types of tumors induced by these agents are, with certain exceptions, generally the same as can be induced in adults by exposure to the same or greater doses of the same agent. A given agent frequently induces tumors of different organ systems in different species, and the nature of the carcinogenic response to any one substance seen in a given species is determined chiefly by intrinsic susceptibility factors peculiar to and characteristic of a given strain and species. "Organotropism" is much less frequently a characteristic of the carcinogen itself than has been generally supposed.

It is important to emphasize that the most effective transplacental carcinogens are direct-acting alkylating agents, which do not depend on enzymes for conversion to a chemically reactive metabolite, or "ultimate carcinogen." The overwhelming majority of chemical carcinogens do require enzyme-mediated metabolism, generally oxidative and O_2-dependent, for generation of an electrophilic, reactive metabolite.[15] In general, the necessary enzymes are present only at low levels, if at all, in fetal tissues until just before birth.[16] In cases where the resulting metabolite is so unstable and short-lived that only negligible amounts reach the fetus from maternal or placental sites of formation, the limiting factor in transplacental carcinogenesis may be the quantity of carcinogenic metabolite which can

be formed in situ in fetal target tissues. If the necessary enzymes are virtually absent, the fetus may actually be less susceptible to the administered agent than adults. The effect of dimethylnitrosamine (DMN) on the rat kidney is a good example.[17]

Neonatal Carcinogenesis. Like the fetus, the newborn rat or mouse is quantitatively more susceptible than adults to certain carcinogens.[18] A number of chemicals, including the antimalarial compound 6-aminochrysene,[19] have proved carcinogenic in neonates after repeated negative tests in adults. Reviews of the utility of neonatal exposure per se for bioassay of suspected carcinogens have concluded, however, that as a general procedure this could not supplant conventional testing.[20,21] A principal reason is that not all tissues appear maximally susceptible at birth, and that not all agents are more effective at this time.

Carcinogenesis in Pre-weanling Animals. In some cases, as reviewed by Toth, animals 2 weeks old were actually more susceptible than either younger or older animals.[20] Aflatoxin B_1, a potent hepatocarcinogen in rats, is not effective in the mouse except during early postnatal life, especially during the first week.[22] Metabolic capacity for conversion of foreign substances into carcinogenic derivatives changes very rapidly during the first month of postnatal life, and in some cases levels of enzyme activity are generated which exceed those in noninduced adult tissues. For example, the mixed-function oxidase activity which activates DMN reaches a maximum activity in rat liver 29 days after birth, or about the time of weaning, and then decreases substantially during the next 3 weeks, remaining relatively constant thereafter.[23] These results would predict that rats should be most susceptible to the carcinogenic effects of this nitrosamine (and conceivably to others as well) between weaning and onset of sexual maturity. A corollary is that rats would be rapidly decreasing in sensitivity to this agent at the time they would be introduced into conventional bioassay tests.

INCORPORATION OF PERINATAL EXPOSURES INTO CONVENTIONAL BIOASSAY PROCEDURES

The feature of interest for bioassays common to the entire perinatal period, from conception through weaning, is then usually a quantitative increase in susceptibility to some, but not all, chemical carcinogens. The majority of the compounds known to be more effective perinatally are small organic molecules or their chemically reactive metabolites. Such substances comprise the largest group of compounds likely to be studied in carcinogenicity tests. For the chemically reactive carcinogens, moreover, no radically different types of tumors appear; the organs affected and the histological features of the tumors induced in them are chiefly the same in rodents, whether exposure to the carcinogen has been perinatal or post-weaning. To maximize sensitivity of bioassay protocols by including perinatal exposures, the entire perinatal period--from conception through weaning--should be added to the conventional period of exposure. There is no reason to include only one portion of the perinatal period, prenatal for example, without the others, or to test substances only during the perinatal period without continuing conventional lifetime exposure after weaning.

Apparently qualitative increases in susceptibility--carcinogenic effects in specific organ systems only after perinatal exposure--are very few, such as those of 6-aminochrysene in neonatal mice[19] or DES transplacentally in human beings.[8] In comparison with the far greater number of carcinogens which are active to varying extents both perinatally and in adults, it does not appear reasonable to test new substances for transplacental or perinatal carcinogenicity per se.

It is not fully clear why increased perinatal susceptibility occurs. The rapid rate of cell division in fetal and infant animals doubtless plays a part, since dividing cells are especially sensitive to carcinogens. Because not all tissues are more susceptible, however, other as yet unidentified factors must also contribute. The functionally immature lymphoid and endocrine systems, patterns of xenobiotic metabolism and excretion, and differences in the pharmacodynamics of transport, tissue distribution, and storage of foreign compounds can all be identified as potentially relevant, and in some cases each has been shown to be an important modulator of perinatal carcinogenesis. There is, however, no adequate basis for predicting *a priori* the relative perinatal/postnatal effectiveness of a new carcinogen, and demonstrations of transplacental or neonatal carcinogenesis continue to be empirical. In the following discussion, it will be assumed that, in addition to conventional exposure, test animals will be exposed both *in utero* and during lactation for a combined perinatal/conventional bioassay.

Addition of perinatal exposures to conventional bioassay protocols would affect each of the aspects of those protocols discussed above. The species of choice appears to be the rat, preferably outbred. While mouse and hamster are possible alternatives, and may be the species of choice in some cases, the mouse is particularly prone to nonspecific teratogenesis; food or water deprivation for a relatively short period, which may easily occur if the animals avoid their intentionally adulterated diet or if the test substance depresses appetite, may increase the likelihood of abnormal development in the offspring. This rarely occurs in rats. Hamsters are notoriously poor mothers under the best of circumstances, and are likely to be frustrating to work with under bioassay conditions. Whichever species is used, F_1 hybrids should be used with caution and the polarity of the cross precisely defined, since the choice of maternal parent may quantitatively influence tumor yield.[24,25]

The number of animals required for a given level of confidence is no different if perinatal exposures are introduced, but the manner of their selection is markedly different and considerably more complex than the simple random distribution from a pool of young adults that suffices for conventional bioassays. Variations in exposure and consequent effect are to be expected both within and among litters, and since the mother is the individual treated, the litter, rather than any individuals in it, is the valid statistical unit. Within a litter, even in the absence of genetic differences which must inevitably occur in random- or outbred lines of animals, individual offspring develop at slightly different rates and to different weights at parturition, depending on their location along the uterine horn, the number of fetuses in that horn and in the total litter, and to some extent on the age of the mother, and whether or not the litter is her first. Different gravid females may vary in their dietary intake (and hence dose), in their rate of metabolism of the test compound, and in the

rates at which the compound crosses the placentae; and after birth, the degree of exposure during lactation may vary from pup to pup within litters, and between litters as well. In the presence of any litter effects associated with transplacental or lactational exposure--which in the case of an unknown compound must be presumed to exist--the random selection of one male and one female pup from each litter will maximize the sensitivity of any statistical methods for assessing the results. This should be done at weaning, prior to which litters should be culled (also at random) to a maximum of eight pups during the first postnatal week in order to insure adequate milk supplies (and comparable dosing). Furthermore, since a significant number of litters can be expected to consist of all one sex, it is necessary to begin with more breeding females (dams) than the number of offspring of a given sex one expects to continue to test after weaning. It has been calculated (D. Krewski, unpublished) that, if the probability of obtaining at least one live pup of a given sex at weaning in any litter is 0.75, approximately 70 dams would be required to yield, on the average, 50 litters having pups of both sexes from which one male and one female could be selected to supply the conventional 50 test animals of each sex, and in any particular study the chances of obtaining a minimum of 50 such litters is only about two out of three. Although first litters from very young dams are generally smaller and potentially less satisfactory than second or subsequent litters, this difference disappears if the dam is somewhat older when first bred, and first litters can generally be recommended from dams mated at 12 weeks of age.

Dose selection is also much more complicated, and is currently a matter of discussion and debate. This is because the toxicity of some substances in embryonic, fetal, and/or neonatal rodents may be significantly greater or less than in adults. If toxicity during the entirety of the perinatal period, and during pregnancy, is the same or less than in nonpregnant adults, there is no difficult decision to be made, as the toxic limit is defined by the tolerance of the dam. The MTD, determined in weanlings as in conventional testing, can then be used as a basis for dose selection once preliminary testing on pregnant animals and their offspring has confirmed that this is the case. A problem arises when the conventional MTD in adults is excessively toxic to either the pregnant or lactating female or her young, and must be reduced, at least during the perinatal period and for the highest dosage level. It is not generally agreed whether:

a. a lower MTD,' determined for the entire perinatal period, should replace the conventional MTD as a basis for the highest dosage level throughout life, thus reducing the magnitude of total lifetime exposure relative to that sustained under conventional conditions;

b. the conventional MTD should be used for dose selection as usual for the post-weaning period, and dosage should be reduced as necessary during the perinatal period at any or all dosage levels at which this is found necessary by independent determination of the perinatal MTD,' applying the same criteria used for MTD determination in weanlings.

The latter choice would maximize the dose, and would seem better to serve the goal of detecting potential carcinogenicity. In any case, the labor involved in estimating toxicity is greatly increased, as this must be done both in weanlings and in pregnant females and their litters.

The duration of testing must be the same as in conventional bioassays, 2 years for rats (effectively for the entire lifespan). As latency for tumor development is an aspect of dose response in carcinogenesis, there is no reason to expect that inclusion of perinatal exposures will allow shortening the period of observation. To do so would cancel any potential increase in sensitivity that perinatal exposure might provide for the detection of weak carcinogens.

Standard statistical procedures can be used for analysis of tumor incidence rates if only one pup of each sex is selected from each litter since, as discussed above, the observations are then independent. If more than one pup is selected from each litter, this must be taken into account in the analysis of the experiment.[26,27]

Extensive discussions are currently taking place in an effort to achieve consensus on a recommended uniform protocol for perinatal/conventional carcinogenesis bioassays. The Toxicology Forum addressed this issue at its 1978 annual meeting (Washington, D.C., February 1978) and in subsequent working groups, and the National Institute of Environmental Health Sciences is conducting model bioassays in which the results of perinatal plus conventional exposure, along the lines suggested here, will be compared to conventional bioassays under the same conditions. The subject can be expected to evolve rapidly in the next few years.

DISCUSSION

Carcinogenesis, like art, means different things to different people, and a discussion of testing chemicals for this phenomenon would be incomplete without a clear statement of what is meant by "carcinogenesis" in this context. It is especially important to distinguish between (1) definitions which are precisely formulated from observations in controlled experimental systems for the elucidation of mechanisms of carcinogenic action, including "initiator," "promotor," "co-carcinogen," and the like; and (2) results of testing an unknown substance, or mixture of substances, for the purpose of safety evaluation. It is clear that there is no mechanism of action common to all chemicals that affect the incidence of animal tumors. This includes both tumors that occur naturally, and those deliberately induced by exposure to some specific agent. While small organic compounds which are carcinogenic require conversion to a chemically reactive intermediate which can form covalent bonds in nucleophilic addition or displacement reactions, it is clear that some agents--asbestos, for example, or plastic films--must involve a different mechanism.[15] For research purposes, it is very important to distinguish among different classes of agents, and to use pure substances or precisely formulated combinations. Safety evaluation requires bioassay of the article of commerce, which is often a complex mixture. Such distinctions cannot be made from a bioassay alone, and for safety evaluation it is by no means established that one class of agent is intrinsically less significant than another as a potential hazard to human health.

For example, the insecticide DDT has been shown to enhance the incidence of hepatocellular tumors in mice, and was in fact studied in multigeneration bioassays in two different strains which included both perinatal

and conventional exposure.[28,29] The mouse liver cell tumor has been shown to be an excellent index tumor for detection of carcinogenic activity; a positive correlation exists between the capacity of a chemical to induce liver tumors in the mouse and its capacity to induce tumors at any site in the rat or hamster, and most known human carcinogens induce this neoplasm in mice.[30] Phenobarbital has also been shown to induce liver cell tumors in mice,[31] and to enhance hepatocarcinogenesis by 2-acetylaminofluorene in rats when given subsequent to the aromatic amine.[32] It has not been reported to induce hepatocellular tumors in rats in the absence of a second agent. Subsequent experiments on DDT and phenobarbital strongly indicate that in the rat liver these compounds act as promoting agents,[33] in a fashion comparable to that of croton oil and other promoting agents on mouse skin,[34] and it is very likely that at least a portion of the hepatocarcinogenic effect observed in mice given DDT was a manifestation of promotion. In the absence of intentional pretreatment with another agent, this distinction cannot be made, however, and the observation of increased liver cell tumor incidence is sufficient to classify DDT as a carcinogen for purposes of safety evaluation.

Another point often disputed in the evaluation of bioassays concerns the malignancy of the tumors seen. In some protocols only malignant tumors have been considered meaningful.[1] This practice cannot be justified, and in more recent protocols all tumors are considered in evaluating results.[4] There is no chemical agent that has been unequivocally shown to induce benign tumors exclusively. Furthermore, evaluation of malignancy is conventionally done by histological examination alone, relying on the criteria of anaplasia, invasion of adjacent structures, and metastasis at the time the particular tumor-bearing animal under examination was necropsied. This fails to take into account the biology of that class of tumors of which the lesion is an example, especially the degree of likelihood that a histologically benign growth may acquire overtly malignant properties over time as it grows and evolves. Tumors originating from the same cell type often behave differently in different species, and a knowledge of the biology and histology of human or rat or any other tumors of, for example, the hepatocyte, cannot be directly extrapolated to another species. The crucial role of the pathologist is to distinguish neoplasia, irrespective of degree of malignancy, from hyperplasia or other responses to injury of a nonneoplastic nature.

For purposes of safety evaluation, a purely phenomenological definition of carcinogenicity--more properly, oncogenicity or tumorigenicity--is appropriate. An agent is to be considered a carcinogen (oncogen) if, to a statistically significant degree in comparison with concurrent controls, it:

a. increases the proportion of tumor-bearing animals;

b. increases the multiplicity of tumors in tumor-bearing animals;

c. decreases the latency period for tumor development.

Not all toxicologists currently accept this interpretation or those previously expressed in this discussion; the reader may refer to published documents for alternative arguments.[1,2]

Finally, the unique feature of transplacental and, to a lesser extent, of all perinatal carcinogenesis is the fact that it occurs in immature, differentiating tissues. However, the overwhelming majority of transplacentally induced rodent tumors are morphologically indistinguishable from histogenetically comparable neoplasms in animals treated with carcinogens after weaning. This suggests that potentially neoplastic, incompletely differentiated cells of many kinds can continue to differentiate to a considerable degree, and allow investigators to study a continuum of varying response to model carcinogens throughout prenatal and postnatal life in many tissues. A uniquely transplacental carcinogen might have any of a number of properties, if it exists at all. It might behave like other carcinogens but require metabolic alteration by an enzyme only transiently present in a developing tissue. Alternatively, a particular stage of morphogenetic differentiation may be uniquely sensitive to some carcinogens. The carcinogenic specificity of the nonsteroid estrogen DES for the vagina, an estrogen-sensitive tissue, in the human fetus[8] is mimicked to a significant degree in the neonatal mouse, where it has been shown that DES, in common with steroid estrogens and other nonestrogenic steroid hormones, specifically and irreversibly alters normal morphogenesis in the vagina.[35] DES may be an example of this kind of action, which, if detected in bioassays, would not only demonstrate nonspecific carcinogenic activity, but, by the unique nature of the results, provide an organ-specific prediction of the potential consequences of human exposure.

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TABLE I

REPRESENTATIVE TRANSPLACENTAL CARCINOGENS

Direct-acting alkylating agents

- Methylnitrosourea
- Ethylnitrosourea
- n-Propylnitrosourea
- n-Butylnitrosourea
- Methylazoxymethanol
- Dimethyl sulfate
- Diethyl sulfate
- Propane sultone
- Methyl methanesulfonate

Enzyme-dependent alkylating agents

- Dimethylnitrosamine
- Diethylnitrosamine
- 1,2-Diethylhydrazine
- Azoethane
- Azoxyethane
- Procarbazine (N-isopropyl-α-(2-methylhydrazino)-p-toluamide)
- Cycasin (methylazoxymethyl-β-D-glucoside)

Polynuclear aromatic hydrocarbons

- Benzol[a]pyrene
- 7,12-Dimethylbenz[a]anthracene
- 3-Methylcholanthrene

Miscellaneous

- Urethan (ethyl carbamate)
- Aflatoxin
- Diethylstilbestrol
- 4-Nitroquinoline-N-oxide

GENETIC TOXICOLOGY--RELEVANT STUDIES WITH ANIMAL AND HUMAN SUBJECTS

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INTRODUCTION

The emergence within the past few years of a new area of toxicology, chemical mutagenesis, is a result of the development of a variety of testing procedures as well as recognition of the importance of the problem of chemically induced genetic damage. Those procedures for identifying chemical mutagens are carried out in systems representing different orders of biological complexity. Generally, nonmammilian systems (excluding higher plants) can detect intragenic mutations while animal systems detect gross chromosomal aberrations reflected as specific types, such as nondisjunction and translocation. It is important to evaluate these test systems for their ability to detect mutagenic-carcinogenic activity of direct acting compounds and of agents which require host metabolism for activation. Of special importance are procedures that can be carried out in man, which can serve as an advance warning system for potential mutagens and/or carcinogens to which a significant part of our population is exposed.

This paper reviews:

- The objectives of mutagenicity testing,
- The inadequacies of bacterial procedures for realizing these objectives, and
- Presently available animal procedures in this special area of toxicology.

In addition, it assesses present capabilities to perform a variety of tests (on human subjects) that can serve as an advance warning system for the presence of mutagenic carcinogenic agents.

OBJECTIVES OF MUTAGENICITY TESTING

The first objective of testing chemicals in mutagenicity assays is the establishment of their capability, if any, to induce heritable genetic effects in man. With the possible exception of studies designed to detect an increase in spontaneous abortion rates, conventional epidemiological studies are of questionable value in determining an altered phenotype in the human population as a result of chemical insult.[1] Hence, nonhuman and even nonmammilian test systems have been developed to resolve this question. Since gametogenesis involves both mitotic and meiotic processes, assessment of mutagenicity ideally should be done on cells that undergo

meiosis, i.e., germinal cells. However, cells other than germinal cells have proved to be very useful indicators for particular genetic alterations.

The second objective is the quantitation of the risk of mutagenicity to man. Since many compounds with potential genetic activity cannot be eliminated from use in the environment, quantitative assessment is necessary for the prediction of acceptable or tolerable exposure levels. So far, mutagenic risk has been quantified in relation to background mutation rates. This approach has been used for both ionizing radiation and chemical mutagens similar to the assessment of the risk from chemical carcinogens. For carcinogens, however, the assessments are sometimes made in absolute rather than in relative terms.

The third objective is the use of the information derived from mutagenicity testing for predicting other toxicological properties, particularly the potential for inducing cancer. The assessment of the usefulness of mutagenicity tests for predicting carcinogenic activity requires considerable efforts to establish satisfactory correlations and to minimize the possibilities of obtaining either false positive or false negative results. This implies that information generated on mutagenic-carcinogenic potential of various chemicals should be derived from the best available in vitro and, more importantly, in vivo methods.

THE INADEQUACIES OF BACTERIAL (IN VITRO) PROCEDURES AS INITIAL SCREENS

The need to test a large number of products for mutagenic activity suggests that simple economic tests with bacteria should be the initial tests for these products. Development of microsomal activation procedures to detect active metabolites and the use of multiple tester strains suggest that these tests are ideal for screening large numbers of chemicals. The simplicity of these procedures, however, must be considered in relation to the objectives of mutagenicity testing. It should be apparent that in vitro procedures cannot be used for the quantitation of the risk of mutagenicity to man. How adequate are these microbial procedures in respect to other objectives of mutagenicity testing, namely for predicting heritable genetic damage in man as well as identifying potential human carcinogens?

The use of in vitro microsomal preparations combined with microorganisms or other indicators as an exclusive primary screen is an approach whose deficiencies must be recognized. An in vitro microsomal activating system cannot reflect the complex dynamic processes that are carried out in the intact animal. Indeed, it is impossible to devise a standard in vitro activation system that can be used generally to screen potential carcinogens. Even if such an in vitro system could activate all compounds that are metabolized by hepatic enzymes, the fact that many materials are either potentiated or detoxified by other routes (e.g., intestinal flora) would argue against the use of the system as an initial screen test. In addition, the important class of chemicals that induces nondisjunction by affecting spindle mechanisms, one of the most important categories of genetic abnormalities, would be missed by bacterial studies.

In both scientific and science-news publications, statements have appeared that would suggest that a high percentage (e.g., 85% or greater) of all chemical carcinogens will be mutagenic in one in vitro system: the Salmonella/microsome system. These statements rely on several studies on groups of carcinogenic and noncarcinogenic chemicals. Actually, the reported correlations between carcinogenicity and mutagenicity in Salmonella range from 63% to 93%.[2,3,4] (See also the chapter of Steve Rinkus and M. S. Legator, this volume.) It is important that the reported correlation studies be seen in their proper perspective. In retrospect, it was the lack of oxidative N-dimethylation and N- and aromatic-hydroxylation in bacterial systems that provided the initial impetus to resort to postmitochondrial fraction in mutagenicity testing. These reactions are performed by the mixed-function oxidase of the endoplasmic reticulum, which itself is found as microsomes as a result of the disruptive nature of the isolation procedure. These enzymes have in common a heme protein, cytochrome P-450--so called for the characteristic shift of the absorption maximum of this protein when it binds carbon monoxide. P-450 in the liver can exist as several subspecies of the cytochrome, which have different substrate and inducer specificities, and can be separated electrophoretically by size. In general, these enzymes are nonspecific, and are readily induced by treatment with lipophilic substrates, the known number of which exceeds 200. Consequently, one chemical can induce the enzymatic activity for the metabolism of many others.

This property was exploited by Garner et al.[5] and Ames et al.[6] in their preparation of S-9 fractions: Microsomal enzymes were induced by adding phenobarbital to the drinking water of the animals whose liver would be taken. Later, after investigating for the optimum procedure to induce these enzymes, Ames recommended Aroclor 1254, a mixture of polychlorinated biphenyls, over phenobarbital.[7] More recently, Matsushima et al. have shown that a mixture of phenobarbital and 5-, 6-benzoflavine can serve as a safe substitute for Aroclor 1254, which is carcinogenic in animal testing.[8]

Both of these later recommendations are based on experiments with the same chemical types that originally had led to the innovation of S-9, i.e., nitrosamines, aromatic amines, and polycyclics. Hence, the development of S-9 has essentially concerned only a few reactions for a few chemical types. Accordingly, the ability of in vitro systems supplemented with S-9 to detect these chemicals has been rather consistent. However, the converse also appears true; chemicals, other than those types with which the testing has been "standardized," that are metabolized in vivo to their active forms, are not as consistently detected in such in vitro testing. In general, poorly activated types include:

- Azonaphthol dyes,
- Carbamyl and thiocarbamyl compounds,
- Benzodioxoles, and
- Symmetrical hydrazines.

Benzene is an excellent example of an important product in energy technology that would not have been detected by microbial tests with or without activation.[9]

In the area of energy and energy-related products, the variety of chemical structures would strongly argue against the use of microbial procedures as an initial screen. The crux of the matter is not whether *in vitro* systems did predict the carcinogenicity of AF-2 or whether they have found mutagenic activity in such things as hair dyes and the air of cities with pollution problems; rather, it is that in a systematic screening of diverse products, certain mutagens will not be detected *a priori* as a result of the aforementioned inadequacies of *in vitro* systems when only such systems are employed for the qualitative identification of mutagens/carcinogens.

The problem with qualitatively testing for mutagens using only *in vitro* systems is the false negative. Such inadequacies are intrinsic to *in vitro* systems, be they microbial or combinations thereof. By necessity, mutagenicity screening must rely on a battery of primarily *in vivo* systems. Toxicologists and other interested individuals should not be lulled into a false sense of security and assume that the chemicals are not mutagenic when a compound or chemical mixture is found to be inactive by existing microbial procedures. At the present state of the art, bacterial and *in vitro* procedures cannot be used for risk assessment and are of limited value for detecting presence of mutagens or carcinogens.

ANIMAL TESTING AND COMBINED TESTING PROTOCOL

In an in-depth study of chemical mutagens, one would first select those test systems that have the capability of detecting the various types of deoxyribonucleic acid (DNA) alterations at the level of the genome as well as chromosomal level. The tests should have the potential to detect active metabolites activated by tissue enzymes or the intestinal flora of the host. In principle, with an unknown chemical or mixture of chemicals, one would start with the best available animal systems, including those tests that evaluate metabolic products of the intact host. Since there is no single test for detecting chemical mutagens, a complete testing protocol would utilize a battery of tests carried out in the intact animal. The integration of the results from these systems should offer the optimum opportunity for identifying mutagens that are potential hazards to man. The subsequent studies of an active compound would rely on refining procedures to isolate and identify the active compound and, subsequently, to characterize the genetic lesions induced by the chemicals under study. This approach is contrary to a tier approach, which uses *in vitro* systems and then advances to animal tests. It is important to realize that available procedures for mutagenic evaluation are less time-consuming, less expensive, and probably more meaningful than many tests that are available and presently used in other areas of toxicology. Indeed, if one employs a battery of tests in animals to detect compounds that cause point mutations and chromosome aberrations, including nondisjunction, the total cost would be only a fraction (approximately one-third) of what is presently allotted for a single carcinogenic evaluation.

Commonly used practical procedures for detecting and characterizing various types of genetic lesions include:

- Detection of premutational lesions: DNA repair studies in experimental animals.
- Detection of point mutations: host-mediated assay and body-fluid analysis in experimental animals, using various indicator organisms.
- Detection of chromosomal change in experimental animals:
  - Dominant lethal test
  - Translocation studies
  - Micronuclei test
  - Direct cytogenetic analysis with both meiotic and mitotic cells
  - Sister chromatoid exchange studies.

While collaborative studies have rarely been conducted in the field of toxicology, it is noteworthy that in the field of genetic toxicology the dominant lethal test and in vivo cytogenetic analysis already have been subjected to collaborative studies.[10,11] The utilization of all or most of the above procedures should characterize the majority of mutagenic agents. Additionally, these studies, combined with in vitro procedures, will in many instances classify the induced genetic lesion.

A critical examination of the available animal methods suggests that many of these procedures can be incorporated into a single testing protocol. In a recent publication, the utility of this combined testing protocol was demonstrated with the antischistosomal agent hycanthone methanesulfonate.[12] The procedures that were combined into a single animal experiment included metaphase analysis, micronucleus test, urine analysis, and the host-mediated assay. Other routine procedures such as in vivo DNA repair, germinal cell analysis, and alkylation of macromolecules may prove to be compatible with this approach. The use of the combined protocol should facilitate screening a large number of potential mutagenic agents and result in substantial savings in both time and money over usual individual toxicological tests. In terms of the three major objectives of mutagenicity testing, including risk assessment, a battery of animal tests offers the only feasible approach to accomplish these objectives.

STUDIES WITH HUMAN SUBJECTS

The long latent period between chemical insult and the resultant altered phenotype, as well as the high background rate of genetic disease, makes it all but impossible to confirm the induction of genetic abnormalities by specific chemicals. There are, however, a series of noninvasive techniques that can now be used to detect potential mutagens and carcinogens in industrial populations. The industrial environment provides a

unique set of circumstances for detecting and characterizing chemicals that induce mutations and neoplasms. In many instances we can identify employees who are exposed to a variety of chemicals, and in certain instances the levels of exposure are exaggerated over those that can be found in the general population. The procedures that can now be used in studies with human subjects include:

- Cytogenetic Analysis,
- Detection of Mutagenic Agents in Urine,
- Sperm Analysis for Morphological Abnormalities, and
- Y Chromosome Nondisjunction (Extra Fluorescent Y Body).

These procedures can detect a wide range of chemicals that produce chromosomal aberrations or point mutations. Of special importance is the fact that when these techniques are used with human subjects they can serve as an advanced warning system for potential mutagens and/or carcinogens in man. If these procedures had been used to evaluate industrial exposure to carcinogenic/mutagenic substances, chemicals such as 1-2-dibromo-3-chlorpropane (DBCP),[13] vinyl chloride,[14] epichlorohydrin,[15] benzene,[16] and lead,[17] among others, could have been detected. The following is a brief description of these procedures.

CYTOGENETIC ANALYSIS

Since the early 1960's, cytogenetic analysis in animals and humans has played a central role in most programs designed to detect metagenic agents. It is interesting to note that most of the chemicals--for example, industrial compounds that have been identified as carcinogens or chemicals that can induce gene mutations--can be detected by cytogenetic analysis. Chromosome aberrations may indeed comprise a major fraction of the genetic damage induced by mutagenic exposure of mammals, including man. Chemicals that induce mutations in specific loci in a eukaryotic genome invariably have also been found to produce cytological, recognizable chromosome damage.

It has been pointed out that many, perhaps the majority, of mutations induced in mammalian cell systems and detected through an alteration or loss of a given protein are associated with a visible cytological change involving the locus in question.[18] In the past, many of the negative findings in which chromosomal abnormalities were considered within the normal range were based upon the analysis of too few cells, whether animal or human. Utilizing statistical procedures based on the known background rate in a given study, it is possible to calculate the number of cells and the number of individuals that must be analyzed to reduce the beta error.[19] Sufficient sample sizes should be used in a well-designed study so as to detect a twofold increase over the control. When used in conjunction with other techniques described in this report, cytogenetic analysis is a valuable tool for detecting the presence of mutagenic substances in man.

DETECTION OF MUTAGENIC AGENTS IN URINE

In one initial publication on the host-mediated assay, the urine and blood of animals treated with streptozotocin were analyzed successfully for the presence of mutagenic substances as an additional procedure.[20] In this study, the pooled urine and blood of the treated mice were directly spotted on minimal media using G-46 auxotroph of Salmonella typhimurium as the indicator organism. Durston and Ames found that the urine of rats treated with 1.6 mg/kg of 2-acetylaminogluorine demonstrated mutagenic activity in TA1538 tester strain of Salmonella after β-glucuronidase cleavage of the conjugate in the urine.[21]

Commoner et al. also detected mutagenic activity in rats fed 2-acetylaminofluorine as well as p-dimethylaminoazobenzene.[22] Krick et al. reported that following a single administration of 150 mg of cyclophosphamide for two noneoplastic diseases (Sjorgen's syndrome, with associated polyomelitis, and rheumatoid arthritis) mutagenic substances in both blood and urine were detected using the TA1535 strain of Salmonella.[23] The mutagenic activity in the urine was many times greater than that detected in blood.

Patients who received the drug after absence from drug therapy for 2 months showed a level of mutagenic activity higher than control levels, using diploid yeast as the indicator organism. Siebert and Simon tested the urine of a simgel cyclophosphamide treated patient for induction of gene conversion.[24] The patient was treated by infusion for four successive days with therapeutic doses of cyclophosphamide. An increase in gene conversion was found 2 hours after infusion; maximal values were obtained in 5.6 hours; and conversion was low at 23 hours post-infusion. From this study, the authors concluded that the test yielded statistically relevant results with the low doses of drugs used in cancer chemotherapy.

Stolz and Miller found mutagenic activity in urine concentrates of rats dosed by gavage with 200 mg of isoniazid using Salmonella typhimurium.[25] In the same study, isoniazid, as well as its common metabolites, did not increase revertants when directly tested against TA1535 with and without microsomal activation. Bruce et al. have isolated mutagenic substances in feces after various treatments.[26]

More recently, studies have been carried out in parallel experiments evaluating body fluids of experimental animals and humans treated with therapeutic drugs for mutagenic activity, both in vitro and in vivo.[27] The agents selected for this study were the antischistosomal drug, niridazole, and the antitrichomonal agent, metronidazole. With both niridazole and metronidazole, mutagenic activity was detected in mice and, of special significance, in human subjects receiving therapeutic doses of the drugs. With niridazole, mutagenic activity was detected in the urine of a single patient 4 days after treatment. With metronidazole, mutagenic activity could be detected in the urine of all 15 female subjects receiving the drug (750 mg per day) from the first through the ninth day of therapy.

Data derived in these studies from humans and animals with niridazole and metronidazole demonstrate our ability to detect widely used drugs administered at therapeutic levels by this technique. Just as drugs at

therapeutic levels have been detected by this procedure, so also should it now be possible to detect mutagens to which workers are exposed during the working day.

SPERM ANALYSIS FOR MORPHOLOGICAL ABNORMALITIES

The abnormal sperm assay detects agents that lead to an increased frequency of sperm with abnormal heads.[28] The normal head shape is very distinctive, and changes are easily recognized. In some strains of mice and most hybrids between strains, the spontaneous frequency is low. Abnormal sperm may be induced as a result of mutagenic treatment during spermatogenesis. Although the exact mechanism for this effect is not known, the characteristic may be passed on for at least two generations.

Since studies of mice homozygous or heterozygous for various translocations indicate that neither translocations per se, nor their meiotic consequence, are responsible for abnormally shaped sperm, it has been suggested that increases in the frequency of abnormal sperm are a consequence of either point mutations or small deletions. However, sperm abnormalities also may arise following nonspecific factors such as the application of heat to the testes.

Y CHROMOSOME NONDISJUNCTION (EXTRA FLUORESCENT Y BODY)

The fluorescent Y body is a new procedure still in the developmental phase that offers promise for monitoring human populations. It detects Y chromosome nondisjunction in human sperm.[29] The procedure is based on the fact that human chromosomes treated with quinacrine mustard, or its closely related analogues, exhibit a banded pattern of differential fluorescence. The quinacrine-stained Y chromosome is sufficiently bright to be seen through the membrane of the intact sperm (YF body). The presence of gametes containing two YF bodies (YFF) indicates aberrance in meiotic anaphase II; this is a disjunctional error in spermatogenesis in which the male chromosomes have failed to separate. The fact that YFF sperm survive and some are capable of fertilization is shown by the existence of XYY individuals (1 in a 1,000 male births). Previous work with this technique indicated an increase in YFF bodies in human subjects after exposure to either radiation or chemicals, including DBCP.

CONCLUSION

In less than 10 years, a new area of toxicology, often referred to as genetic toxicology, has emerged. The name genetic toxicology describes some of the positive aspects as well as the problems encountered in this unique area of toxicology. Since we are dealing with genetic events, a variety of biological systems that allow characterization of induced genetic lesions are available, and in many instances the molecular event that is induced by DNA-altering substances can be classified. In no other area of toxicology can this type of analysis be performed. The direct participation

of geneticists and molecular biologists in this new area of toxicology has added an important dimension to the entire field. On the other hand, simply because we are concerned with a genetic event, sound toxicological principles and practices cannot be ignored. Pharmacokinetics and metabolism are as important to this specialized area of toxicology as any other. Just as animal studies are the major approach to determining adverse effects that may affect humans, so also are animal studies the major means of meeting the primary objective of mutagenicity testing.

Of the three major objectives of mutagenicity testing--(1) establishment of capability of a chemical to induce heritable genetic effects in man; (2) quantitation of risk of mutagenicity to man; and (3) predicting potential for inducing cancer--microbial systems clearly cannot be used for quantitation of risk to man; and they are of doubtful value for realizing the other two objectives when used in the absence of animal tests. The major reason for utilizing microbial systems or in vitro systems is the alleged correlation between bacterial mutagens and animal (human) carcinogens.

It is interesting to note that although a correlation of 85+% is usually cited, the actual correlation reported in the literature ranges from 62% to 90+%. Even when a high correlation has been reported, the data are suspect for one or more of the following reasons:

- Failure to code samples,
- Nonrandom selection of chemicals,
- Questionable criteria as to what is a carcinogen, or
- Questionable criteria as to what is positive (negative) in microbial tests.

The problem with the bacterial systems (in vitro procedures) resides in our inability to define standard protocols for activation, differences in chromosomal organization, metabolism, and cellular membranes between microbes and mammals. These differences challenge the use of microbial systems such as Salmonella as an initial prescreening test for carcinogenic as well as mutagenic agents. This is not to say that in vitro procedures do not provide useful information about a potentially hazardous chemical but only that these tests should be used in conjunction with more relevant and meaningful tests in experimental animals. The inescapable conclusion is that a chemical that is mutagenic in an in vitro test should be evaluated in animals; and, more importantly, a chemical that is not mutagenic in these in vitro procedures should also be tested further in animals before any firm conclusions can be drawn.

In constructing a battery of tests the following criteria should be applied in selecting the testing procedures:

- Ability to cover all possible genetic lesions,
- Coverage of all likely metabolic products,

- Adequate reproducibility of test results,
- Appropriateness of design, permitting quantitative evaluation, and
- Predictive value for human risk.

The available procedures for point mutations (host-mediated assay, body-fluid analyses) and the direct tests for cytogenetic abnormalities are the presently available procedures that, to a large extent, fulfill the criteria for mutagenicity testing. Many of the available procedures can be combined into a single experiment, thus maximizing the efficiency of the testing protocol. Even when run individually, the commonly used animal tests are short term and economical when compared to chronic toxicological studies.

An exciting approach in the field of mutagenicity testing that has not received adequate attention is our ability to use many of the testing procedures employed in animals to determine potential mutagens/carcinogens in exposed humans. These procedures include metaphase studies, fluid analysis, sperm abnormality, and evaluation for presence of extra fluorescent Y chromosome. If these procedures had been used, many of the chemicals that have been found to be hazardous to man could have been identified earlier. We now have the capability to use these techniques as an advanced warning system, which should lead to the elimination of hazardous substances before their effects occur in man.

A rare opportunity now exists to adequately evaluate the safety of specific industrial chemicals, products, and processes. In attempting to detect and characterize mutagenic/carcinogenic agents, the test objectives should be clearly defined and meaningful, rather than inappropriate. The combination of studies both in humans and animals offers the hope of preventing cancer and genetic abnormalities from environmental contaminants.

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DETERMINATION OF ALKYLATION OF MACROMOLECULES IN MAMMALS AND HUMANS

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Most mutagenic and carcinogenic chemicals are electrophilic agents, or are converted to electrophilic agents by metabolic activation. It is important to find methods that allow quantitative risk assessments of such chemicals. One major difficulty in the extrapolation of risk from experimental organisms to man is the uncertainty of the obtained in vivo dose of the ultimate electrophilic compound.

The work reported here has aimed at the development of one method for determination of in vivo dose, that may be directly applicable to measurements in man. This procedure takes into account the chemical properties of a large category of genotoxic compounds, i.e., their ability to alkylate nucleophilic sites in macromolecules.

The reaction of an alkylating agent RX with a nucleophilic compound Y can be described as follows:

$$RX + Y \xrightarrow{k_y} RY + X \qquad (1)$$

where k_y is the second order rate constant for the reaction. The reaction rate depends both on the nature of RX and on the nucleophilic strength of Y. The rate of formation of the alkylated product RY is given by

$$d[RY]/dt = k_y[RX][Y] \qquad (2)$$

and the degree of alkylation [RY]/[Y] may be calculated from the integrated equation

$$[RY]/[Y] = k_y \int [RX]dt = k_y \cdot D \qquad (3)$$

($\int [RX]dt$ = the dose D). Equation (3) is valid when [RY] is small compared to [Y]. This is normally the case at the low dose levels of RX that are of interest in the context of environmental toxicology. In principle, when the degree of alkylation of any nucleophilic compound and the corresponding rate constant k_y are known, the effective dose D may be calculated using equation (3).

Thus, immediate information on the tissue dose after exposure to an alkylating agent may be obtained from an analysis of the degree of alkylation of specific nucleophilic groups in, for example, proteins or nucleic acids. It is required that during the time of study and within the limits of accuracy desired, the nucleophilic target molecule and its alkylated product should not be subjected to turnover, repair, or similar effects.

Methods for determining the extent of reaction with the genetic material in experimental animals by use of radiolabeled compounds are available. Assessment of the degree of alkylation in man requires the development of nonradioactive methods using samples acquired from the individual with a minimum of risk.

The possibilities for using hemoglobin from red blood cells as a dose monitor have been explored by Ehrenberg and co-workers in a series of experiments on mice using directly alkylating agents,[1,2,3] as well as agents that need metabolic activation.[2,4,5] After treatment of the animals with the radiolabeled compounds, the degree of alkylation of quanine-N-7 of DNA and of cysteine-S and histidine-N-1 and -N-3 in proteins from different organs were determined parallel to the determination of alkylation of cysteine and histidine of hemoglobin. It was shown that for small, rapidly diffusing and sufficiently stable molecules, such as ethylene oxide (biological mean-life about 12 minutes[1,2]) and methyl methanesulfonate (biological mean-life about 40-50 minutes[3]), the erythrocyte dose is representative to doses of other organs, including the liver and the testes. For more unstable compounds, such as the reactive metabolites from vinyl chloride[5] and dimethylnitrosamine,[2] the gonad doses were considerably lower than the erythrocyte dose.

It has been demonstrated experimentally that alkylated products of hemoglobin (alkyl group being methyl or hydroxyethyl) have a high stability *in vivo*, and that the alkylation per se does not influence the lifetime of the erythrocytes.[2,3] This stability provides the basis for the use of hemoglobin alkylation to integrate doses of alkylating agents over a long period of time. At prolonged exposure the degree of alkylation of nucleophilic groups in hemoglobin increases according to the equation[2,3]

$$[RY]_{acc}/[Y] = at - at^2/2t_{er} \qquad t \leq t_{er} \tag{4}$$

where $[RY]acc/[Y]$ is the accumulated degree of alkylation after t days of observation, a is the daily increment of the degree of alkylation and t_{er} is the lifetime of the erythrocytes. After constant exposure over a period of time corresponding to t_{er} the degree of alkylation will reach a plateau value[2,3]

$$[RY]_{acc}/[Y] = a \cdot t_{er}/2 \tag{5}$$

Thus, in the mouse the accumulated degree of alkylation after 40 days will be 20 times the daily increment and correspondingly, in man the degree of alkylation will amount to 63 times the daily increment 126 days after onset of exposure.

The accumulation of alkylated groups in hemoglobin and the relatively large amounts of hemoglobin that may be isolated from one blood sample and analyzed, together with an application of sensitive analytical techniques, make it feasible to determine the small quantities of alkylated amino acids that may be formed as a consequence of exposure to genotoxic compounds in the environment.

This type of analysis has been applied to estimate the *in vivo* dose, and to assess the risk connected with exposure to ethylene oxide in a

sterilization plant using this gas.[6] A careful monitoring of the ethylene oxide levels in the air was undertaken during the summer 1976.[7] Blood samples were collected at the end of 1977, i.e., they reflected the exposure during the period August-November 1977. However, no alterations in the working conditions were carried out in the meantime.

The alkylated product 3-N-(2-hydroxyethyl)histidine was isolated from samples of 1-1.5 g of hemoglobin after hydrolysis and ion-exchange chromatography. Radiolabeled 3-N-(2-hydroxyethyl) histidine was added before the hydrolysis to provide an internal standard for quantitation. Half of the material was analyzed on a Durrum D-500 amino acid analyzer. To the other half, a known amount of deuterium-labeled 3-N-(2-hydroxyethyl)histidine was added, the samples were esterified with methanol-HCl, acetylated with heptafluorobutanoic anhydride, and quantitated by means of gas chromatography mass spectrometry. The accumulated degree of alkylation of the N-3-position of the histidine of hemoglobin from two control persons and five exposed workers given in nmoles 3-N-(2-hydroxyethyl)histidine per g of hemoglobin (GC-MS determination), and the estimated average daily exposure in ppm/hr were: (a) 0.05, 0 (b) 0.05, 0 (c) 0.5, 36 (d) 3.4, 44 (e) 3.6, 180 (f) 6.0, 180 (g) 13.5, 220, respectively. The analysis on the Durrum amino acid analyzer gave consistent results. The limits for quantitation are at present 0.05 nmole/g hemoglobin with the combined gas chromatography mass spectrometry and about 0.8 nmole/g hemoglobin with the amino acid analyzer.

The rate constant for the reaction of ethylene oxide with the histidine-N-3 of hemoglobin was determined to $0.14 \cdot 10^{-4}$ l (g hemoglobin)$^{-1}$hr^{-1} by exposure of human erythrocytes to ethylene oxide *in vitro*. The daily increment of the degree of alkylation was calculated from the accumulated degree of alkylation according to equation (5) and inserted together with the rate constant into equation (3) to give the average daily *in vivo* dose for each worker. The relationships between external exposure and *in vivo* dose was found to be $(44 \pm 26) \cdot 10^{-3}$ $\mu M \cdot hr/ppm \cdot hr$.[6]

The *in vivo* dose determined accordingly takes into account the features of the human metabolism, i.e., the rate of uptake, detoxification and excretion, and forms a basis for an estimation of the degree of alkylation of DNA (equation (3)).

Work in progress with the compound methyl bromide indicates that, due probably to the lipophilic character of this compound, the degree of alkylation of DNA is much smaller than expected from reaction kinetic data, and that a 'correction factor' should be introduced when estimating DNA-alkylation from hemoglobin data.[8] Other factors that modify the alkylation pattern and factors that affect the biological response include: steric factors, charge, bifunctionality, high reactivity, interactions with other compounds, and repair mechanisms.

Continued studies aimed at an evaluation of such factors and further development of the techniques to determine the degree of alkylation of hemoglobin should provide a basis for meaningful risk estimates for a large number of mutagenic compounds.[9,10,11]

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SPONTANEOUS ABORTION STUDIES: ROLE IN SURVEILLANCE

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The usefulness of spontaneous abortion studies in surveillance and monitoring is just beginning to be recognized. Because this is still a relatively unfamiliar and unexplored territory, the first part of this paper gives an overview of the epidemiology of spontaneous abortions, and especially of those aspects relevant to surveillance. Next, we take a look at some evidence we have for thinking spontaneous abortions are influenced by environmental exposures, and we also examine a real-life epidemic that was noticed in our abortion-based surveillance system. From this experience, we have compared the appropriateness of using one or another outcome associated with spontaneous abortions to monitor changes over time. We show, more generally, that adding definition to the outcome greatly enhances the power of a monitoring system. In concluding, we visualize a three-tiered monitoring system, which will link findings in spontaneous abortions, at amniocentesis, and in births.

EPIDEMIOLOGY

Spontaneous abortions (pregnancies that terminate by the 28th week) comprise about 15% of recognized conceptions. In addition to these, it is conjectured a number of unrecognized conceptions terminate very early. For the moment we shall ignore this unknown.

Sometimes the product of conception that aborts is a well-formed fetus, sometimes a malformed one, or a growth disorganized embryo; sometimes only the fetal membrane can be seen enclosing an empty sac. The karyotypes of fetal cells often show abnormalities of chromosomal number or structure. The concentration of chromosomal abnormalities in spontaneous abortions exceeds that found in live births about 100-fold (TABLE I).

The aneuploidy includes many forms never seen, or rarely seen at term (trisomy 13 and 18), as well as those (like trisomy 21) well known in the newborn. In fact, the range of anomalies encountered was hardly suspected until revealed by studies of spontaneous abortions carried out over the past 10 years.

Because spontaneous abortions contain so many anomalies, and of so wide a variety, we have conceptualized spontaneous abortion as a maternal screening device which rejects anomalous conceptions. FIGURE 1 helps to concretize this thought.[1] "Anomaly" in the diagram refers to karyotypic abnormalities.

The incidence in abortions of eukaryotypic abnormalities is less well established, but like aneuploidies they too seem to be in general increased in concentration, and to represent a wider range of malformations than at birth.

FIGURE 1 includes the possibility that functionally and structurally normal fetuses will sometimes be found among the spontaneously aborted products of conception. We distinguish r_a, the probability that an anomalous fetus will abort, from r_n, the probability that a normal fetus will abort. It is presumed that with r_n, some maternal factor interferes with retention of the fetus or, possibly, there is a maternal-fetal interaction.

The frequency of anomalies at birth could be caused by a change in the production of anomalies (denoted as p), by insensitive screening (r_a), and by nonspecific screening (r_n). Depending on which process is affected, the effects at birth will be of course quite different.

The concentration and the range of anomalies give leverage to surveillance systems based on spontaneous abortions, because of the resulting increase in "power" over systems based on term births. Power is the probability that an effect of a specified size, if present, will be detected.[2] Power depends on four elements: (1) concentration, and among term births most malformations are fortunately rare; (2) the size of the effect, and most exposures, also fortunately, will have a rather small effect; (3) the size of the type I error set for the study; and (4) the sample size. We have shown elsewhere that if studies can be conducted on abortions as compared to term births far smaller samples are required to demonstrate a small effect[3] (see also, reference 4 and TABLE II).

A third property of spontaneous abortions, only now beginning to be exploited, is that the product of conception can be examined grossly, microscopically, and biochemically, as well as karyotypically. Tissue can also be stored for subsequent study. For the epidemiologist, this means that the dependent variable can be refined, another accession to analytic strength. In time, we can hope that the cause of a spontaneous abortion will be assigned with at least as much specificity as the cause of death. The origin of the chromosomal error, with respect to the parent, and likely timing involved, can be deduced in some cases.[5] Even at present, when we classify a case by karyotype, gross morphology, and period of gestation, this yields, at its crudest, a matrix of 5 x 6 x 6. To give each particular karyotype an individual, instead of a group, identity, for instance, makes the matrix much more complex. In a monitoring system based on spontaneous abortions one may count all events, as we do in crude mortality rates in a population, but one may also count specific entities, and derive the type of statistics used in proportional mortality ratios.

In effect, if the suspected mechanism is postulated, and the dependent variable defined, the sensitivity and the specificity of surveillance will be greatly enhanced. We shall illustrate this point below, from our ongoing work.

A fourth property of spontaneous abortions, which is relevant to their usefulness in surveillance from a public health perspective, we refer to as "lead time." Because the abortion occurs typically at about the third to fifth month post-conception, it can provide an earlier warning signal than a system that relies on births. We will return to this point too. Here we note that in a system such as ours, based on hospital cases, the stage of gestation at which abortion takes place varies from 4 weeks after the last menstrual period to 28 weeks, with a mean around 13 weeks.

There are two objections which some see as limiting the usefulness of spontaneous abortions in surveillance. One argument is that anomalies are only of importance if they come to term, and that those that never do so do not have implications for public health planning, although they may have for biology. We agree that the malformations that come to term, and may survive, have importance over and above the contributions they make to the elucidation of these causes. We would in no way, however, deny the general significance of any disturbances in reproduction, all of which need to be detected, and, if possible, prevented. A cogent counter to this objection will be the demonstration that the factors leading to anomalies in spontaneous abortions are the same as the factors leading to anomalies at term. Although, as yet, the evidence in support of such similarity is still insufficient for a firm statement, there is every reason to anticipate it will be forthcoming.

A second objection made is that all we know, thus far, about spontaneous abortions is based on hospital patients, and is thus of limited generality. Current studies are obliged to ignore unrecognized conceptions, as well as those that are recognized by the women involved, but who do not come for care. While it is true that almost all studies have been hospital based, the data we have show remarkable consistency from one hospital to another, and even from one country to another. This may turn out to be a less troublesome objection than it appears.

DETECTING THE INFLUENCE OF AN ENVIRONMENTAL EXPOSURE

Over the past 4 years, we have identified all women with the diagnosis of spontaneous abortions who came for care to any one of three hospitals.[6] We interview all who consent (about 2,000 have consented so far), as well as a selected group of women who carry their pregnancies beyond 28 weeks (controls). Whenever possible, the abortus is examined systematically, including karyotyping.

This approach gives us two analytic tools: comparison of cases with spontaneous abortions with controls, and comparisons among cases with spontaneous abortions. The second comparison can sometimes provide a validation of the case-control comparison, because, unlike the case-control study, the within-case comparisons are free of bias in the response that could be

attributed to a particular outcome. Until the fetal tissue has been cultured and karyotyped (at least 3 weeks after the interview), no one (not the patient, the interviewer, nor the laboratory team) knows the nature of the karyotype. As an example, consider the finding that the relative risk of having an abortion is raised, if conception occurred when an intrauterine device was in place. (This finding, established in other studies, was confirmed by our study.) Intuitively, one would expect the excess in abortions to be of chromosomally normal fetuses, and so it turned out.

Our initial analyses have already detected one environmental exposure, maternal smoking during pregnancy, which was more common among cases than among controls, with an odds ratio of 1.8.[7] Among those cases using the public services at the medical centers, there is an excess of chromosomally normal fetuses. Referring back to FIGURE 1, we infer that smoking affects r_n, the risk of spontaneously aborting a chromosomally normal fetus.

In monitoring changes over time in the incidence of congenital malformation, problems of recognition, of recording, and of statistical inference have troubled epidemiologists for some years.[8,9] Most of these difficulties still have to be dealt with when the outcome is spontaneous abortion. As an example, early last year we noticed an apparent excess of trisomic conceptuses among women who reported the last menstrual period occurring during December 1976 and January 1977.[6]

There are several ways of testing the statistical significance of this excess. On the lower part of FIGURE 2, the histogram shows the numbers of trisomies found in each month of conception. On the upper part of the figure, we have plotted the findings by the method known as the cumulative summation technique.[8,11] By this method, when the excess rises above the line known as the decision boundary, an alerting signal is given. In these data the chances that such an alert will occur by chance is only 1 in 500 months. Therefore, we took this rise as probably meaningful.

In retrospect, we examined a number of other outcomes available to us (total number of abortions, number of early abortions, number of disorganized fetuses, etc.) to see if they would have reflected the increase in trisomy. However, for none of these would the alerting signal have been given. If the purpose of the monitoring system is to detect a rise in aneuploidy, then no method other than one which provides karyotypes will be sensitive enough.

For the examples given, it becomes clear that the information gained from studying spontaneous abortions, like that from term births, depends on the suspected agent, the suspected pathogenetic process, and the suspected outcome. Smoking affected r_n, the risk of spontaneously aborting normal fetuses, and was detected in the case-control study. Whatever caused the excess in trisomies did not affect r_n, and could not be detected by a single count of abortions.

Recently, an increase in spontaneous abortions was reported in wives of vinyl chloride workers.[12,13] As an exercise, and assuming reports of a relationship were to be confirmed, we considered the possible pathogenetic

process. Biologically, we considered three possibilities by which the agent could have acted through sperm:

1. There was interference with meiosis and fertilization, leading to polyploidy, or to nondisjunction.

2. Vinyl chloride caused dominant mutations, expressed as nonviability in karyotypically normal fetuses, which accounted for the excess abortions.

3. Vinyl chloride acts as a clastogen, causing chromosomal breaks and rearrangements in the products of conception. As we show in TABLE III, an excessive rate in abortions of the scale reported by Infante et al.,[12] if it was indeed caused by a clastogen, would be apparent after karyotyping only 12 specimens from an exposed population. (See also reference 3 for a fuller discussion of this example.)

A THREE-TIERED MONITORING SYSTEM

Finally, we would like to take the case of the trisomy epidemic a little further. As we mentioned, it was noted about a year ago, and we were at a loss as to how to act. Colleagues at the Center for Disease Control had had no other indications of anything untoward. The next step was to follow the pregnancies of the "affected" conception cohorts. Some women who were in the older age groups had had amniocentesis, and a number of laboratories made their results available to us. In FIGURE 3, the continuous black line shows the trisomies present in spontaneous abortions, and the broken line shows the trisomies at amniocentesis. At first, it seems that there was a parallel, though lesser, peak of trisomies among amniocentesis cultures. In the fuller series now available to us, the slight rise evident in FIGURE 3 among the cohorts under study is not statistically significant.

However, a recent letter in Lancet reported the birth of five infants with trisomy 13 in the Maryland area, all within the same conception cohorts as our trisomy epidemic.[14] Trisomy 13 is an extremely rare anomaly at birth, occurring in about 1 in 14,000 live births. For the same cohorts in New York City, there were three births with trisomy 13. In both Maryland and New York, the excess is statistically significant. In the trisomy epidemic among spontaneous abortions, however, the excess was accounted for mainly by trisomy 16. There was one trisomy 13 among the spontaneous abortions in the trisomy epidemic; although not statistically significant, only one trisomy 13 had been observed in the 32 months prior to the epidemic.

This puzzling finding brings us back to the question of connectedness between anomalies among aborted conceptuses and anomalies at birth. Trisomy 16 is common in spontaneous abortions, but is never seen in births, or even at amniocentesis. In the trisomy epidemic among spontaneous abortions we saw only one trisomy 13, and one trisomy 21. The functional significance of each type of trisomy is certainly different, for instance as expressed anatomically, and in the probabilities of intrauterine and

postnatal survival. On the other hand, the experiences favoring the production of nondisjunction may not be as different. Thus advanced maternal age is associated with an increase in every kind of trisomy, although there are differences in linearity and slope.[15] If an environmental exposure were to induce nondisjunction at meiosis, it could perhaps do so for any chromosomes or for all chromosomes at random, or for those chromosomes with some similar properties. Thus trisomy 16 and 13 could both have this vulnerability, although only trisomy 13 survives to give the evidence at birth. To detect the link with trisomy 16 one would have to look among spontaneous abortions.

In conclusion, studies of spontaneous abortions, while still crude and imperfect indicators of reproductive failure, give promise that they could play a considerable role in detecting reproductive hazards to men and women in the workplace.

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TABLE I

PREVALENCE (PER 1000) OF CHROMOSOMAL ANAMOLIES IN SPONTANEOUS ABORTIONS, INDUCED ABORTIONS, PERINATAL DEATHS, AND LIVE BIRTHS

	N	Prevalence of Karyotypic Anomaly per 1000
Spontaneous Abortions		
Gestation <18 weeks		
Lauritsen (15)	255	549
Gestation ≤28 weeks		
Current study	395	296
Creasy et al. (16)	941	305
Induced Abortions		
Kajii (17)	2,782	17
Perinatal Deaths		
Machin (18)	500	56
Live Births		
Berger (19)	24,448	5

SOURCE: Reference #3

TABLE II

OUTCOME VARIABLES AND STATISTICAL POWER: ESTIMATES OF RELATIVE RISK AND SAMPLE SIZE NEEDED TO DETECT A CHANGE IN THE FREQUENCY OF A GIVEN OUTCOME IN COHORTS OF PREGNANT WOMEN, WITH A POWER OF 80 AND ASSUMING THAT THE PROBABILITY OF AN ANOMALY AT RECOGNIZED CONCEPTION IS DOUBLED IN THE EXPOSED GROUP

	Relative Risk	Sample Size Needed for Each Exposed and Unexposed Group
Chromosomal defects in newborn: Diagnosed on appearance:	2.0	22,843
Chromosomal defects in newborn: Diagnosed on systematic karyotyping:	2.0	4,542
Incidence of spontaneous abortion:	1.3	901
Prevalence of chromosomal anomalies in abortions (4-28 weeks):	1.5	161
Prevalence of chromosomal anomalies in abortions (4-15 weeks):	1.4	133

SOURCE: Reference #3

TABLE III

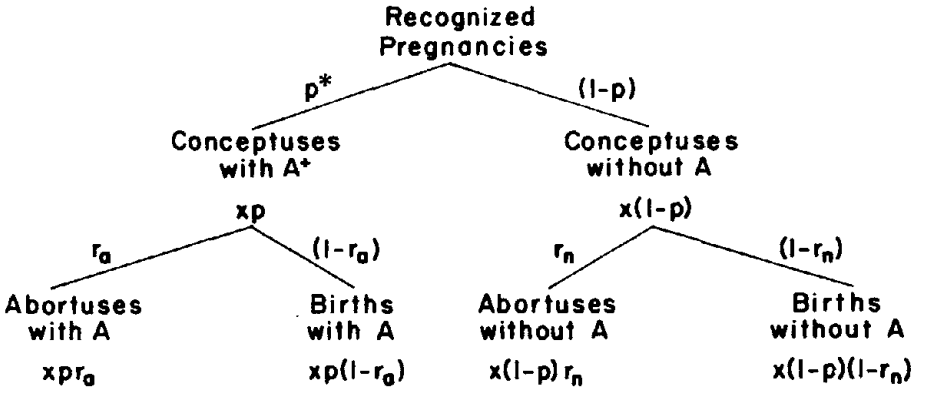
SAMPLE SIZE AND PATHOGENESIS: ASSUMING A RELATIVE RISK OF 1.8 FOR SPONTANEOUS ABORTION INCIDENCE IN MATES OF MEN EXPOSED TO VINYL CHLORIDE

(= .05, Two Tailed Test, Power = .90)

	Percent Distribution in Affected Cells		
Pathogenesis	Predicted in Exposed	Expected in Unexposed	Sample Size Required
Clastogen	45	.64	12
Normal karyotype: dominant mutation: intact empty sac or growth disorganized embryo	47	4	17
Meiosis, sperm viability, or fertilization	62	32	56
Excess normal karyotypes	82	68	198

SOURCE: Reference #3

Diagram showing relationships between incidence of anomalies, fetal loss and birth defects among recognized pregnancies



*p could be taken as a function of gestational time

+for the moment only one "A" per fetus is assumed

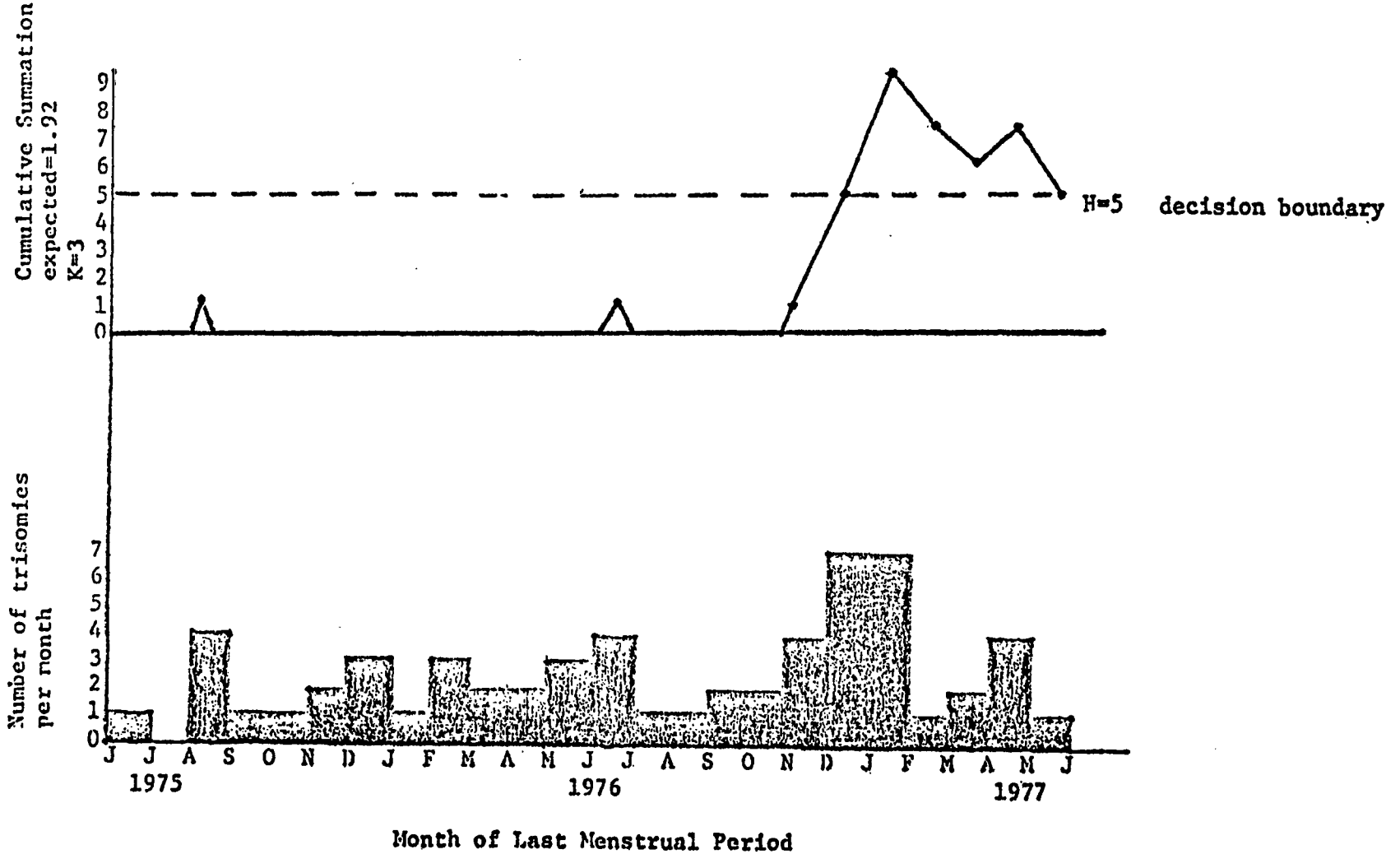
SOURCE - Stein et al Spontaneous abortion as a screening device American Journal of Epidemiology, 1975, 102(4) 275-290

FIGURE 1

Spontaneous abortion as a screening device. The effect of fetal survival on the incidence of birth defects.

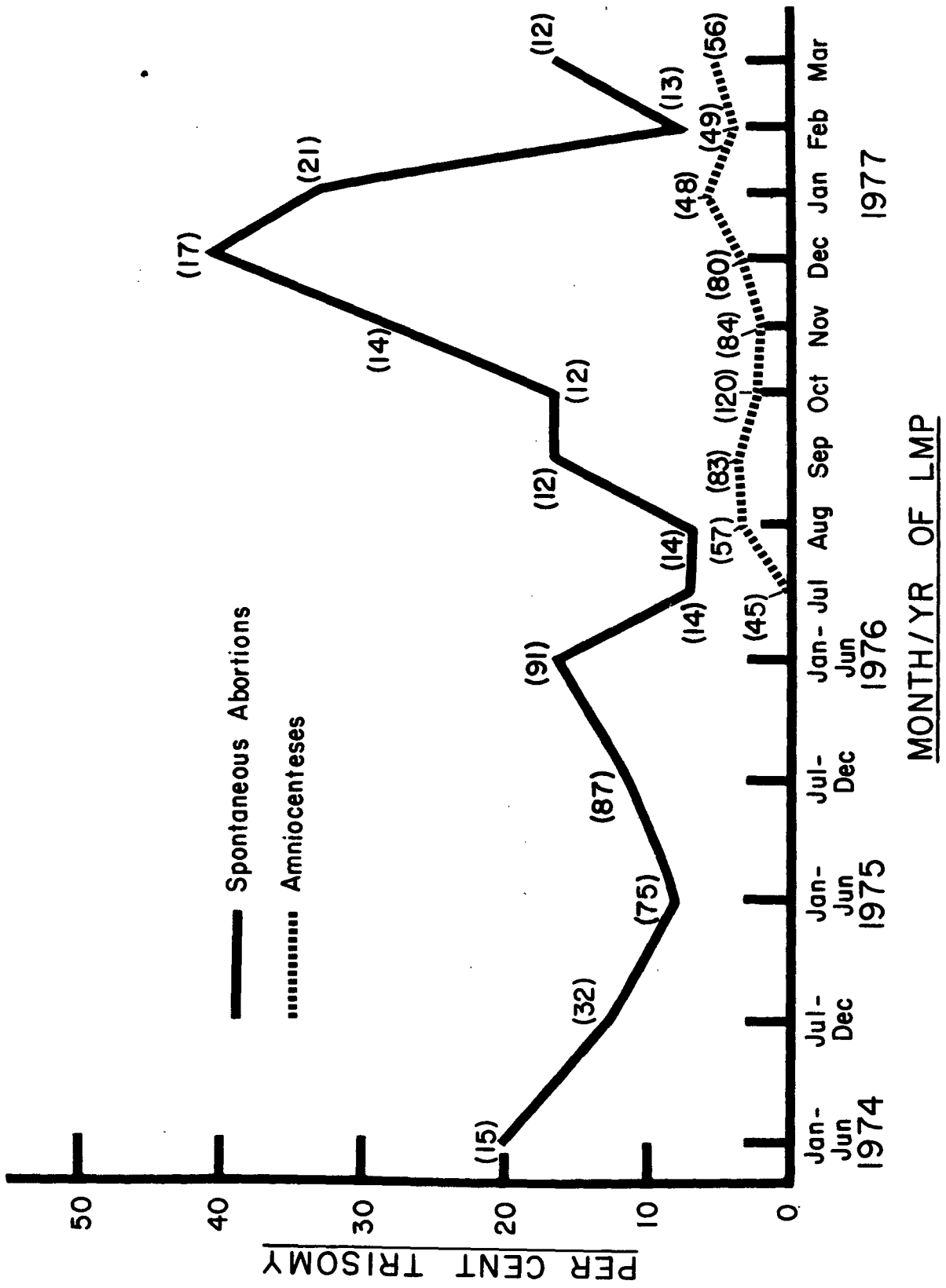
FIGURE 2

Frequency of trisomy among spontaneously aborted conceptions in three New York City Hospitals and Cumulative Summation Plot



SOURCE: Reference #7

FIGURE 3



SOURCE: Reference #7

MONITORING INDUSTRIAL POPULATIONS BY CYTOGENETIC PROCEDURES

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INTRODUCTION

Industrial workers exposed to potentially hazardous substances offer us a way to study the effects of these substances in humans. Workers are usually exposed to higher concentrations of the agents than the general population. These higher exposures may be used to determine whether the agents are causing detectable physiological changes in the workers. Such information may then be used to protect the general population as well as the worker from the agent(s). Cytogenetic monitoring of populations has been used to uncover genetic hazards, e.g., irradiation,[1] vinyl chloride,[2] epichlorohydrin.[3]

Why should we be concerned about cytogenetic changes? There are two main reasons. One, individuals showing increased levels of cytogenetic abnormalities have an increased chance of having a neoplastic disorder; and two, these same individuals have an increased chance of having a child with birth defects. Individuals with increased levels of cytogenetic abnormalities have an increased chance of having a neoplastic disorder. This is best seen in the atomic bomb survivors of Hiroshima and Nagasaki where survivors have shown an increase in chromosomal aberrations as well as a dramatic increase in the incidence of leukemia.[1] There are many other examples that can be used to demonstrate this relationship between cytogenetic abnormalities and cancer. To name a few, leukemia in x-irradiated and ankylosing spondylitis and benzene-exposed workers, lymphoma and leukemia after treatment with cytoxin for rheumatoid arthritis, lung cancer in uranium miners, liver cancer in thorotrast-exposed persons and in vinyl chloride workers, osteogenic carcinoma in luminous dial painters, and visceral cancers after treatment with methotrexate for psoriasis.[4]

Individuals with increased levels of cytogenetic abnormalities have an increased chance of having a child with birth defects. This can be seen in populations with a high level of background radiation, where individuals have an increase in chromosomal aberrations as well as an increase in the incidence of Down's Syndrome in their offspring.[5]

I would like to limit the remainder of this presentation to examples of the cytogenetic monitoring of industrial workers exposed to chemicals.

METHODOLOGY

The methodology used in culturing peripheral lymphocytes, including the collection of pertinent patient data, tissue culture procedures,

techniques of observation, nomenclature and classification of aberrations, has been described in detail elsewhere.[6]

Proper interpretation of obtained data must include consideration of the background rate of aberrations, and the number of cells analyzed per individual. FIGURE 1 is a hypothetical curve of the repeated analysis of an individual with 15% of his cells showing aberrations. If we were to repeatedly examine samples of 25 cells from this individual, the range of values obtained would be from 0 to over 40% aberrations. As we increase the sample size to 50 cells, we see that the range of values obtained would be from 2 to 32% aberrations. With a sample size of 200 cells, the range of values obtained would be from 8 to 22% with most of the values between 12 and 18%. Finally, with a sample size of 2,000 cells, the range of values obtained would be from 12 to 18%. As seen in FIGURE 2, as we decrease the true aberration rate to 3%, we shift the probability of detection of aberrations to the left side of the figure. Thus, with samples of 25 cells for analysis, there is greater than a 45% chance of finding a patient with no aberrations when the patient really has a true aberration rate of 3%. Again, repeated analyses with a 200 cell sample size approximates the true aberration rate, while analyses with a sample size of 2,000 cells accurately describes the true aberration rate. If we further reduce the true aberration rate to 1% (FIGURE 3), we see that sample sizes of 25 or 50 cells are insufficient. A sample size of 200 still approximates the true aberration rate, while a 2,000 cell sample size accurately describes the true aberration rate. FIGURE 4 is an actual plot of data from over 1,300 nonexposed individuals screened cytogenetically. Notice how closely the theoretical curves of FIGURES 2 and 3 approximate the actual data from nonexposed individuals. The greater spread of the percentage of abnormal cells is a reflection of the heterogeneity of the general population.

In summary, it appears that on an individual basis, at least 200 cells per analysis are necessary to properly estimate the cytogenetic abnormalities of any one patient.

EXAMPLES OF CYTOGENETIC STUDIES

A. Benzene

Benzene is a clear, colorless, noncorrosive, highly flammable liquid with a low boiling point and a high vapor pressure. In 1976, over 11 billion pounds were produced in the United States, with the petrochemical and petroleum refining industries producing 94% of the U.S. total. Only 11 other chemicals and only 1 other hydrocarbon (ethylene) are produced in greater tonnage in the United States.

We have studied workers exposed to benzene.[7] Cytogenetic evaluation of peripheral blood lymphocytes from 52 workers exposed to low levels of benzene (less than 10 ppm) has revealed an increase in aberration rates as compared to that of a 44-person group seen for preemployment examinations. As seen in TABLE I, 200 cells per individual were scored for the presence of cytogenetic aberrations. These included chromatid and chromosome breaks

(deletions), chromosome breaks, marker chromosomes (rings, dicentrics, translocations, and exchange figures), and total abnormal cells. On a cellular basis there appears to be no difference in the percentage of chromatid breaks and total abnormal cells between the two groups. However, the benzene workers have twice the percentage of chromosome breaks, and three times the percentage of marker chromosomes. Comparison of the distribution of results on an individual basis is shown in TABLES II and III. Both benzene workers and preemployment individuals were divided into those showing no chromosome breaks and those with one or more per 200 cells.

There was a highly significant difference in the distribution with almost twice the percentage of workers having cells with chromosome breaks (TABLE II). Also, the distribution of individuals with both chromosome breaks and marker chromosomes was compared for the two groups (TABLE III). Again, there was a highly significant difference in the distribution with a 10-fold increase in the percentage of benzene workers having both types of aberrations.

Additional analysis was done by separating the workers into three groups based upon their exposure to benzene. Comparison of the distribution of individuals with both chromosome breaks and markers showed that less than 3% of the nonexposed individuals showed these aberrations, while 33% of the workers exposed to greater than 2.5 but less than 10 ppm benzene revealed such aberrations (FIGURE 5).

B. Epichlorohydrin

Epichlorohydrin is a colorless liquid at room temperature; it is insoluble in water. It is used as a solvent for natural and synthetic resins and in the manufacture of epoxy resins, pharmaceuticals, insecticides, etc. Over 550 million pounds of epichlorohydrin were produced in the United States in 1975.

We have studied workers exposed to epichlorohydrin.[8] Cytogenetic analyses of peripheral blood lymphocytes from 93 workers exposed to epichlorohydrin have shown an increase in aberration rates as compared to that of a 75-person group seen for preemployment examination. Two hundred cells per individual were scored for the presence of chromatid and chromosome breaks (deletions), marker chromosomes (rings, dicentrics, and translocations), severely damaged cells (those containing 10 or more aberrations), and total abnormal cells. As seen in TABLE IV, cells from epichlorohydrin workers had a marked increase in all five categories of aberrations as compared to those from preemployment individuals. For statistical evaluation, Chi-square analysis of the distribution of individuals rather than cells was used. Comparison of the distribution of results on an individual basis for chromatid breaks is seen in TABLE V. Both epichlorohydrin workers and nonexposed individuals were separated into those showing no chromatid breaks, those with 1 to 6 breaks, those with 7 to 12 breaks, and those with more than 12 breaks per 200 cells. There was a highly significant difference in the distribution with almost four times the percentage of exposed workers having more than 12 breaks. Similar analyses for the distribution of individuals with chromosome breaks, severely damaged cells,

and abnormal cells are seen in TABLES VI, VII, and VIII. Again, there were highly significant differences in the distribution with 9 times the percentage of epichlorohydrin workers having more than 4 chromosome breaks, almost 12 times the percentage of workers having severely damaged cells, and over 5 times the percentage of workers with more than 12 abnormal cells.

SUMMARY

In conclusion, industrial populations, because of their high potential for exposures, can be used for cytogenetic studies in an effort to protect the health of the workers and that of the public. Attention to methodology is of prime importance. Individual cytogenetic studies should consist of at least 200 cells per person in order to properly estimate the chromosomal aberration profile of the individual. Finally, through the implementation of proper techniques, benzene and epichlorohydrin have been shown to induce cytogenetic abnormalities in workers exposed to these substances.

Acknowledgment

The author wishes to thank Dr. Elbert Whorton, Associate Professor and Director, Division of Biometry, Department of Preventive Medicine and Community Health, the University of Texas Medical Branch at Galveston, who supplied the theoretical data used in FIGURES 1-3.

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TABLE I

CYTOGENETIC SUMMARY OF 52 WORKERS EXPOSED TO BENZENE (7)

	Workers	Controls
Number of Individuals	52	44
Number of Cells	10,400	8,800
Chromatid Breaks	1.0%	1.1%
Chromosome Breaks	0.67%	0.35%
Marker Chromosomes*	0.19%	0.06%
Abnormal Cells	1.6%	1.4%

*Rings, Dicentrics, Translocations, and Exchange Figures

TABLE II

DISTRIBUTION OF CHROMOSOME BREAKS RELATED TO BENZENE EXPOSURE (7)

Group	Number in Group	Individuals With No Chromosome Breaks (%)	Individuals With Any Chromosome Breaks (%)
Controls	44	59.1	40.9
Benzene Workers	52	26.1	73.1

$X^2 = 10.24$ P = less than 0.005

TABLE III

DISTRIBUTION OF CHROMOSOME BREAKS AND MARKERS RELATED TO BENZENE EXPOSURE (7)

Group	Number in Group	Individuals Without Both Chromosome Breaks and Markers (%)	Individuals With Both Chromosome Breaks and Markers (%)
Controls	44	97.7	2.3
Benzene Workers	52	76.9	23.1

$X^2 = 8.96$ P = less than 0.005

TABLE IV

CYTOGENETIC SUMMARY OF 93 EPICHLOROHYDRIN WORKERS AND 75 NONEXPOSED INDIVIDUALS (8)

	Number of Observations	
	Nonexposed Individuals	Exposed Workers
Individuals	75	93
Cells	15,000 (100%)	18,600 (100%)
Chromatid Breaks	323 (2.15%)	807 (4.34%)
Chromosome Breaks	77 (0.51%)	179 (0.96%)
Marker Chromosomes*	12 (0.08%)	24 (0.13%)
Severely Damaged Cells	2 (0.01%)	23 (0.12%)
Abnormal Cells	357 (2.38%)	791 (4.25%)

*Rings, Dicentrics, and Translocations

TABLE V

DISTRIBUTION OF CHROMATID BREAKS IN EPICHLOROHYDRIN WORKERS AND NONEXPOSED INDIVIDUALS (8)

Group	Number In Group	Percent of Individuals With ____ Chromatid Breaks			
		0	1-6	7-12	Greater Than 12
Nonexposed	75	17.3	60.0	17.3	5.3
Exposed	95	5.4	52.7	21.5	20.4

$X^2_{(3)} = 13.35$ P = less than 0.005

TABLE VI

DISTRIBUTION OF CHROMOSOME BREAKS IN EPICHLOROHYDRIN WORKERS AND NONEXPOSED INDIVIDUALS (8)

Group	Number In Group	Percent of Individuals With ____ Chromosome Breaks			
		0	1-2	3-4	Greater Than 4
Nonexposed	75	53.3	30.7	14.7	1.3
Exposed	93	26.9	50.5	10.8	11.8

$X^2_{(3)} = 18.43$ P = less than 0.005

TABLE VII

DISTRIBUTION OF SEVERELY DAMAGED CELLS IN EPICHLOROHYDRIN WORKERS AND NONEXPOSED INDIVIDUALS (8)

Group	Number In Group	Percent of Individuals With ____ Severely Damaged Cells: 0	Greater Than 0
Nonexposed	75	98.7	1.3
Exposed	93	84.9	15.1

$X^2_{(1)} = 9.62$ P = less than 0.005

TABLE VIII

DISTRIBUTION OF ABNORMAL CELLS IN EPICHLOROHYDRIN AND NONEXPOSED INDIVIDUALS (8)

Group	Number In Group	Percent of Individuals With ____ Abnormal Cells: 0	1-6	7-12	Greater Than 12
Nonexposed	75	12.0	57.3	26.7	4.0
Exposed	93	2.2	51.6	24.7	21.5

$X^2_{(3)} = 15.87$ P = less than 0.005

FIGURE 1

Hypothetical curves for the repeated analysis of an individual with 15% of cells showing aberrations

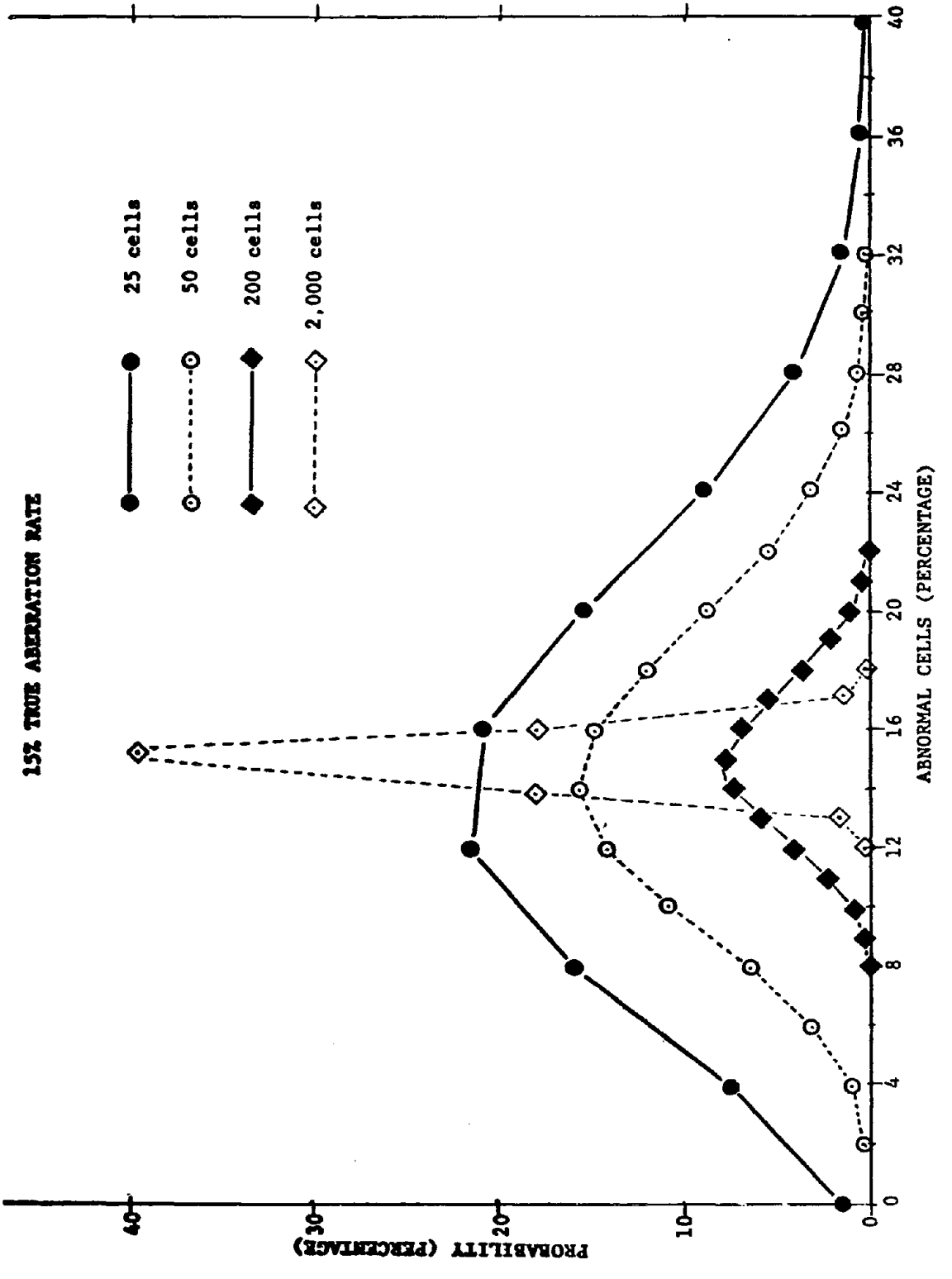


FIGURE 2

Hypothetical curves for the repeated analysis of an individual with 3% of cells showing aberrations

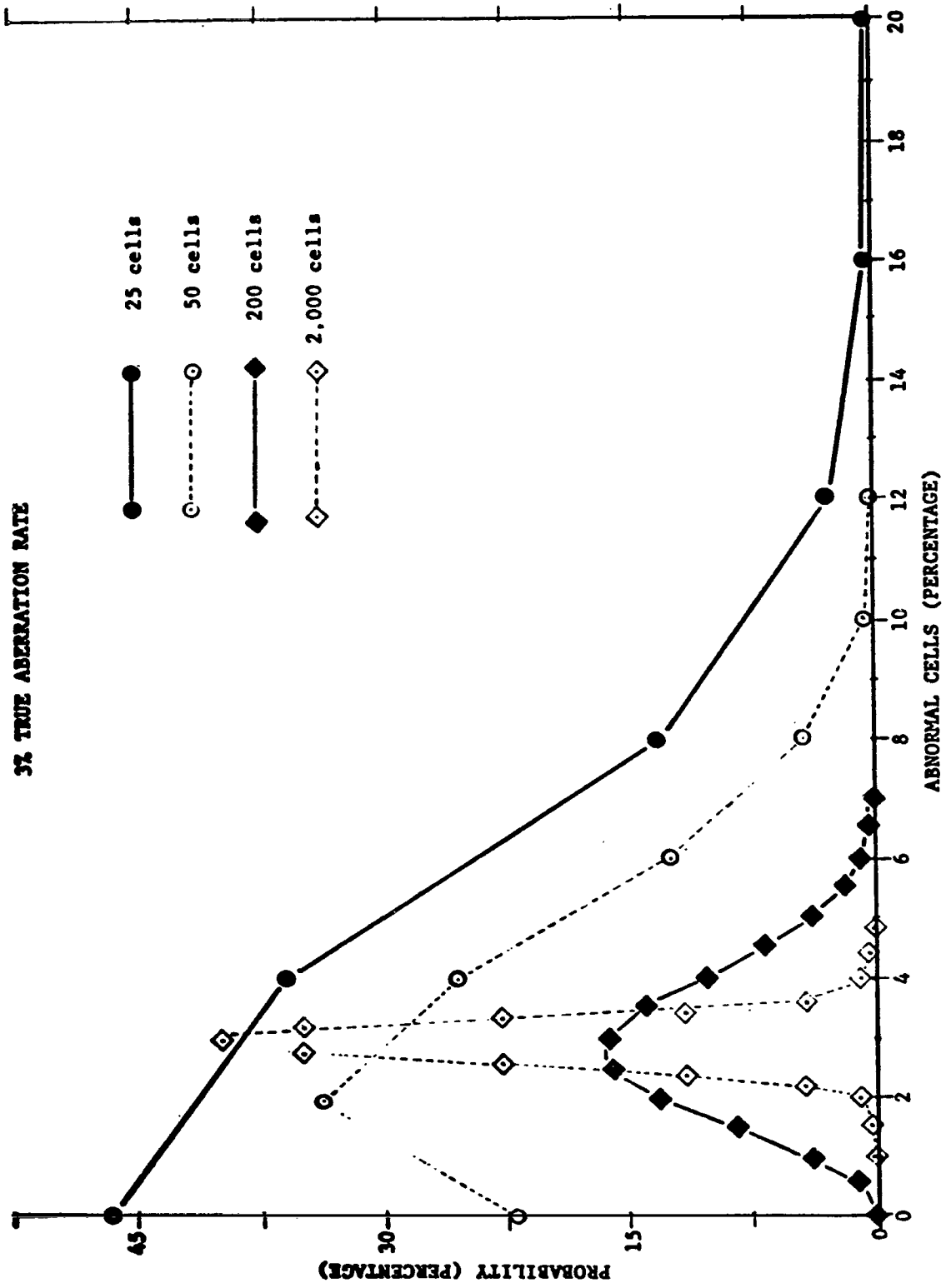


FIGURE 3

Hypothetical curves for the repeated analysis of an individual with 1% of cells showing aberrations

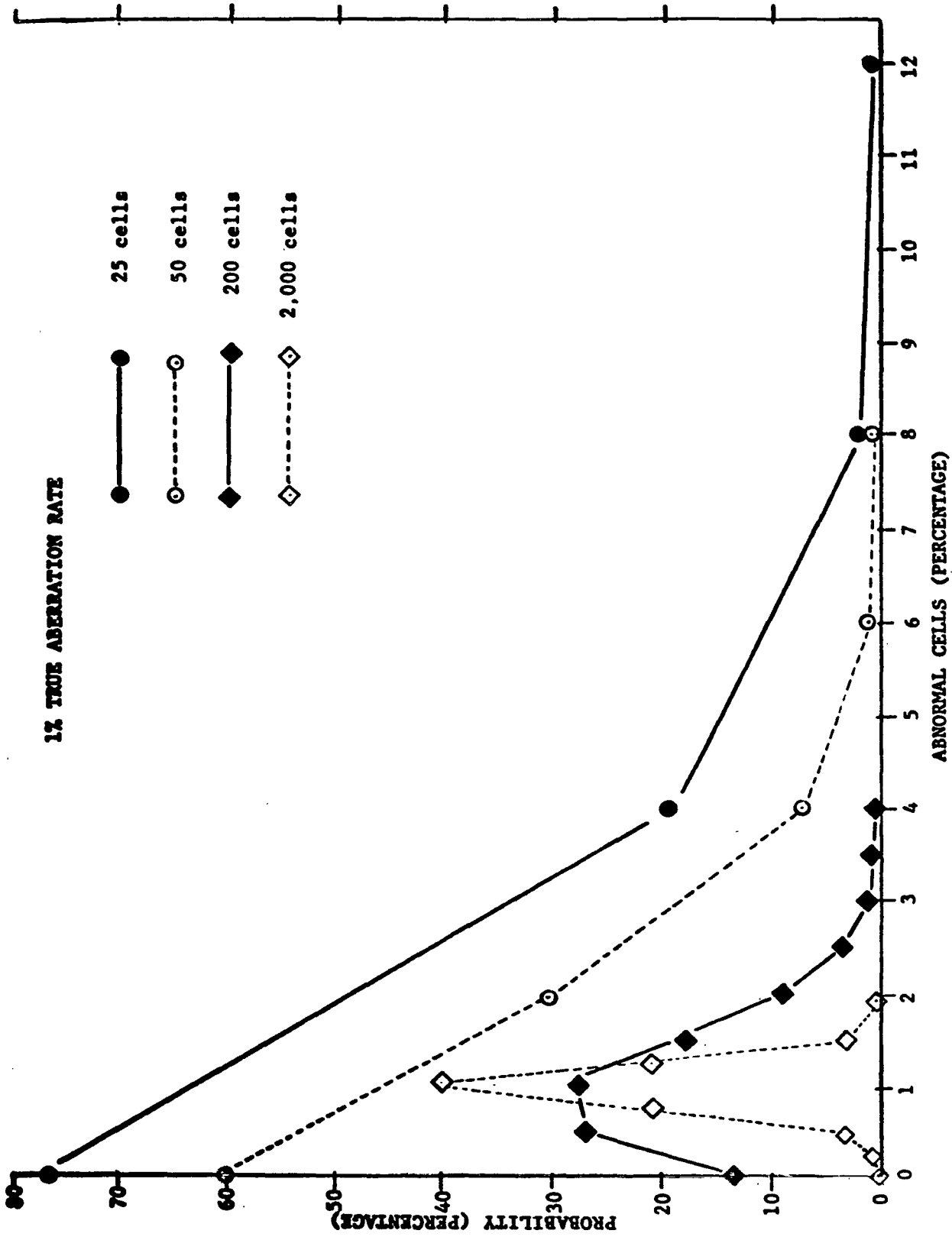
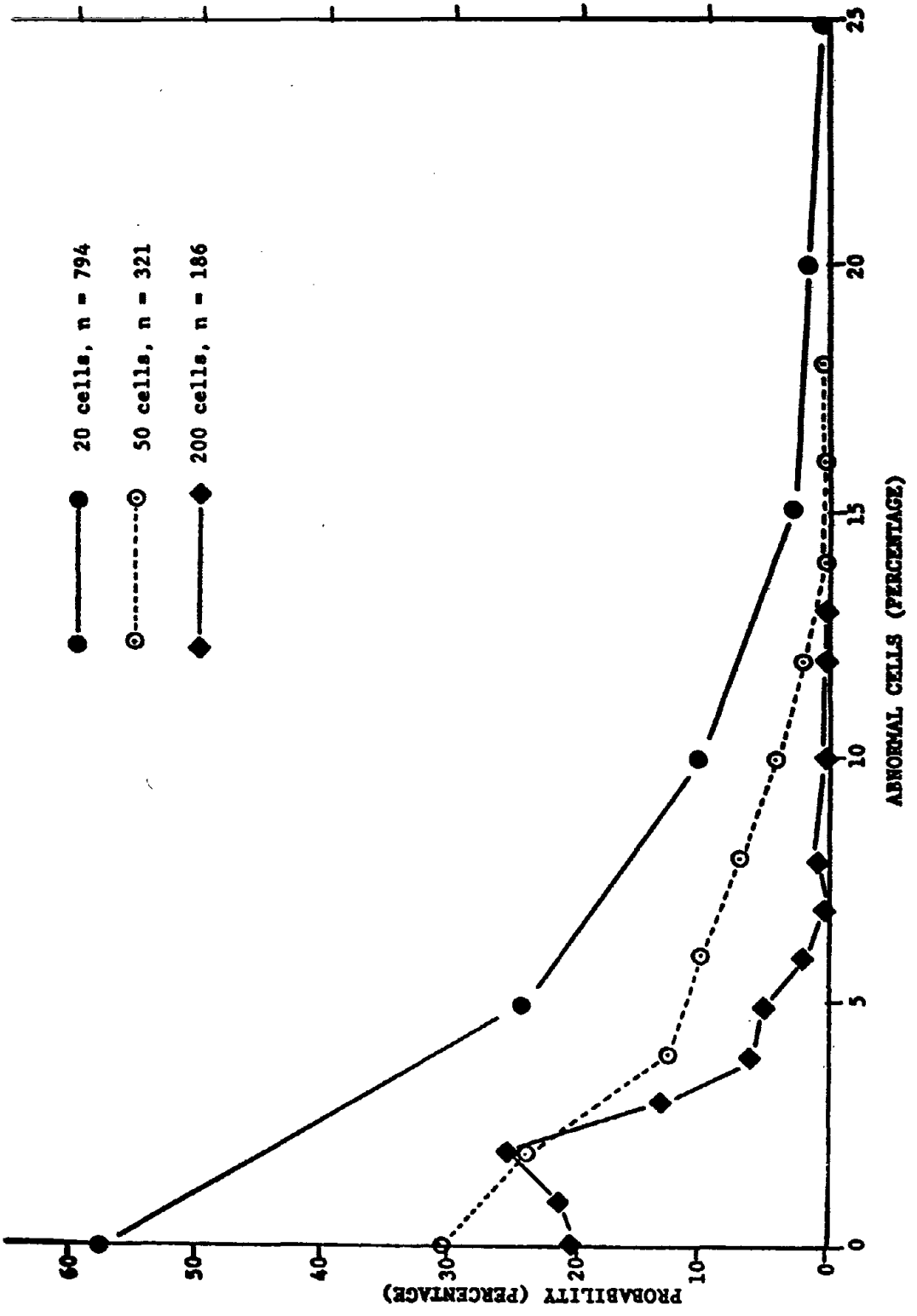


FIGURE 4

Actual curves for the analysis of 1,301 nonexposed individuals as a function of sample size



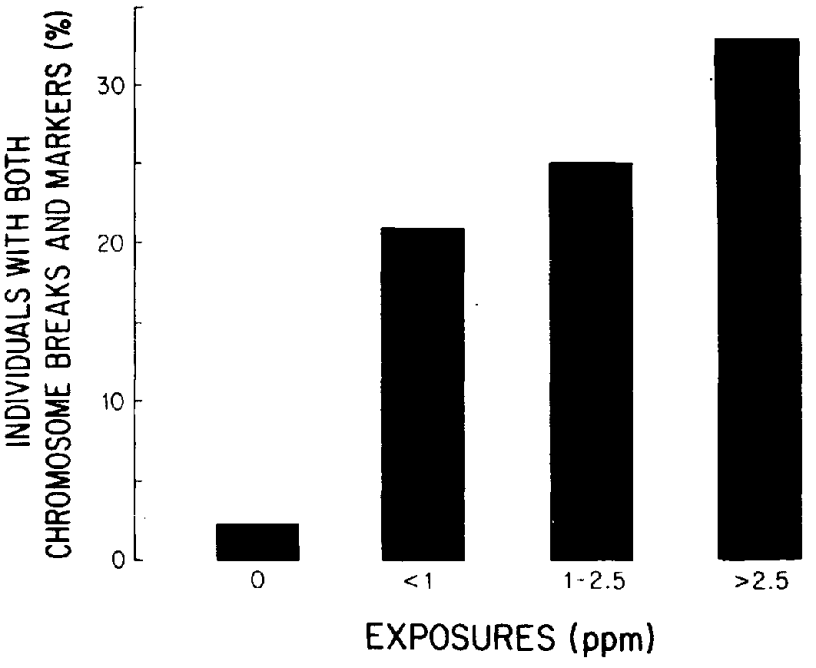


FIGURE 5

Comparison of the distribution of individuals with both chromosome breaks and markers as a function of exposure to benzene (7)

MONITORING Y CHROMOSOMAL NONDISJUNCTION IN HUMANS WITH THE YFF SPERM TEST*

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Mutagens are agents which affect a breakdown in the transmission of the informational content of DNA. The effects of these agents upon subhominoid test systems can generally be measured by scoring readily accessible end-points such as death, survival, or fertility. Monitoring mutation in man himself poses many problems which are not easily overcome because of the moral and ethical considerations, and because of the paucity of human tests and lack of information concerning our knowledge of human mutation.

The overall progression of mutagenesis as it applies to man is outlined in FIGURE 1. DNA mutates in some fashion; the cell can then deal with this mutation in one of three ways: (1) it can die, (2) it can repair the error, or (3) it can persist and become part of the genome which is transmitted to any daughter cells should the cell divide.

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This mutation can affect either gametic or somatic cells. If the mutation is somatic, three situations can occur: First, if the somatic cells are in a fetus, the aberration will not perpetuate itself because the mutation did not affect gonadal cells, hence the aberration is self-limiting. Second, somatic cells can mutate to form clones which can result in malignancies. Third, nonproliferative type mutations can result in a general increase in total cellular mutations which can be characterized as aging.

If the mutation affects gametic cells, the pathways are very different. First, the cell can be eliminated via natural meiotic selection which results in sterility if the selection is 100%. On the other hand, if the abnormality persists in a viable form and fertilization occurs, the outcome can end only in abortion or abnormal offspring. This propagation of genetically unfit offspring constitutes a significant load on the human gene pool which should be monitored.

Gametic cells are subject to several types of basic mutation as indicated in FIGURE 2. First, point mutations can occur which can be classified as either (a) base pair, or (b) frameshift type. Second, mutagens which affect chromosomes do so in two general ways: They can alter (a) the structure, or (b) the number of chromosomes (nondisjunction). Clinical studies have shown that some 0.4% of all living births display chromosomal numerical errors,[1] and it is estimated that over one-third of early spontaneous abortions contain similar aberrations.[2]

Since the incidence of chromosomal nondisjunction in man is high, a major part of our research has been to evaluate the human reproductive system with respect to nondisjunction.

The identification of individual human chromosomes was made possible with the discovery by Caspersson et al. that human chromosomes treated with quinacrine mustard or its closely related analogues exhibit a banded pattern of differential fluorescence.[3] Zech[4] and Vosa[5] both noted that the distal portion of the long arms of the Y chromosome fluoresces brightly when stained in this manner, and further studies[6-9] showed that quinacrine-stained Y chromosomes will fluoresce brightly enough to be identified in the interphase nuclei of many types of human tissue. The same phenomenon involving the Y chromosome of human spermatozoa was described by Barlow and Vosa[10] who observed that the fluorescent effect was bright enough to be seen through the membranes of intact sperm. (See FIGURE 3.)

The DNA quantitation studies of Sumner et al. leave little doubt that the spermatozoa which show one quinacrine-fluorescent spot are Y chromosome-bearing.[11] However, these investigators were not completely convinced as to the significance of spermatozoa with two quinacrine-fluorescent spots.[12] We believe that the identification of two fluorescent bodies within a spermatozoon indicates the presence of two Y chromosomes. (See FIGURE 4.) Studies of buccal smears have demonstrated the presence of two quinacrine-fluorescent spots in many cells from some male patients; the XYY status of each was confirmed by lymphocyte culture karyotype.[13] As part of an investigation to use the YFF phenomenon to monitor nondisjunction in humans, a method has been developed to detect changes in the frequencies of YF and YFF bodies in sperm.

MATERIALS AND METHODS

Each specimen was collected by masturbation into a sterile siliconized glass bottle, and immediately brought to the laboratory for slide preparation. A drop of semen was placed upon a slide, and another slide was pressed firmly against the first. The two slides were pulled apart to produce a uniform film. The slides were dried horizontally in a dust-free environment for 24 hours. The dried material was fixed in absolute methanol for 15 minutes and, subsequently, stained in a 0.5% aqueous solution of quinacrine dihydrochloride (Sigma Chemical Co., St. Louis) for 20 minutes. The slides were drained and rinsed for 1 minute, first with tap water, and then with distilled water. The slides were mounted in an aqueous citric acid-phosphate buffer solution (pH 5.5), and left undisturbed until the time of evaluation approximately 3 hours later. Two independent scorers (MCB and RWK) read the slides under fluorescent microscopy. Each observer scored approximately 500 sperm per patient. It was decided that only sperm of normal appearance would be scored. Each sperm was required to be unremarkable in size and shape, to be intact, and to have an attached tail. In addition, each fluorescent body was required to fall within the intact membranes and to form a distinct point of light. Adherence to these strict criteria eliminated some F positive sperm from consideration but helped reduce false positive scoring. Case 1 (FIGURE 5) was a donor whose semen was examined over a period of 21 months. Each of the 38 samples was examined by the two independent scorers. Notice the close correlation between the two scorers. As can be seen, there are sporadic changes in these YFF values; therefore, serial sampling is necessary to establish a baseline.

Case 6 (FIGURE 6) was a 22-year-old patient with metastatic osteogenic sarcoma. Two semen samples were collected prior to a standard 3-month course of Adriamycin. The YFF values for this patient showed a three-to-four-fold increase less than 1 month after starting the chemotherapy. (The average time needed to detect nondisjunctional errors in anaphase II of the spermatogenic cycle is between 4 and 6 weeks.) YFF values remained elevated in the next five samples. At this point, however, the patient's condition deteriorated, and further cooperation became impossible. Case 7 (FIGURE 7) was a physician who went on fluoroscopy service at the point indicated by the arrow. At 3 to 5 weeks post-exposure there is a sharp increase in the frequency of YFF sperm observed. Case 8 was a 32-year-old research scientist with intestinal amebiasis who underwent diagnostic radiation 9 days before starting on a 10-day regimen of Flagyl. YFF values for this patient showed a doubling 5 weeks after radiation exposure as can be seen in FIGURE 8. Note that only one observer (MCB) scored these slides.

Certainly one criticism of the technique is that the incidence of single F bodies is considerably below the expected 50%. TABLE I shows data from five normal individuals including the donor with 38 serial samples (Case 1). Notice that the combined single F body mean is 16.6%, and the combined double F body mean is 0.7%.

One would expect approximately 50% of the sperm to carry a Y chromosome and other investigators have reported the incidence of double F bodies at 1.3%. The differences may be explained, in part, by our strict scoring criteria; however, the low incidence of single F bodies was troublesome.

It was subsequently suggested that the staining time be increased from 20 to 40 minutes. Further, the quinacrine stain is now commercially available, and is a considerably better grade of stain than the experimental stain used when this investigation was initiated in 1973. The rinse time was also decreased to 30 seconds. With these modifications in staining procedure, the incidence of sperm with single F bodies is approximately 40% as shown in TABLE II. The incidence of single F body sperm ranged from 40.0% to 42.6% with a mean incidence of 41.1%. The incidence of double F body sperm ranged from 0.9% to 1.4% with a mean incidence of 1.1%.

TABLE III shows data from a 28-year-old with seminoma (a solid testicular tumor of germinal epithelial origin) who was receiving X-ray therapy (Case 14). Notice that the single F body range varies from 38.5% to 52%, while the double F body range varies from about 1.0% to 6.0%. FIGURE 9 (Case 14) shows the incidence of double F body sperm plotted against time. The initial sample was collected prior to serial X-ray therapy. Note that 20 to 30 days after initiation of treatment there is a clear five- to six-fold increase in the percent of sperm containing double F bodies.

TABLE IV shows data from a group of 20 men who have experienced occupational exposure to dibromochloropropane (DBCP). There were originally 32 semen samples collected; however, 12 of the samples were aspermic and analysis was not possible. We were able to obtain only a single semen sample from each individual. Note the incidence of sperm with a single F body ranged from 36.3% to 46.3% with a mean incidence of 41.5%. The incidence of double F body sperm ranged from 1.8% to 5.3%, with a mean incidence of 3.8%.

Unequivocal assessment of these data cannot be made because the length of employment, age of the individual, and estimated dose were not available at this writing. It should be noted, however, that the mean incidence of double F body sperm in these DBCP workers was significantly higher than the mean incidence of double F body sperm in unexposed males by chi-square testing with one degree of freedom ($x = 26.8$; $p < 0.001$). Hence, as a group, the DBCP workers showed an elevation in sperm with double F bodies. We believe that the data presented here clearly establish the consistency between two observers in scoring the same sample. This method can be utilized in a screening program to monitor the exposure of human males to mutagenic substances.

At the present time, there is no way--aside from testicular biopsy--to monitor for gametic mutation in the human male. The noninvasive method presented here requires only masturbation to collect the specimen, and permits rapid evaluation of a nondisjunctional event in mature spermatozoa. It is not unreasonable to assume that an agent which increases Y chromosomal nondisjunction (frequency of YFF sperm) will increase the probability of autosomal nondisjunction as well. Thus, the finding of an elevated level of sperm with double F bodies among workers exposed to DBCP suggests that further study should be undertaken to determine whether the YFF monitoring system could be used in the occupational setting to detect mutagenic response to substances that cause chromosomal nondisjunction.

In view of the gametic changes observed in the 4 patients and 20 occupationally exposed individuals presented here, it may be prudent for men

who have a history of radiation exposure, industrial chemical exposure, and/or chemotherapy, and who are contemplating reproduction, to be evaluated for evidence of Y-Y nondisjunction before attempting conception. In the event of pregnancy when increased YFF values are observed, genetic counseling and chromosomal analyses by amniocentesis should be made available.

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TABLE I

YF AND YFF FREQUENCIES IN FIVE NORMAL INDIVIDUALS AS SCORED BY AUTHORS M. C. BENGE (1) AND R. W. KAPP (2). COMBINED VALUES ARE GIVEN IN THE LAST TWO COLUMNS. FIVE HUNDRED SPERM OR MORE PER EJACULATE WERE ANALYZED BY EACH OBSERVER UTILIZING THE 20 MINUTE STAINING TIME AS DESCRIBED IN THE TEXT.

Case No.	No. of Ejaculates Analyzed		Observer MCB (1) YF (%)	Observer MCB (1) YFF ± S.D.	Observer RWK (2) YF (%)	Observer RWK (2) YFF ± S.D.	Combined YF (%)	Combined YFF ± S.D.
1	38		17.8	1.0 ± 0.79	17.0	0.8 ± 0.80	17.4	0.9 ± 0.80
2	11		16.4	0.5 ± 0.54	16.7	0.4 ± 0.34	16.6	0.4 ± 0.45
3	6		14.5	0.7 ± 0.71	14.7	0.3 ± 0.30	14.6	0.5 ± 0.56
4	7		16.7	0.7 ± 0.26	15.9	0.5 ± 0.56	16.3	0.6 ± 0.43
5	8		17.9	0.7 ± 0.80	12.1	0.5 ± 0.58	15.0	0.6 ± 0.69
Total	70	Mean	17.2	0.9 ± 0.73	16.0	0.6 ± 6.68	16.6	0.7 ± 0.71

TABLE II

YF AND YFF FREQUENCIES IN FIVE NORMAL INDIVIDUALS AS SCORED BY AUTHOR R. W. KAPP (2). FIVE HUNDRED SPERM OR MORE PER EJACULATE WERE ANALYZED UTILIZING THE 40 MINUTE STAINING TIME AS DESCRIBED IN THE TEXT.

	No. of Ejaculates		Observer RWK (2)		
Case No.	Analyzed		YF (%)	YFF (%)	±S.D.
9	2		41.4%	1.3%	±0.35
10	2		40.1%	1.3%	±0.35
11	2		41.5%	1.40%	±0.14
12	2		40.0%	0.9%	±0.18
13	6		42.6%	1.1%	±0.17
Total	14	Mean	41.1%	1.1%	±0.25

TABLE III

SPECIMEN YF AND YFF FREQUENCIES IN CASE 14 WHO WAS RECEIVING X-RAY THERAPY FOR TREATMENT OF SEMINOMA AS SCORED BY AUTHOR R. W. KAPP (2). FIVE HUNDRED SPERM OR MORE PER EJACULATE WERE ANALYZED UTILIZING THE 40 MINUTE STAINING TIME AS DESCRIBED IN THE TEXT.

Case 14

Observer RWK (2)

Date of Sample	YF (%)	YFF (%)
8/23/76	47.5	1.3
8/30/76	44.0	1.0
9/5/76	42.0	1.4
9/12/76	47.0	4.0
9/15/76	52.0	5.0
9/17/76	39.0	4.0
9/21/76	43.0	6.0
9/22/76	44.0	6.0
9/24/76	45.0	4.0
9/29/76	48.0	5.0
10/1/76	44.0	4.0
10/4/76	44.5	6.0
10/11/76	46.5	4.5
10/29/76	38.5	5.0
11/1/76	46.0	4.5
11/12/76	39.0	3.0
11/16/76	40.0	3.5
12/1/76	45.5	3.3

TABLE IV

YF AND YFF FREQUENCIES IN 20 INDIVIDUALS OCCUPATIONALLY EXPOSED TO DBCP AS SCORED BY AUTHOR R. W. KAPP (2). FIVE HUNDRED SPERM OR MORE PER EJACULATE WERE ANALYZED UTILIZING THE 40 MINUTE STAINING TIME AS DESCRIBED IN THE TEXT.

Case No.	No. of Ejaculates Analyzed	Observer RWK (2) YF (%)	YFF (%)
15	1	44.5	3.3
16	1	41.3	2.8
17	1	42.3	5.0
18	1	40.0	4.5
19	1	41.5	3.8
20	1	46.3	3.8
21	1	42.5	2.0
22	1	40.3	2.8
23	1	40.3	1.8
24	1	37.3	4.0
25	1	41.3	4.3
26	1	36.3	2.0
27	1	40.5	5.0
28	1	40.0	5.3
29	1	43.3	5.3
30	1	39.0	3.5
31	1	43.0	4.0
32	1	46.0	4.0
33	1	39.5	4.5
34	1	44.8	4.0
Total Industrial Cases Examined	20	41.5 ± 2.65 (S.D.)	3.8 ± 1.07 (S.D.)

FIGURE 1

Schematic representation of mutagenesis as it applies to man.

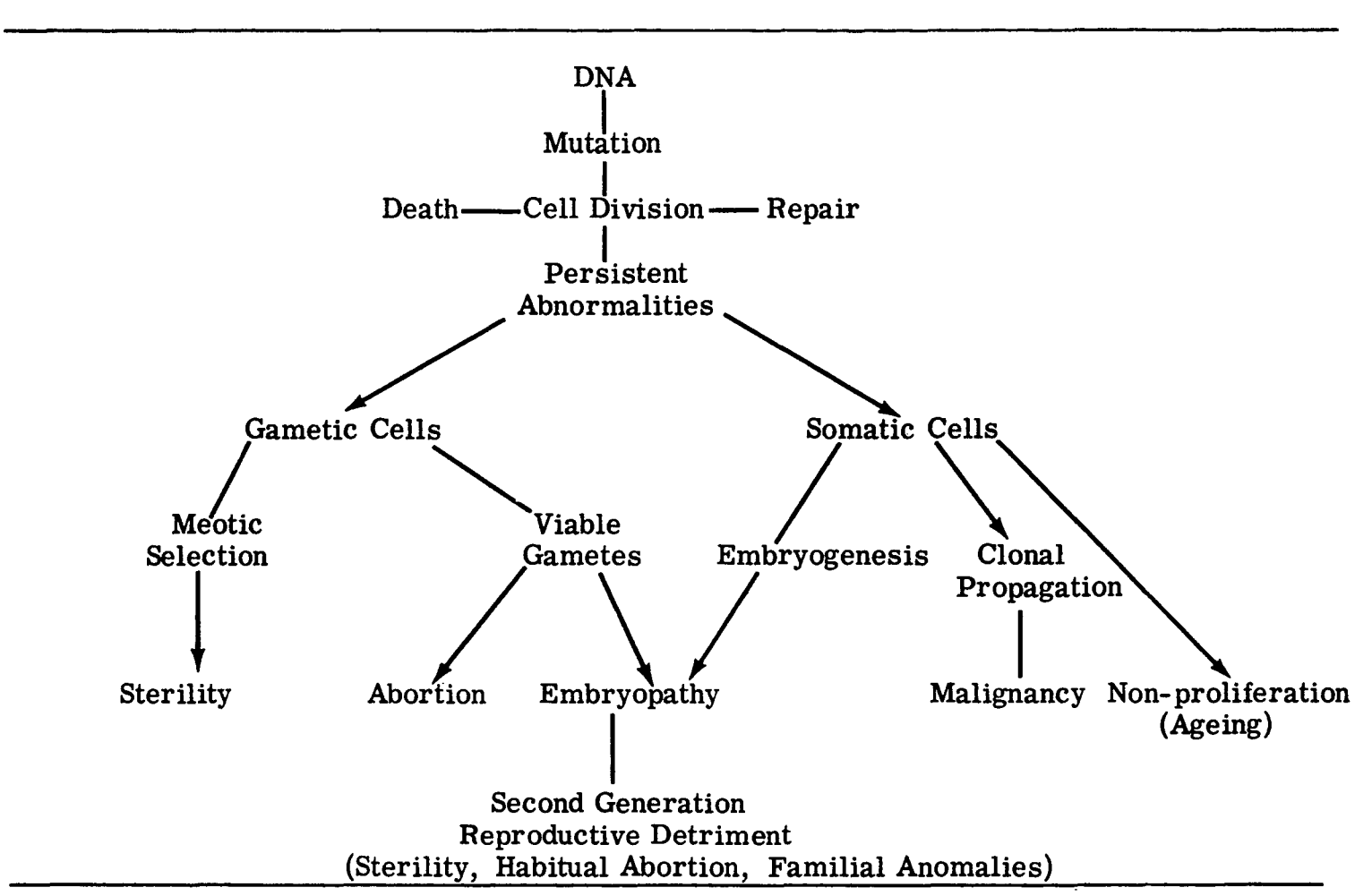


FIGURE 2

Basic types of gametic mutation.

TYPES OF GAMETIC MUTATION

1. Point Mutations

A. Base Pair Substitutions

B. Frameshift Type Mutations

2. Chromosomal Mutations

A. Structural

B. Numerical

FIGURE 3

Single fluorescent F body in human sperm. (YF)

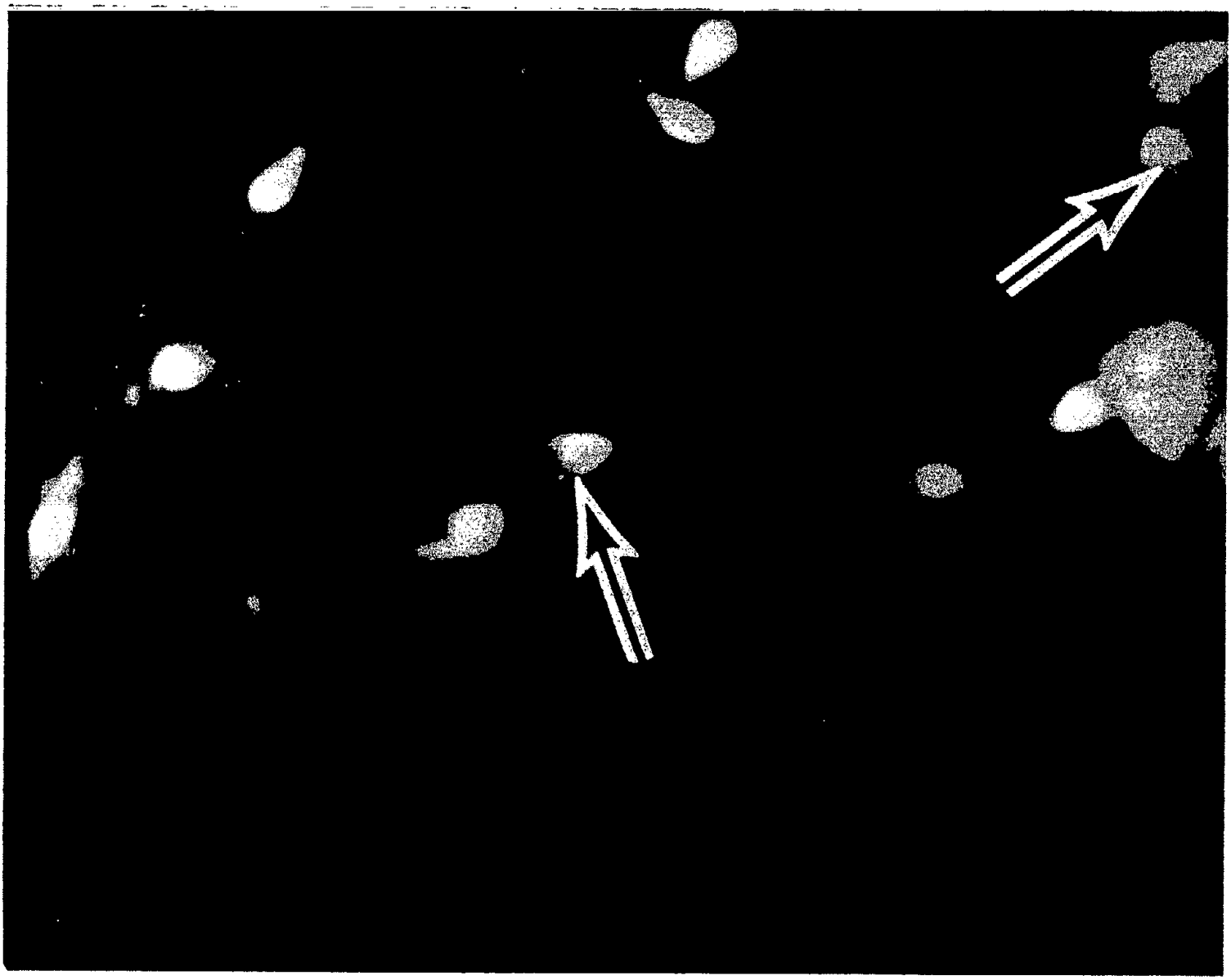


FIGURE 4

Two fluorescent F bodies in human sperm. (YFF)

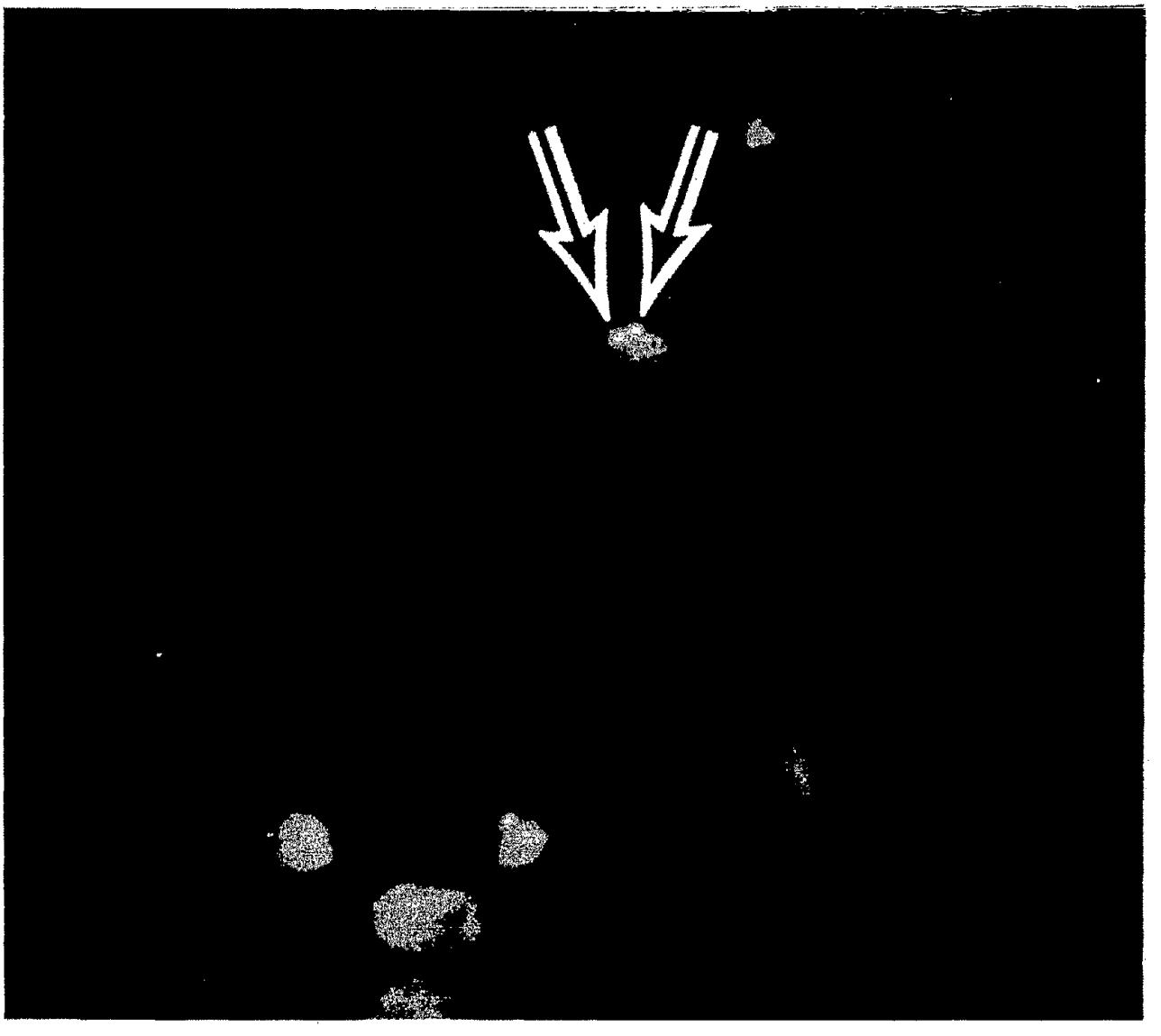


FIGURE 5

YFF scored by two observers in 38 sperm specimens from a donor over a 21-month period. (Case 1, see text for details)

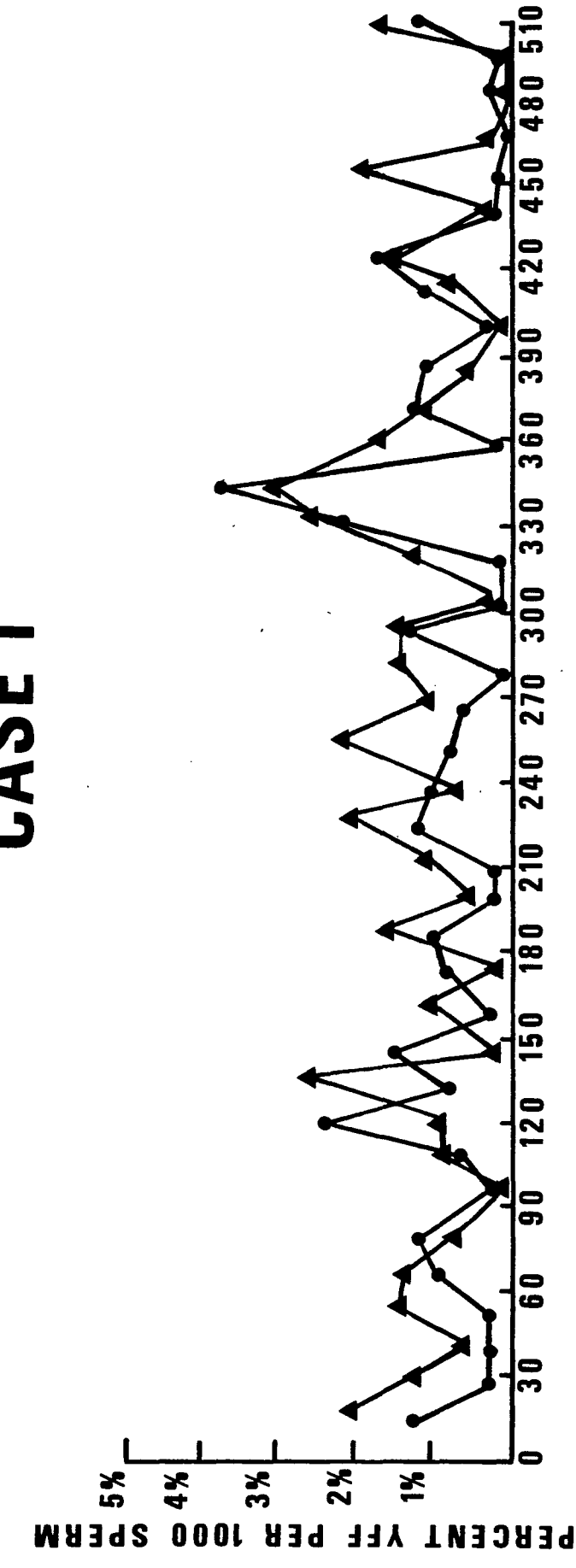


FIGURE 6

YFF scored by two observers in 9 sperm specimens over an 8-week period from a patient being treated for metastic osteogenic sarcoma. (Case 6, see text for details)

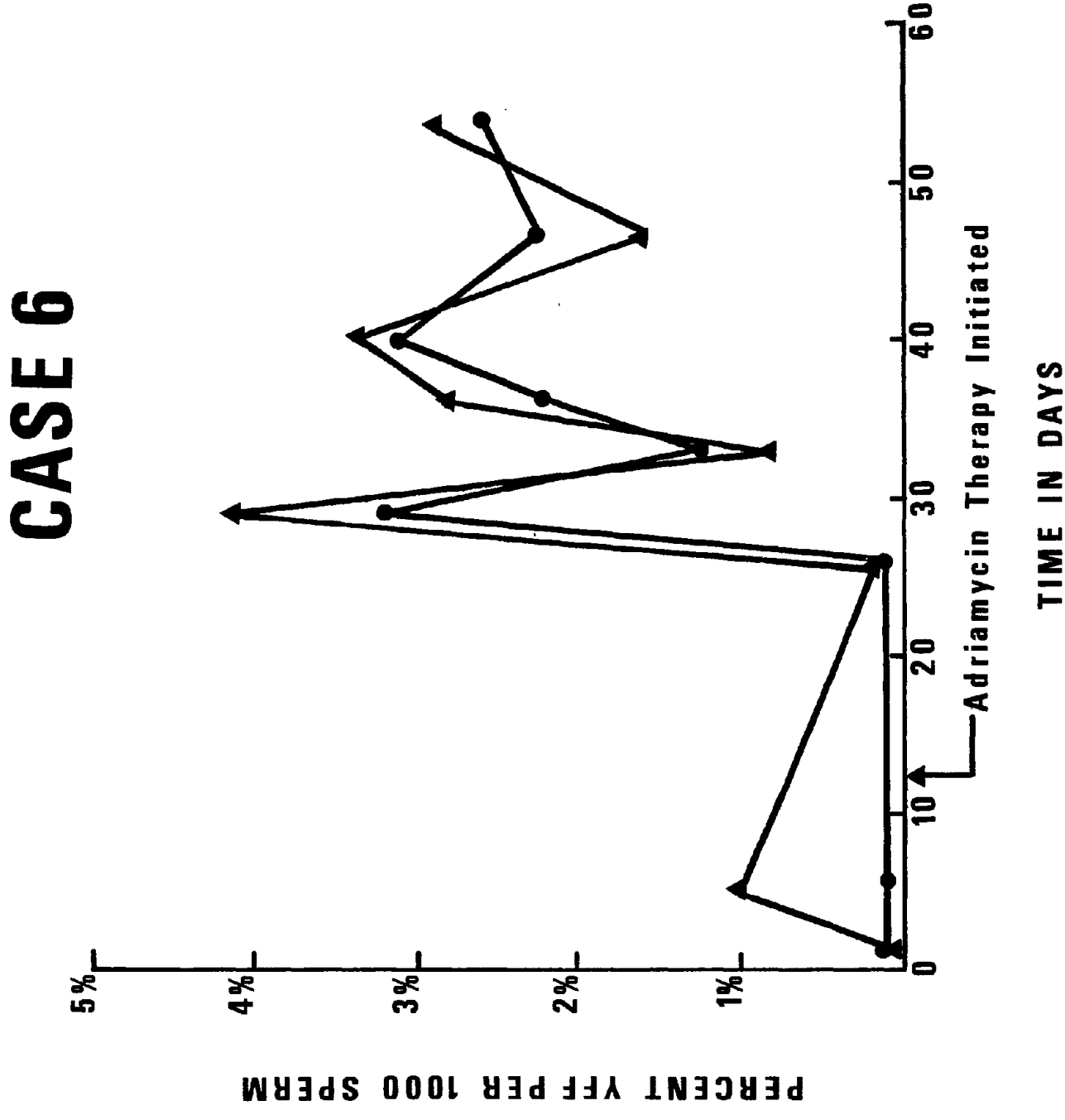


FIGURE 7

YFF scored by two observers in 8 sperm specimens over a 4-month period from physician who received occupational fluoroscopy. (Case 7, see text for details)

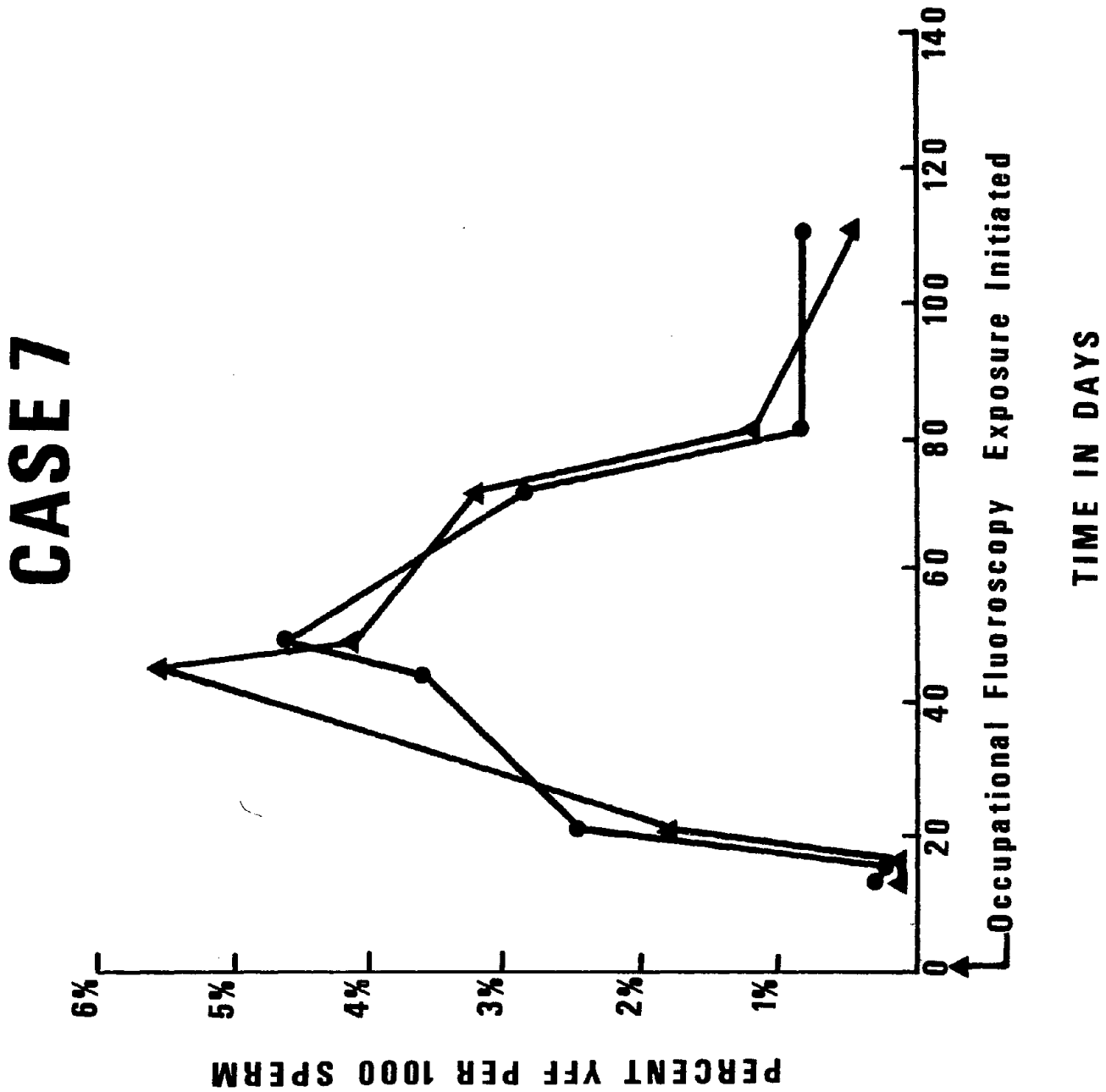


FIGURE 8

YFF scored by a single observer (MCB) in 8 sperm specimens over a 4-month period from a patient being treated for intestinal amebiasis. (Case 8, see text for details)

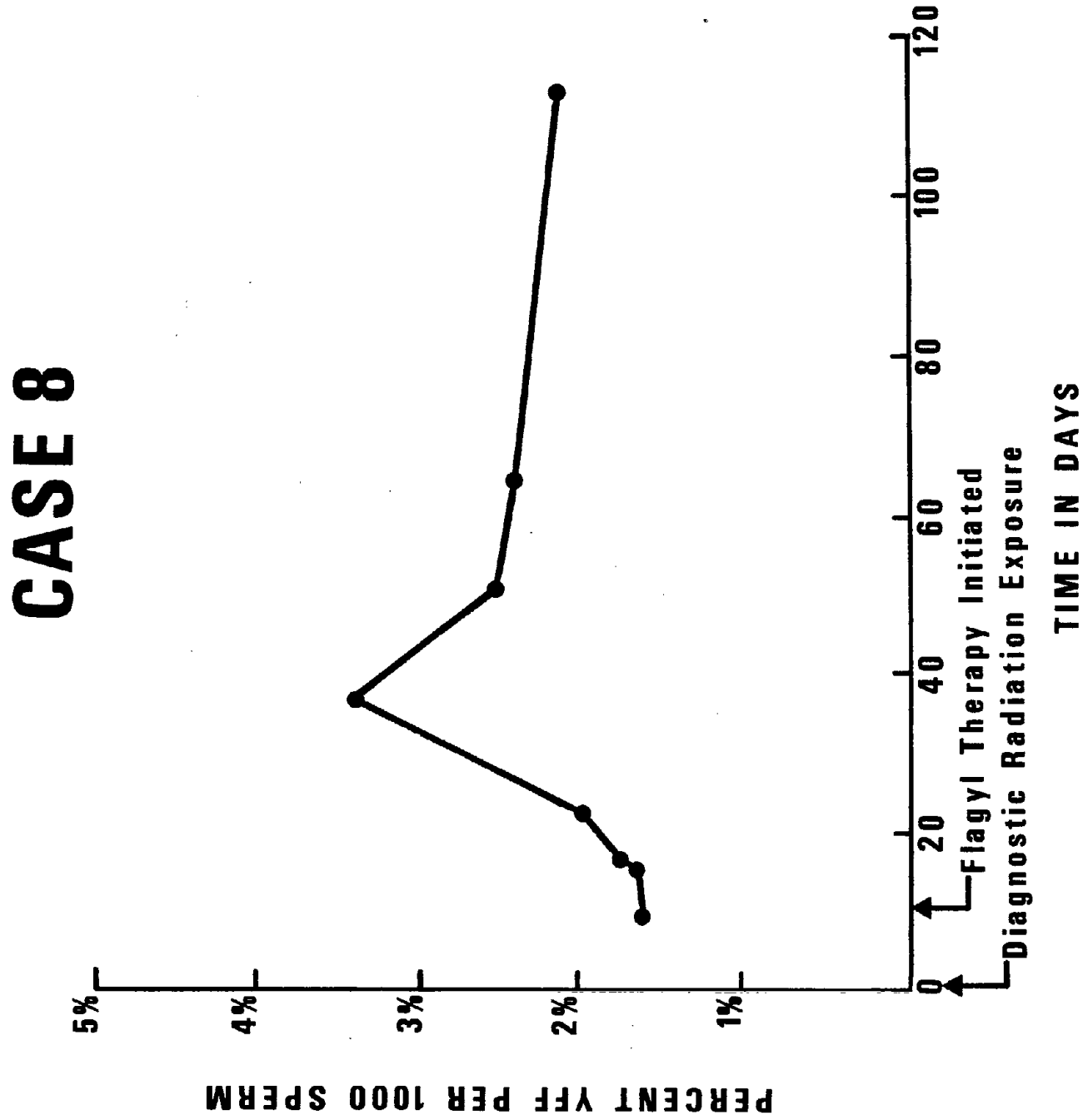
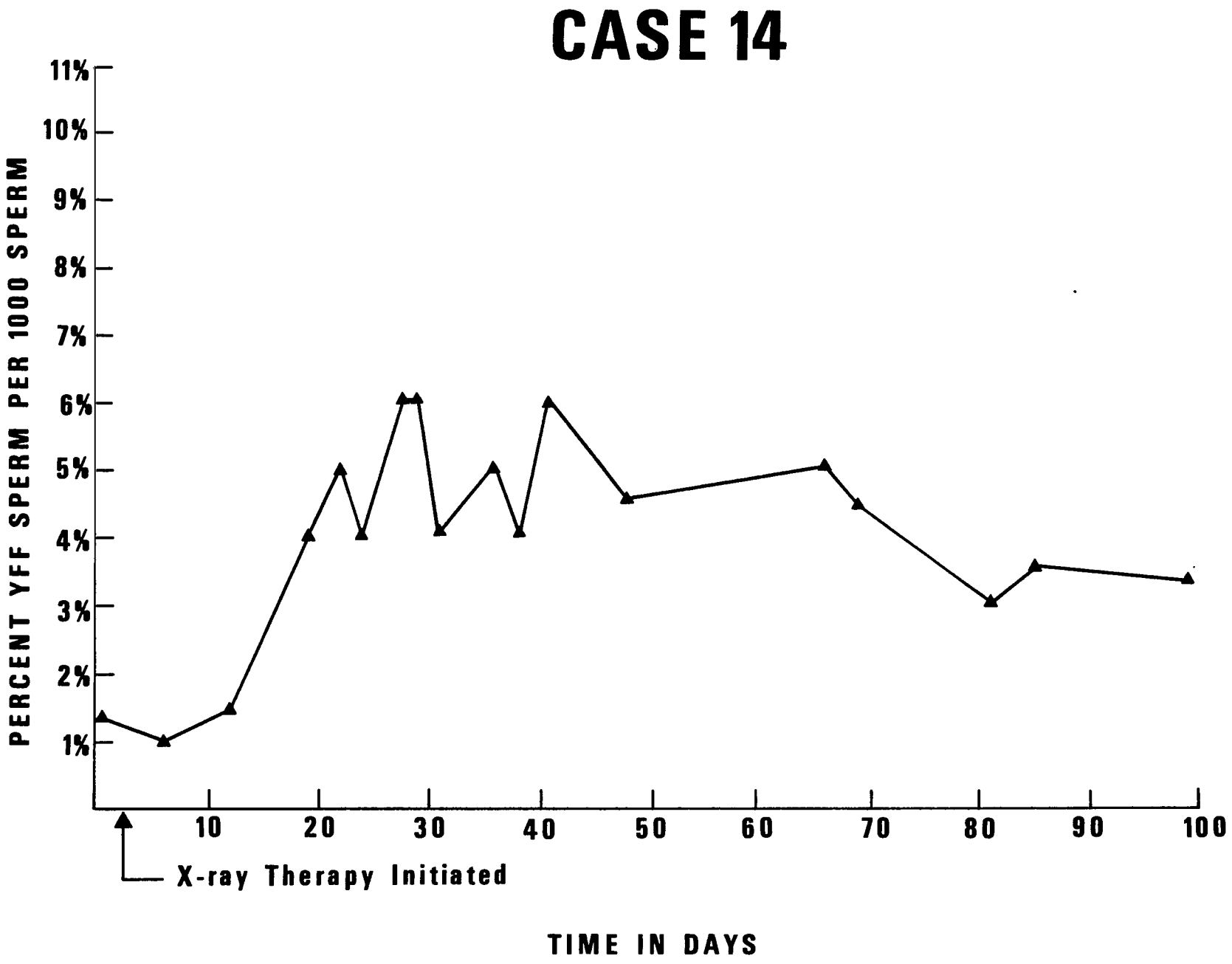


FIGURE 9

YFF scored by a single observer (RWK) in 18 sperm specimens over a 3½-month period from a patient being treated for seminoma. (Case 14, see text for details)



HUMAN SEMEN ASSAYS FOR WORKPLACE MONITORING

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INTRODUCTION

Decades of human semen studies have yielded compelling evidence that sperm can be used to assess reproductive potential and diagnose pathology. With these studies as background, the small number of detailed semen studies of men exposed to physical and chemical agents points with optimism to the application of human semen assays as efficient, effective means to monitor for reproductive hazards in the workplace. Sperm are the most accessible of human gonadal tissue, and provide a means of monitoring exposure-induced changes in the human testes, changes which may result in infertility and increased frequencies of genetically abnormal gametes. The focus on semen has precipitated the development of new semen bioassays which use older conventional andrological methods, as well as recently developed high speed flow and scanning methods for automated cytological analyses. Here we overview the status of these sperm assays for workplace surveillance, give suggested procedures and examples of use, as well as evaluate their effectiveness. We also briefly describe the available mouse models of induced semen changes and discuss the importance of these models for evaluating the genetic implications of findings in human semen.

CONVENTIONAL ASSAYS OF HUMAN SEMEN

Visual studies of semen and microscopic analyses of sperm have a long history in fertility diagnosis in many species, especially domestic animals and man.[1-5] Recently, much interest has focused on the demonstrations that exposure of men to physical or chemical agents can induce marked changes in sperm count,[6-8] sperm motility,[9] and sperm morphology.[10-15] These studies suggest that the analysis of human semen based upon conventional parameters (i.e., sperm count, motility, morphology) may be a very useful means of assessing human spermatogenic injury induced by physical and chemical agents.

Numerous studies have shown the utility of conventional sperm assays for assessing changes in the testes induced by external agents. These studies have been generally applied to human populations in one of two main ways: in cross sectional samplings where one or two semen samples are collected from many men in both control and exposed groups, and in longitudinal samplings, where repeated samples are collected from a small number of individuals before, during, and after exposure. The following are brief descriptions of these two applications of the conventional semen techniques as they might be used for workplace surveillance.

1. Cross Sectional Samplings

Since between-male variability in semen characteristics is high even among fertile and presumed healthy men, rather large numbers of subjects are required to establish differences between control and exposure groups. For example, in a study of 60 healthy and presumed fertile hospital and research staff (Wyrobek, Watchmaker, Cohen, and Brodsky, unpublished results), we observed a mean and standard deviation of 40 ± 12% abnormally shaped sperm. Therefore, to detect a 6% increase in percent abnormal sperm in similar men exposed to some noxious agent would require about 70 persons in control and in test groups, i.e., about 140 males per study (significance at a 0.05 level, with a 90% certainty, Dan Moore III, personal communication). The detection of smaller changes would require even more men per group. If the high frequency of vasectomies in some groups is also considered, as well as the unavailability of some men due to personal beliefs not allowing semen collection, numbers needed in planning a study are even higher. Cross sectional studies are therefore typically large, i.e., 170 in the study of smoking effects[12] and 200 in the study of lead effects.[15]

a. Procedure

Based on published examples of cross sectional semen samplings and our own experience in the implementation of these techniques, we suggest the following steps as a preliminary framework for planning a cross sectional semen study in the workplace.

1. Identifying populations of men at high risk of exposure to testes-active agents

Groups of men can be initially identified as "at risk" if they are potentially exposed to any agent, or analogue, with positive mutagenicity, carcinogenicity, teratogenicity, or testicular toxicity in any mammal. The size of the workforce potentially exposed to the agent as well as the likelihood of high exposures must, of course, also be considered. Identification of groups, however, may be even more direct, as shown in the study of men exposed to dibromochloropropane, a study which was initiated by worker complaints of unintentional childlessness.[8]

2. Recruiting donors

Numerous methods for recruiting volunteers are possible (e.g., an announcement at general meetings, the circulation of a form letter, etc.). We have found that irrespective of the method of introduction, there is no substitute for an individualized session between the project director and the prospective donor on matters of information, clarification, and motivation.

3. Assigning males to exposure groups

This may be done by comparing exposed males to males not exposed (e.g., plant workers versus front office personnel). Assignment into dose groups may also be possible in certain studies. Viczian[12] grouped males by number of cigarettes smoked per day for more than a year, and Lancranjan, et al. grouped men according to the level of lead in the peripheral blood and urine.[15]

4. Collecting semen samples

One complete semen sample per individual is required. Before collection day, each donor is given an instruction form covering the importance of obtaining a complete and fresh sample as well as the accepted methods of collection (i.e., glass bottle or Mylar sheath used with either coitus interruptus or masturbation).[16] All samples are coded on delivery to the laboratory, and all further analyses done as blind studies. Each male should also complete a coded questionnaire to determine his work history for the past year (place and type of work, as well as chemicals handled frequently), his medical history (including bacterial and viral infections especially in the past 3 to 6 months, history of varicocele, as well as medications and drugs taken during the past year), his personal habits (smoking, alcohol, as well as sauna and hot bath usages), and fertility data (number of children, their ages and general health).

5. Analyzing semen samples

Methods for conventional semen analyses, sperm count, motility, and morphology are generally simple and rapid, but require that fresh semen be analyzed and slides prepared within a few hours after collection.[4,16] We routinely determine sperm counts by hemocytometer, motility by visual observation under a light microscope, and prepare air-dried smears for morphology within 2 to 4 hours after semen collection. Air-dried sperm smears can be stored for an extended period of time (we have kept slides for 2 months), and then fixed, stained, and scored for sperm morphology at a later date, and in a different laboratory. We typically score 500 sperm per individual and classify each sperm into 1 of the 10 categories shown in FIGURE 1. The entire procedure of semen analyses usually averages to about 30 minutes per semen sample.

6. Statistical treatment of data

Data for control and exposed groups are tested for normalcy and for significances of group differences using standard statistical tests.[17] One can also compare the control and exposure groups by the frequencies of males that show less motility, lower sperm counts, or more abnormal sperm than some generally accepted, within laboratory standards for asthenospermia, hypospermia, and teratospermia. With this approach, one would plot against exposure levels the proportion of men in each group that falls outside the limits of these standards (see FIGURES 2 and 3).

b. Examples

Several examples of the use of cross sectional sampling of semen as a method of evaluating the effects of occupational exposure on the testes have been published. The two most complete studies are those of Lancranjan, et al.[15] on lead workers, and Popescu and Lancranjan on men with long-term exposure to ionizing radiation.[14] As illustrated in FIGURE 2, the percent of workers showing seminal defects (i.e., reduction in sperm motility, reduction in counts as well as increases in sperm abnormalities) is related to the mean levels of lead in the blood and urine. This study was undertaken because lead is known to cause testicular damage in animals, and large numbers of men are involved. One hundred men with mean occupational

exposures of 8.5 years in a battery storage plant were studied, as well as 50 men who worked in annex workrooms of the plant. An additional group of 50 men not working in the plant were used as unexposed subjects, i.e., controls. Men were grouped by the amount of lead detected in peripheral blood and urine, as well as other measures of lead toxicity. Semen samples were obtained from 89 volunteers (59%) of the workers, and from all 50 control volunteers. The proportion of men with semen defects was determined for each exposure group; they show dose-related effects (FIGURE 2). Changes in sperm shape abnormalities clearly showed the largest effects due to lead exposure.

Grouping men by dose is a major difficulty in any cross sectional study. In a study of 72 healthy volunteers with long-term low-level exposure to ionizing radiation (i.e., radiologists, X-ray technicians, industrial technicians working with gamma sources, and uranium miners), Popescu and Lancranjan approached this problem by grouping all exposed workers and comparing them to 42 controls.[14] The results, plotted in FIGURE 3, again illustrate the effectiveness of monitoring with seminal analyses. Effects of radiation were most noticeable as changes in motility and sperm abnormalities.

c. Effectiveness

A cross sectional study can be done rather quickly, and may yield valuable information on environments hazardous to the human testes. A major disadvantage is that it cannot be generally applied to all sizes of occupationally exposed groups. Large numbers of males per group are required unless expected effects are very large. Furthermore, a cross sectional study gives virtually no information about the cause of individual seminal effects, since observed defects may be due to causes other than the exposure in question, such as other adult or intrauterine exposures or inherited defects. For example, as shown in FIGURE 4, Viczian's data suggest that quantities of cigarettes smoked may affect sperm counts and sperm abnormalities in a semen study.[12] In appropriate situations, however, cross sectional semen studies may provide an invaluable approach for identifying and ranking environments that may pose hazards to the human testes.

2. Longitudinal Sampling

Although semen characteristics of healthy, presumed fertile men show marked variabilities, within person variabilities are known to be much smaller. This is especially true for semen cytology.[18] MacLeod, for example, studied one man for 12 years and found a strikingly consistent pattern of percent abnormal sperm.[11] In a 6-month longitudinal analysis of 119 men who were evaluated for fertility, Sherins et al. observed high between-male variabilities, and high within-male constancy of sperm parameters.[19] Their study also showed that, for each individual, seminal cytology was the most invariant seminal parameter.

MacLeod's studies show a constancy of the overall percent abnormal sperm within an individual, and a constancy of the types of abnormalities seen.[10] In a series of 15 semen samples collected from the same individual over a 7-month period, MacLeod found 67 ± 8% of the sperm with shape

abnormalities (mean ± standard deviation based on scoring 100 sperm per specimen) with two-thirds of the abnormalities consistently of the tapering types (see FIGURE 1). In another individual, he sampled 16 collections over a 2-month period, and found 53 ± 7% with four-fifths of these abnormalities consistently of the small type.

These findings provide a strong basis for longitudinal studies. In contrast to a cross sectional study in which very large groups of males were needed because of the high variabilities among the males of any control group, longitudinal studies require numerous semen samples from smaller numbers of control and exposed individuals. In principle, induced testicular effects are determined by comparing semen quality before, during, and after the exposure interval. In this way, each individual serves as his own control, and very few people are required. For example, MacLeod compared the effects of two dosage levels of the drug, cyproterone acetate, by studying only a single male per dose.[11] Since we expect workplace exposure to be generally less marked, we suggest that small groups of males per exposure dosage (perhaps 5 to 10) with an equal number of concurrent control (i.e., unexposed men) should be sufficient in initial studies.

a. Procedure

The following is a suggested outline of major considerations for designing a longitudinal semen study, and is based on published studies as well as our own experience.

1. Identifying suitable groups of men

In concept, groups of men should be identified before exposure to agents potentially active in the testes, so that preexposure semen samples can be obtained. Since this is not always possible, the longitudinal approach to semen sampling will be often limited in its correct application. However, it may be possible that semen samples could be taken as part of pre-employment physicals, or before assignment to a potentially hazardous work area. Recruiting volunteers is simplified by requiring fewer men, but is slightly confounded by the longer duration of the study and the necessity of repeated samplings. Men may be assigned to exposure groups, as was discussed for cross sectional studies.

2. Collecting and analyzing the semen samples

Each semen sample should be accompanied with a simple questionnaire to detect changes in occupational, medical, and relevant personal factors since collection of the last sample. Format of the questionnaire and the steps in the semen analyses are the same as in the cross sectional sampling techniques already discussed.

3. Data analyses

Semen from persons with major changes in medical and relevant personal factors should not be included in analyses. Statistically significant changes in motility, counts, and morphology in repeated semen samples should be monitored in each individual and compared among men both within and between exposure groups.

b. Examples

The published applications of longitudinal semen studies have been confined to persons exposed to experimental drugs.[10,11,13,20,21] Morse, et al. reported a detailed longitudinal study of the effects of 200 mg of cyproterone acetate per day on sperm concentration, seminal fluid volume, testicular cytology and levels of plasma, and urinary ICSH, FSH, and testosterone in six normal men.[13] This agent showed a striking effect on the germinal epithelium as seen by changes in testicular cytology as well as seminal sperm counts (FIGURE 4A) and seminal cytology (FIGURE 4B). As shown in FIGURE 4, for one of the six men, the effects of daily exposures on sperm counts and morphology were not seen until about 10 weeks after the beginning of exposure. Both parameters show marked changes that persist for about 25 weeks after the end of the 20-week exposure period. Sperm morphology, however, showed less variability before exposure and smoother rates of change than the rather erratic curve of sperm counts. MacLeod published the results of a longitudinal study of the effects of 25 weeks of daily exposures of cyproterone acetate on two men each receiving 100 and 200 mg per day, respectively (FIGURE 5).[11] When compared to the study of Morse, et al. the small differences seen in the times of onset of semen defects in the men receiving the 200 mg daily dose may be due to small differences in age, method of exposure, or medical histories of these men.[13]

MacLeod also reported a composite study of the effects of N'-bis(dichloroacetyl) 1,8-octane diamine, an experimental antispermatogenic compound, on sperm counts, motility, and morphology of three normal men.[10] As shown in FIGURE 6, effects on all semen parameters were seen by about 5 weeks after the beginning of daily treatments, and persisted for about 4 months after the end of exposure.

Although the overall patterns of induced defects in these examples are similar, differences are seen in the times of onset after the initiation of exposure, and the times required after the cessation of exposure before improvement is apparent. While these studies do not permit an accurate estimation of an optimal sampling frequency for workplace monitoring, inspection of FIGURES 4 to 6 suggests that sampling frequencies of greater than once every 2 months or so might have been insufficient to detect the transient semen defects induced by these agents. This would suggest that monitoring for induced transient effects similar to the ones seen in these studies would require sampling frequencies of approximately once every 6 to 8 weeks. The detection of semen defects in persons with chronic or very severe acute exposures, however, may permit longer intervals between samplings. These questions need further study.

c. Effectiveness

Although a longitudinal study requires more time than a cross sectional study, it is designed to yield useful data for both the epidemiologist and the volunteers in the study. For the epidemiologist an exposure environment may show similar defects in a number of men, clearly implicating the environment as harmful to the testes. Furthermore, unlike the cross sectional study, the repeated semen samples of the longitudinal study may also aid in quantitating the magnitude of the exposure for each

individual. MacLeod's study of cyproterone acetate (FIGURE 5), for example, shows that for two men, each receiving a different dose, the man exposed to the higher daily dose showed the more severe semen defects with earlier onsets.[11]

3. Genetic and Fertility Implications of Induced Semen Changes

Considerable animal and human data suggest that reduction in sperm count, reduction in sperm motility, and increase in sperm abnormalities correlate with reduced fertility.[1,4,16] Three lines of evidence from mice strongly suggest that the induction of abnormal sperm also may be linked to increased production of sperm with heritable genetic abnormalities.[22] First, there is considerable evidence that sperm shaping and the production of abnormal sperm is polygenetically controlled by autosomal as well as sex-linked genes, suggesting that any agent that affects sperm shaping is interfering with a system that has strict genetic controls.[23,24] Second, in studies with over 60 mutagens and nonmutagens, it was found that the mutagens generally induce sperm abnormalities while nonmutagens generally show no effect.[25,26] Third, in a compelling study with three agents known to induce sperm abnormalities in exposed males, it was shown that sperm abnormalities may be transmitted to the male offspring of the exposed mice.[22,27]

Some evidence also supports the link, in humans, between the induction of sperm shape abnormalities and the production of sperm with heritable genetic abnormalities. Numerous studies have suggested a relationship between pathological semen (including abnormal sperm shapes), and the likelihood of spontaneous abortions and stillbirths.[14,28] Although no human fertility and offspring data are yet available for the examples of occupational exposure shown in FIGURES 2 and 3, some murine data are available for lead. When mice were exposed to lead they showed semen defects, including abnormally shaped sperm. When mated to unexposed females, the males yielded an increased proportion of offspring with defects in sperm shaping.[22] This study suggests that mice exposed to lead produced sperm with abnormal shapes, as well as sperm with heritable genetic abnormalities, with the latter resulting in the observed defects in the offspring. A similar pattern may be applicable to human smokers. The increased sperm abnormalities found in smokers by Vizcian[12] may be associated with the correlation between paternal smoking and the likelihood of stillbirths and congenital abnormalities found by Mau and Netter in a study of 5,200 pregnancies.[29]

The link between induced changes in human sperm morphology and the production of genetically abnormal gametes is further strengthened by a correlation we have found between the induction of sperm carrying double Y bodies (see paper by Kapp, this volume) and the induction of sperm abnormalities (TABLE I). Kapp showed that exposures to clinical doses of Flagyl (metronidazole) induced elevated frequencies of sperm showing double Y bodies. Double Y bodies are thought to be due to meiotic nondisjunction of the Y-chromosomes. We evaluated several of the sperm samples of this individual for changes in sperm morphology, and found a similar time course of changes. Both our data and that of Kapp show a near doubling of spontaneous values at about 30 days after the beginning of Flagyl therapy.

Based on these murine and human studies, we believe that any agent, or environment, that induces defects in the human semen (i.e., changes in counts, motility, morphology, or double Y bodies) should be considered as an anti-fertility agent which may raise the probability of producing sperm with heritable genetic abnormalities.

MOUSE MODELS AND THE EVALUATION OF HUMAN SEMEN

A major advantage to initiating wide-scale semen studies in exposed humans at this time is the availability of a well-documented mouse model. This mouse model (both the Fo and F_1 sperm tests), and the human semen assays, may be integrated as follows:

1. Use of the Mouse Fo Sperm Test: A Screen for Agents That May be Hazardous to the Human Testes

The Fo sperm test is a very easy and rapid assay.[25] It requires about 20 mice per agent (with the LD_{50} known), and only a few hours of work to generate a 5-point dose response curve. Typically five groups of four mice each receive five daily intraperitoneal injections at graded doses of the test material diluted in dimethylsulfoxide or water (vehicle). We have used doses of 1/2 LD_{50}, 1/6 LD_{50}, 1/18 LD_{50}, and 1/54 LD_{50}, as well as unexposed animals as controls. Thirty-five days after the first injection, each mouse is sacrificed, both epididymal sperm counts and percent abnormal sperm are determined, and the results plotted as dose response curves.

Large numbers of agents to which the human testes may be exposed in the workplace may be readily tested with this assay. Positive results may be used to focus attention on these workplace environments that are likely to be most hazardous for the human testes.

2. Use of the Mouse F_1 Sperm Test: A Means of Evaluating the Fertility and Genetic Implications of Findings on the Human Assays

Findings with human semen assays may show certain workplace environments to be hazardous to the testes. Factors such as (a) the size of the potentially exposed worker group, and (b) the cost of reducing human exposures may necessitate more accurate evaluation of the potential for infertility and production of genetically defective gametes. The mouse F_1 sperm studies, although less rapid, and more costly, than the mouse Fo sperm test, may provide an effective approach to this problem.[22] For example, mice can be exposed to "human-like" situations of exposure (e.g., inhalations, 8 hours per day) at various doses, including those much higher than those found in the workplace, and mated to unexposed females during the presterile period. Approximately 200 F_1 offspring at each dosage level should be analyzed for semen defects, especially for changes in sperm morphology. Agents would be scored as positive if they show increased proportions of offspring with semen defects. Comparisons of Fo and F_1 dose response curves from the mouse with the human results might then be used to better estimate the likelihood of human infertility and defective offspring.

DEVELOPMENT OF FLOW CYTOMETRIC ASSAYS OF HUMAN SPERM

Numerous advances in flow cytometry of sperm strongly suggest that these methods may soon become effective tools in the analyses of induced changes in the DNA content and shape of human sperm. Exposure of the testes to mutagens results in the production of sperm with abnormal morphology, and may increase the variability of individual sperm DNA content as a result of blastogenesis or meiotic nondisjunction. Sperm are normally haploid and noncycling, and therefore should be uniform in DNA content except for differences in the sex chromosomes, or for differences due to nondisjunction or blastogenesis. Use of flow systems to measure DNA content offers the major advantages of measuring hundreds of sperm per second with unique statistical precision and sensitivity.[30]

1. Development in DNA Measurements by Flow Cytometry

a. Early studies with mouse sperm

Sperm DNA content and dry mass have been extensively studied by X-ray methods, by quantitative electron microscopy, and by absorption-scanning cytophotometry.[31] The general conclusion is that the DNA content or total dry mass of single sperm are distributed symmetrically with rather large coefficients of variation (CV)* of about 10% although some studies[32] on bull sperm measurements by ultraviolet absorption techniques and guinea pig sperm, measured by Feulgen cytophotometry, report CVs for the measurements of between 3% and 6%.[33] These methods are extremely tedious, however, because they require measurements by scanning of individual sperm on slides. When flow cytometry is used to measure the DNA content of sperm after staining by the fluorescent acriflavine Feulgen procedure,[34] a very asymmetric distribution consisting of a narrow peak with a prominent shoulder to the right of the main peak is found.[35] This particular shape of distribution is seen with sperm from 13 mammalian species. The high refractive index and flat shape of the normal sperm head produce this effect by causing the fluorescence measurements to vary with orientation of the sperm head in the measuring chamber. Thus in the configuration of the illustration and detection optics shown in FIGURE 8 both DNA content and sperm shape appear to influence the measurements.

b. Detecting chromosomally abnormal mouse sperm

Variability in DNA content of sperm from F_1 hybrids between the laboratory mouse (Mus musculus) and the tobacco mouse (M. poschiavinus) has been determined in our laboratory.[36] These F_1 hybrid mice are known to be heterozygous for seven metacentric Robertsonian chromosomes which show

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*Assuming a normal distribution, the CV, expressed as a percent, is equal to

$$\frac{\text{standard deviation} \times 100}{\text{mean}}$$

irregular segregation at the first mitotic division leading to increased production of sperm with abnormal DNA content. This has been confirmed by microspectrophotometric analyses of Feulgen stained sperm[37] and ultraviolet absorption of unstained sperm.[38]

For flow cytometric analysis, populations of nuclei from late pachytene spermatocytes, round spermatids, epididymal sperm, and spleen cells were obtained by centrifugal elutriation, and acriflavine-Feulgen stained.[39] Fluorescence intensity distributions were obtained and analyzed to determine mean fluorescence intensities and CVs. The mean intensities of corresponding cell types from M. musculus and from the F_1 hybrids were the same, indicating identical average DNA content. The average CV of fluorescence peaks from nuclei from the pachytene spermatocyte, spermatid, sperm, and spleen cells from the laboratory mouse were about 5% as were the CVs for the fluorescence peaks from spleen cells and pachytene nuclei from F_1 hybrids. However, in the post-meiotic nuclei from spermatids and sperm in the F_1 hybrids, abnormal meiotic segregation resulted in an increased variability of 6% in the amount of DNA in the sperm, and these differences were easily detected with flow systems.

c. Early studies on human sperm

Sarkar, et al. used flow cytophotometry to demonstrate that the sperm of four out of five human donors segregating balanced translocations showed an increased variability in DNA content.[40] In samples from the subjects heterozygous for the translocations, the mean DNA value fluctuated rather unpredictably over a wide range, and the standard error of the mean was about 10 times higher than that for 15 randomly chosen donors without detected chromosomal disorder. These measurements were complicated by the use of a flow cytometer with orthogonal geometry similar to that shown in FIGURE 8. Broad, asymmetric peaks with CVs ranging from 9% to 20% were obtained from sperm from normal donors. Similar findings were made in our laboratory (FIGURE 9A) until a flow chamber of novel design was put into use, and instrumental artifacts were reduced.

d. Improving resolution: technical and biological factors

Until recently, we routinely showed large CVs when measuring sperm from human donors (FIGURE 9A). Dean, et al.,[41] in our laboratory, have developed methods to hydrodynamically orient mammalian sperm in the sample core, and thereby considerably reduce the CV. A wedge-shaped tube positioned inside the standard simple-tube causes the formation of a thin, ribbon-shaped sample core, and a corresponding orientation of flat sperm heads in the plane defined by the flow axis and the two tips of the wedge-shaped tube. With hydrodynamic orientation of sperm we now routinely obtain measurements with CVs of about 4% for human sperm (FIGURE 9B).

In a 1977 publication we proposed to continue to improve the resolution to the point where we could measure differences between X- and Y-chromosome bearing sperm.[42] Based on chromosome length measurements, the differences in DNA content between X-bearing spermatids of the mouse which carry 19 autosomes plus the X chromosome and the Y-bearing spermatids which carry the autosomes plus the Y chromosome, is expected to be 3.4%. We

calculated that a CV of less than 1.5% is necessary to resolve two such populations of sperm in a semen sample from a mouse, and that such a small CV should permit detection of variability in sperm DNA content caused by blastogenic and nondisjunction events.

Recent advances in fluorometry have been achieved for mouse testis cells by pepsin treatment of the cells, staining with ethidium bromide and mithramycin, and incubation with RNase.[43] Meistrich, et al.,[44] combined this advance in cytochemical technology with centrifugal elutriation,[39] and a new generation, high-precision flow cytophotometer to resolve two classes of round spermatids in mice, differing in mean DNA content by 3.5%.[45] This difference is consistent with the difference expected between the DNA content of X- and Y-chromosome bearing spermatids. The area under the peak with lower DNA content represented 52% of the haploid cells, the other peak represented approximately 48%. In samples sent to us by Meistrich we have confirmed these results in an epi-illumination flow cytometer. Despite improved resolution, two classes of mature sperm are not yet seen by us, or by Meistrich.

Distinguishing X- and Y-sperm on the bases of differing DNA content will require further improvements in instruments, methods of cell preparation, and procedures for quantitative staining. Identification of human Y-sperm with selected fluorochromes is already possible (as described elsewhere in this volume by Kapp), and perhaps fluorescent markers, such as those used with the YFF sperm test (double Y body), can also be quantitated with flow cytometry.

e. Detecting effects of external agents

Data is just beginning to appear in the literature to show the usefulness of flow cytometry as a method to monitor radiation and drug effects on spermatogenesis of mammals. Hacker, et al. treated mice with adriamycin or irradiated mice with doses ranging from 10 to 1,500 rads of gamma radiation.[46] The animals were killed, along with the controls, at post-treatment intervals varying from 2 to 35 days. Single-cell suspensions of the spermatogenic epithelium were prepared, and the cellular DNA was stained with either ethidium bromide and mithramycin or DAPI. The DNA content of individual cells was measured with an epi-illumination flow cytometer.[45] An initial reduction in the relative number of S-phase and 4C cells was followed by an increase to above normal numbers. A decline in numbers of 2C cells suggested a high rate of cell killing for diploid spermatogonia. Spermatids with abnormal DNA contents were also identified.

2. Two Parameter Flow Cytometry of Sperm: Use in the Detection of Morphologically Abnormal Sperm

Two parameter flow cytometry offers automated detection of abnormally shaped sperm.[47] To implement a screening procedure, particularly one overviewing human occupational exposure, automated methods must be developed for the recognition, classification, and counting of abnormally shaped sperm. We have applied two parameter flow cytometry of acriflavine-Feulgen fluorescence and 90° light scatter. The histograms generated are displayed

and analyzed with the interactive computer program SWELL in which replicate two parameter data are pooled, and a mean and a standard deviation are computed for each histogram element.[48] Two parameter data from a test population are compared to the pooled data by calculating the probability that the value for each element of the test histogram is different from the corresponding element of the pool. We have compared pooled flow cytometric measurements of epididymal sperm from 13 SWR/J mice (4% abnormal sperm heads) to those from 8 BALB/c mice (65% abnormal sperm heads). There were significantly ($p < 0.01$) more sperm with high amounts of scattered light and intermediate amounts of fluorescence from each of the BALB/c mice than for the SWR/J pool. We believe this is due to the abnormally shaped sperm. It is consistent with our findings that in flow cytometers, where light collection is orthogonal to laser excitation and to sperm flow, both fluorescence and scatter are sensitive to sperm morphology.[35] Using this approach, we may be able to isolate enriched portions of abnormally shaped sperm from semen samples. If this is true, we will be able, subsequently, to analyze abnormally shaped sperm for biochemical differences.[49] We should also be able to develop automated methods for counting abnormally shaped sperm, and thereby circumvent the onerous task of individual scoring through the microscope.

DEVELOPMENT OF AUTOMATED SLIDE BASED SCANNING SYSTEMS FOR HUMAN SPERM

There is much controversy as to what constitutes normal human semen, especially with regard to sperm morphology. Early attempts to quantitate sperm head morphology of mammals were based on measuring projected dimensions.[50-52] Recent studies with the mouse have shown that sperm morphology of individual sperm can be readily quantitated with a discriminant score made up of selected dimensions.[53] In a similar way, Burgoyne, et al. were able to assign single mouse sperm to the strain of origin with less than a 6% error rate.[54] None of these studies, however, tested the quantitative measurements in distinguishing abnormally shaped sperm, nor have the methods been applied to human sperm.

Irradiation and chemical exposures of the testes increase the frequency of abnormal shape of the sperm head, and induce changes in sperm head size--but the latter are much harder to detect.[22] It seems that the human eye is more effective in detecting subtle shape changes than size changes. In a study with rabbit sperm, which are more "human-like" in morphology than mouse sperm, decreases in size (two dimensional views) were measured at exposure doses of methycholanthrene well below those causing changes in numbers of abnormally shaped sperm (Wyrobek and Gledhill, unpublished results).

Slide based scanning systems are being developed to monitor changes in human sperm morphology (18, David, personal communication). Such systems would be especially effective in making morphology assessments more objective, and in identifying subtle changes that may be induced in an individual receiving low-level chronic exposures.

SUMMARY AND PROJECTIONS

Decades of experience have resulted in the development of rapid and effective semen assays that now can be applied to monitoring the effects of environmental agents on the human testes. The effectiveness of conventional andrological methods (i.e., sperm counts, motility, and morphology) has been well demonstrated in numerous studies of occupational and drug exposures. Recently, Kapp (this volume) has demonstrated the effectiveness of the YFF tests as an assay for sperm with double Y-chromosomes. The methodologies of both the conventional and YFF semen assays have been adapted so that their application can be routine. Available mouse and human data suggest that these assays are effective in identifying agents or exposures that may cause human male infertility, and lead to the increased production of genetically abnormal sperm.

Numerous promising human semen assays are still in the development stages. These include (a) the use of the flow cytometer for the rapid and quantitative cytological analyses of sperm DNA and shape changes, and (b) systems for the automated scanning of sperm smears and the objective, computerized quantitation of sperm morphology.

The effective application and interpretation of human semen assays, however, depend on an understanding of the relationship between the induced semen defects, and associated fertility and genetic implications. These studies are best done in the mouse. For example, the mouse Fo sperm test may be used to screen hundreds of agents commonly found in the workplace, to identify potentially hazardous environments, and to aid in setting priorities for choosing those workplace environments in which human semen should be sampled. The mouse F_1 sperm tests may then be used to evaluate and quantitate the likelihood of associated infertility, and the production of sperm with heritable genetic defects. However, much work still needs to be done with this mouse model, especially in quantitating the dose relationships between effects seen in the Fo test and effects seen in F_1 tests.

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TABLE I

A COMPARISON OF THE INDUCTION OF ABNORMAL SPERM SHAPES AND SPERM WITH DOUBLE Y BODIES (YFF TEST) IN A HUMAN RECEIVING DIAGNOSTIC RADIATION AND THEN FLAGYL (METRONIDAZOLE) THERAPY FOR AMOEBIC DYSENTERY

Time (Days)	Treatment	% Double Y Bodies[a]	Ratio[b]	% Abnormal Sperm[a]	Ratio[b]
0	Diagnostic Radiation	—[c]	—	—	—
10	Begin Flagyl	1.6	1.0	22.3	1.0
17		1.6	1.0	23.6	1.1
18		1.7	1.1	—	—
20	End Flagyl	—	—	—	—
24		2.0	1.3	—	—
38		3.3	2.1	40.3	1.8
52		2.5	1.6	39.0	1.7
66		2.4	1.5	—	—
115		2.0	1.3	—	—

a. Based on 1000 sperm scored for semen collected on specific days. Double Y-bodies scored by Kapp (see paper in this volume) and abnormal sperm scored by us.

b. Ratio of percent for individual semen samples divided by the best estimate of the pre-exposure values, i.e., the day 10 results.

c. Dash refers to unavailability of slides.

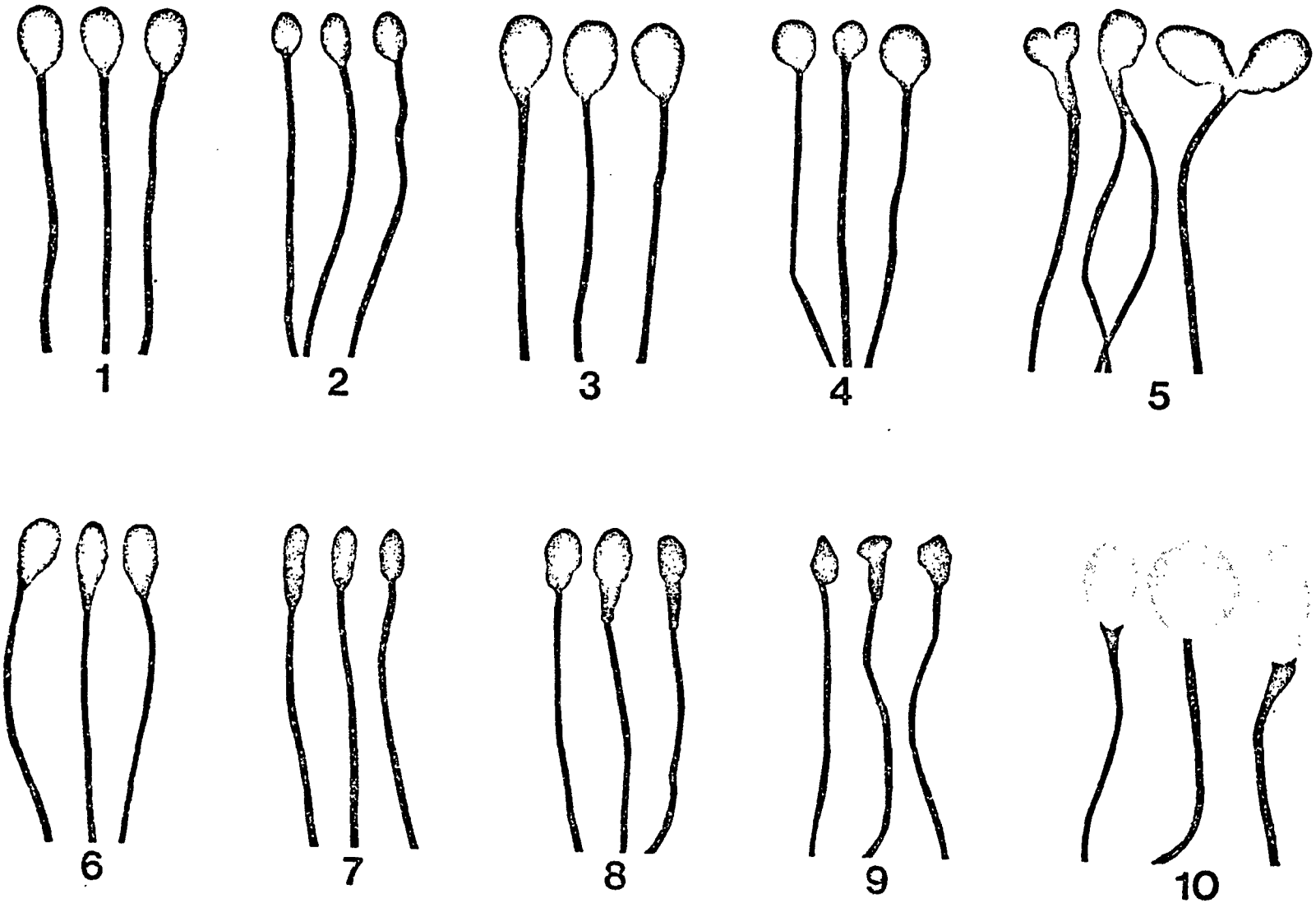


FIGURE 1

Shape variations in human sperm

The head shapes of the sperm in "1" are oval and considered by us as being normal whereas those in "2" to "10" are scored as being abnormal. The sperm in "2" are small, "3" are large, "4" are rounded, "5" are doubles, "6" are narrow at the base of the head, "7" are narrow, "8" are pear-shaped, "9" are amorphous, and "10" are ghost-like.

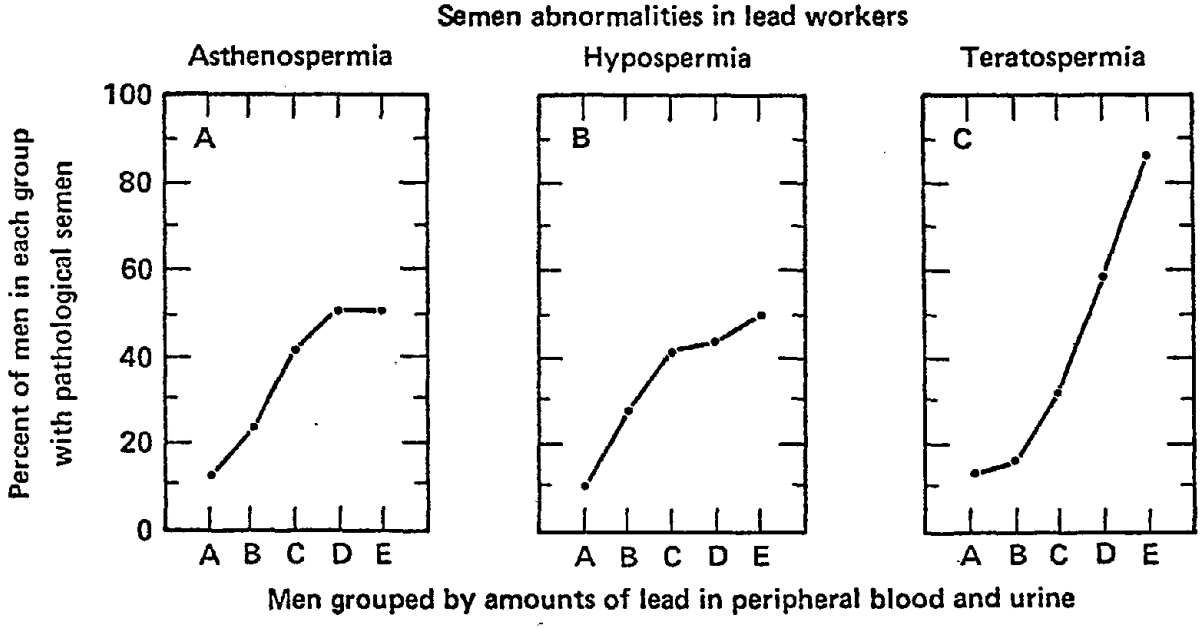


FIGURE 2

Semen abnormalities in lead workers

Two hundred men were divided into five groups of exposure to lead (A to E). Group A is the 50 men who were not exposed to occupationally toxic materials, and who served as controls for the study of the 150 men who worked in a lead battery storage plant (Groups B to E). The 50 men in B were technicians and office workers of the plant. Groups C, D, and E represent 35, 42, and 23 men, respectively, who worked in the plant for an average of 8.5 years. The average blood (µg/100 ml) and urine (µg/liter) levels of lead in each group were 23 and 92 for Group A; 41 and 101 for C; 52 and 251 for D; and 74 and 385 for E. Each data point in the figure represents the percent of men in each group that showed asthenospermia (Panel A), hypospermia (Panel B), and teratospermia (Panel C). Graphs are drawn from the data of Lancranjan, et al.[15]

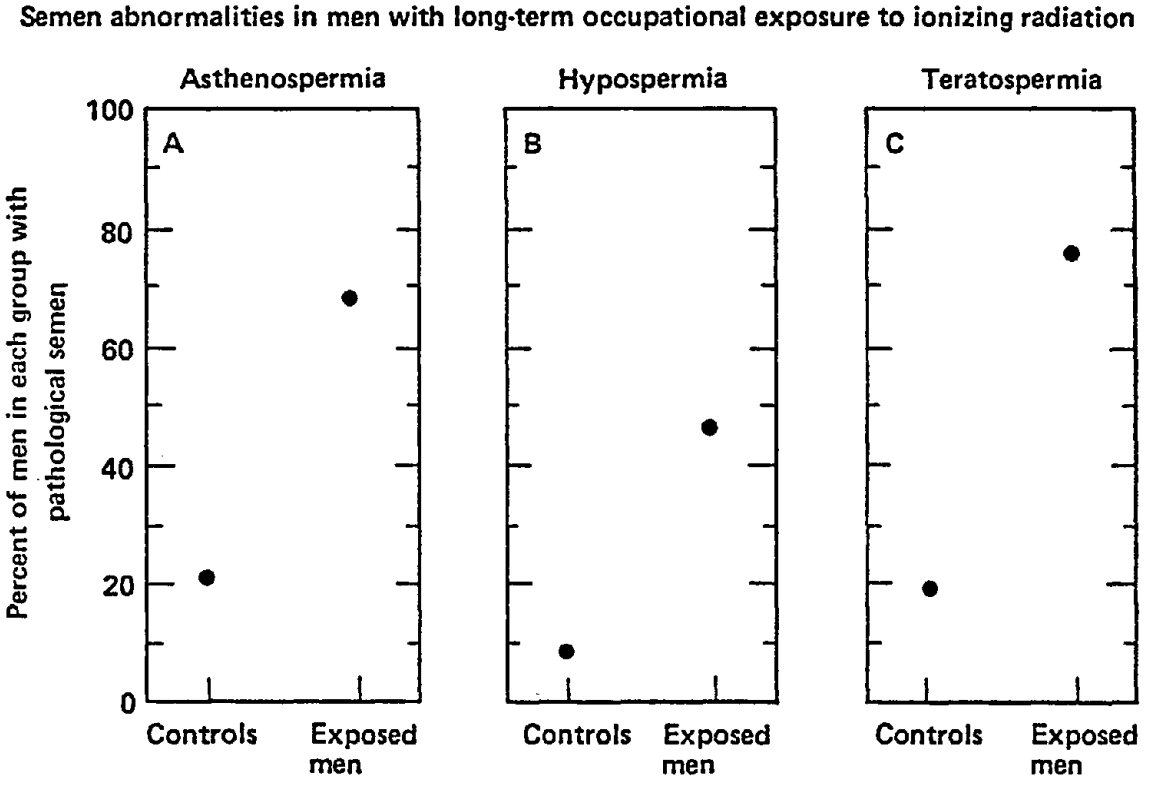


FIGURE 3

Semen abnormalities in radiation workers

The effects of low-level exposure to ionizing radiation on semen abnormalities were studied in 72 healthy volunteers, each with more than 2 years of occupational exposure to radiation (medical radiology, industrial technology, uranium mining). The data points for the "exposed men" represent the percent of men with asthenospermia (Panel A), hypospermia (Panel B) and teratospermia (Panel C). The percent of men with semen abnormalities was also determined for 42 matched molybdenum miners who served as "controls" for this study. Graphs are drawn from the data of Popescu and Lancranjan.[14]

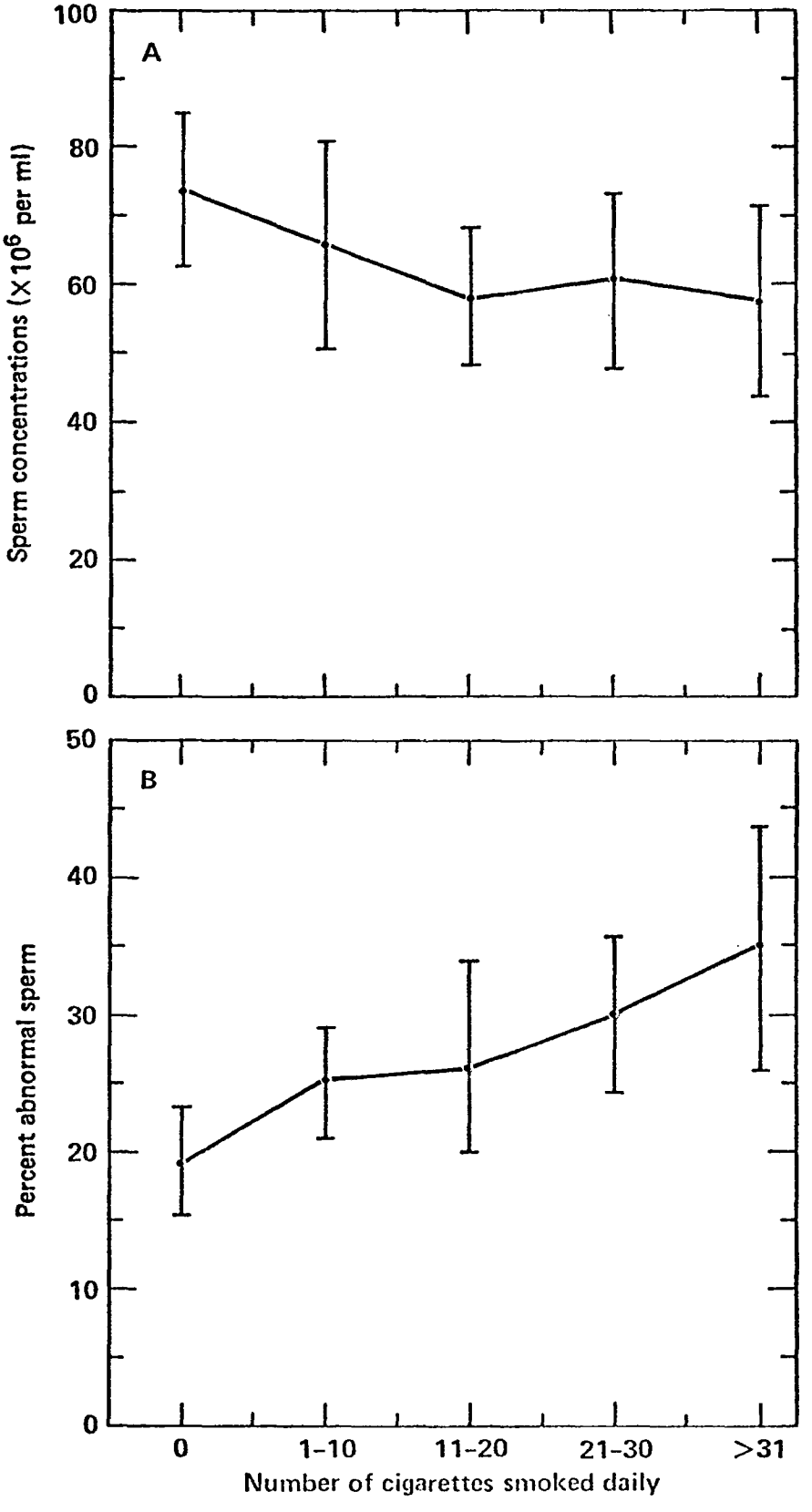


FIGURE 4

Semen abnormalities in smokers

The effects of cigarette smoking on semen abnormalities were studied in 50 nonsmokers and 120 men who smoked either 1 to 10 cigarettes per day for more than a year (22 men), 11 to 20 daily (43 men), 21 to 30 daily (48 men), and more than 31 cigarettes daily (7 men). The points and bars represent the mean value and standard error of the mean for sperm concentration and sperm abnormalities in each of the five exposure groups. Males who smoke more than 21 cigarettes per day show significant increases in sperm abnormalities when compared to the control group. Graphs are drawn from the data of Viczian.[12]

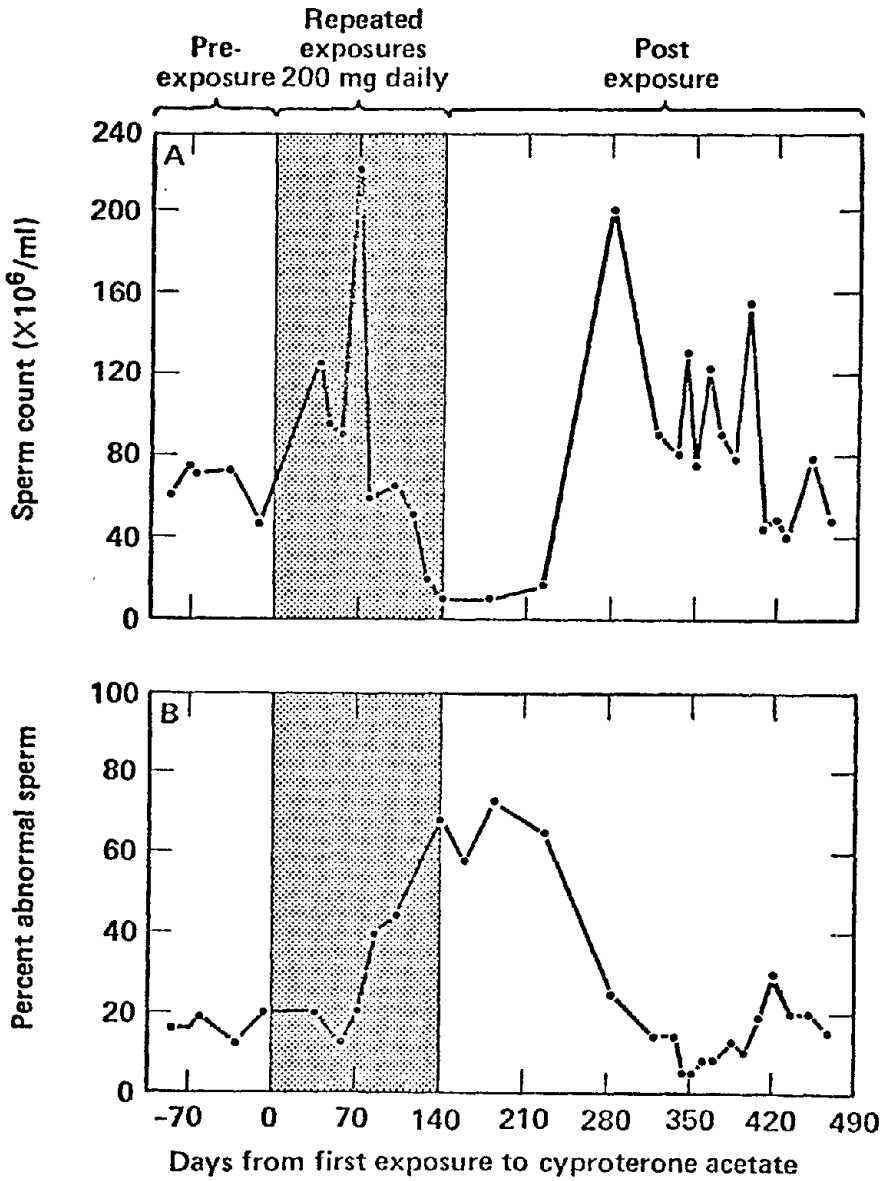


FIGURE 5

Semen abnormalities in a man exposed to cyproterone acetate

Each data point represents the sperm count (Panel A) or sperm abnormalities in individual semen samples collected from a single male receiving, for 20 weeks, 200 mg cyproterone acetate daily, plotted against days before and after the first day of exposure. Each panel is divided into 3 regions: those semen samples collected pre-exposure, those collected during exposure, and those samples collected post-exposure. Graphs are drawn from the data of Morse, et al.[13]

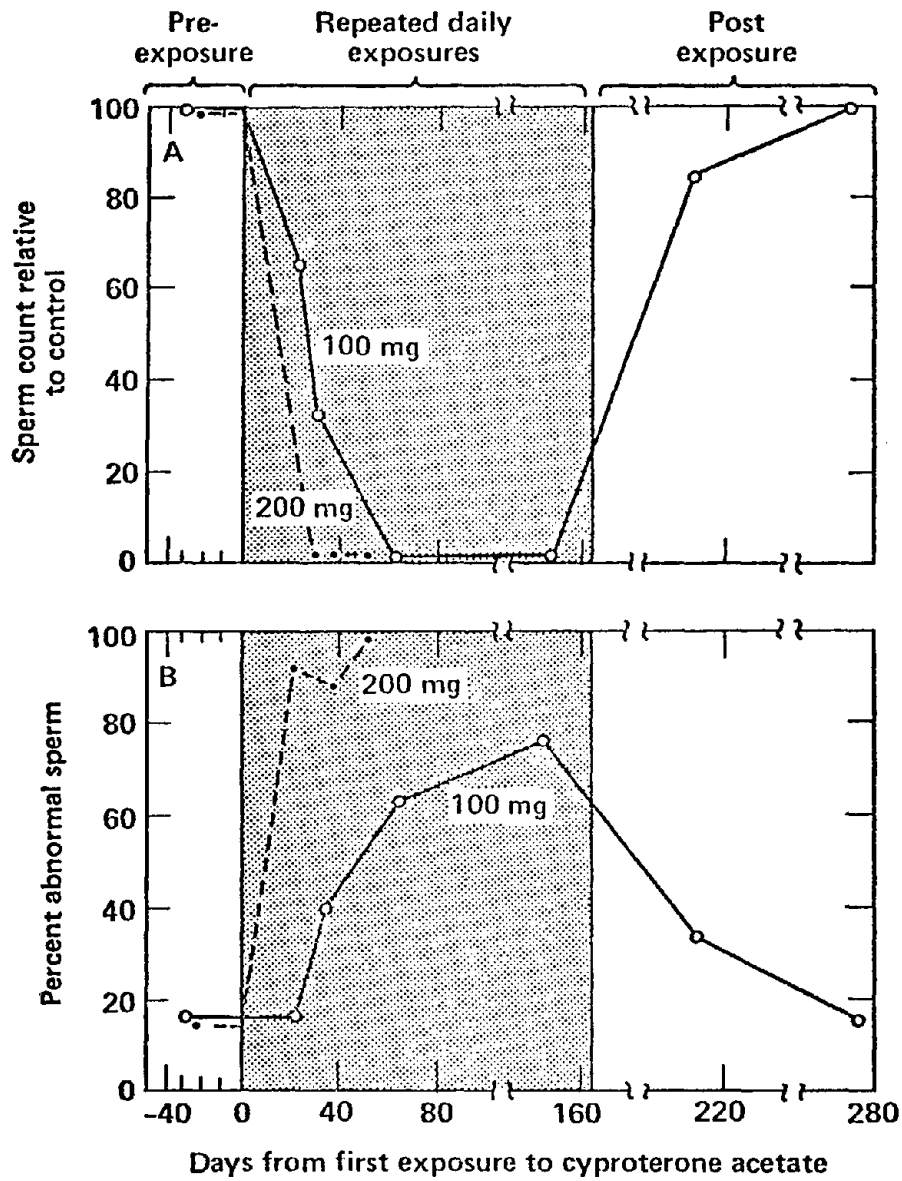


FIGURE 6

Semen abnormalities in two men receiving different doses of cyproterone acetate

Each point represents either sperm counts (Panel A) or percent abnormal sperm (Panel B) for one of two men receiving either 100 mg (open circles) or 200 mg daily (closed circles) of cyproterone acetate. Data for semen samples are divided into 3 regions in relation to the approximate 25-week exposure period, i.e., pre-exposure, during exposure, and post-exposure. Graphs are drawn from the data of MacLeod.[10]

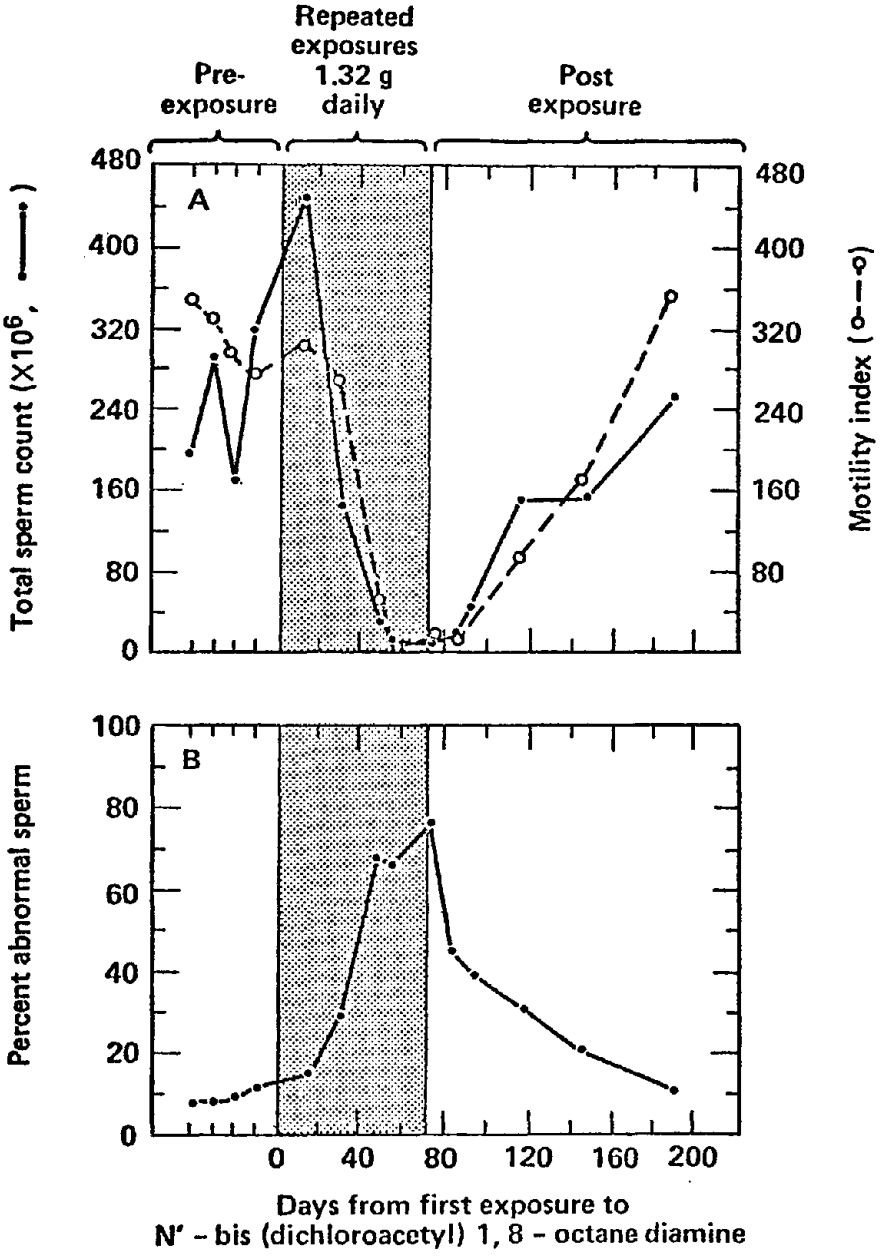


FIGURE 7

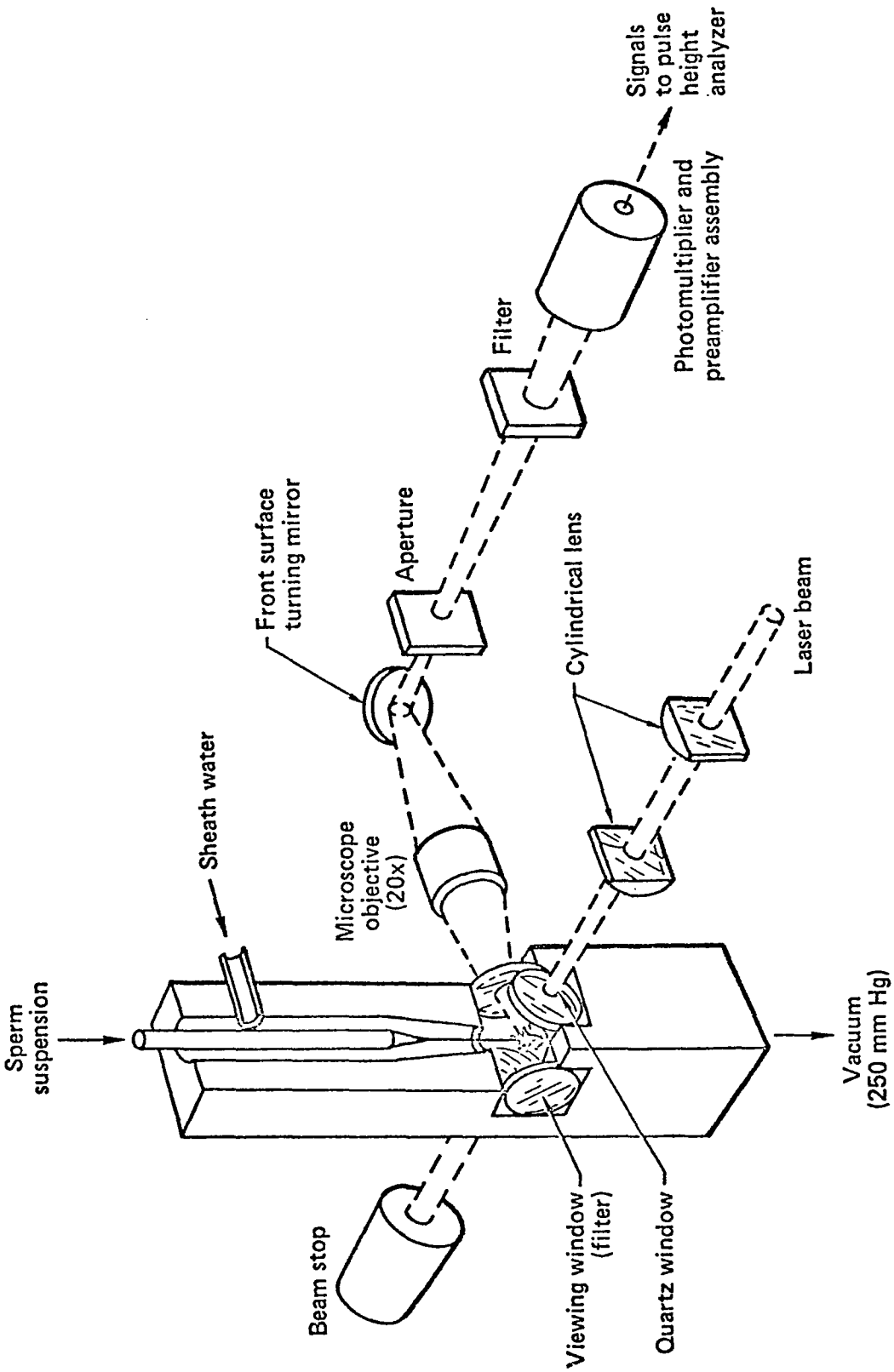
Semen abnormalities in three men exposed to N'-bis(dichloroacetyl) 1,8-octane diamine

Each point represents the average sperm counts (closed circles, Panel A), motility index (as described by MacLeod, 1965, open circles, Panel A), or percent abnormal sperm (closed circles, Panel B) for three men receiving 1.32 grams of this agent daily for about 10 weeks. The samples were collected before, during, and after the exposure period (dotted region in both panels). Graphs drawn from the data of MacLeod.[10]

FIGURE 8

Schematic representation of the Lawrence Livermore Laboratory Flow Cytometer (FCM)

Orthogonal axes of sample flow, laser beam illumination, and fluorescent light detection systems are shown. Redrawn from Van Dilla, et al.[42]



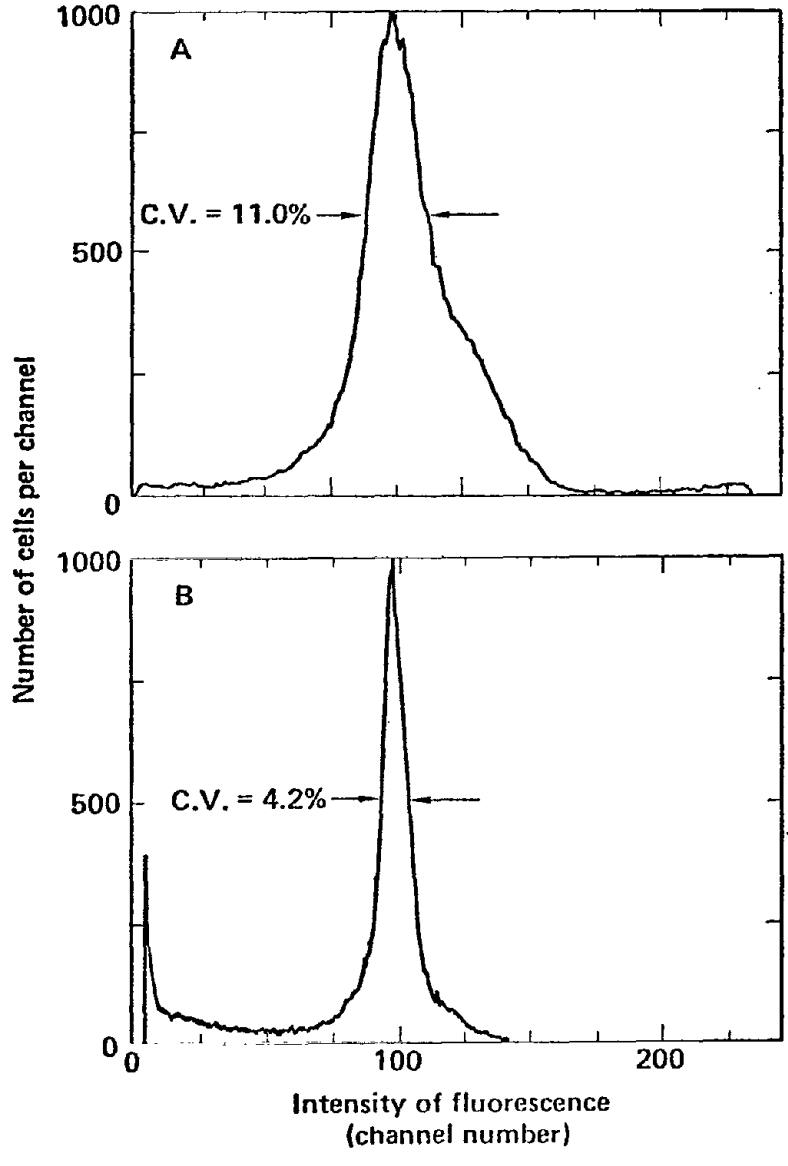


FIGURE 9

The effect of cell orientation on fluorescence distributions for human sperm

A. Randomly oriented human sperm nuclei stained with the acriflavine Feulgen procedure and excited with laser light of 488 nm. B. A beveled sample tube has been used to orient the sperm nuclei so that their plane is orthogonal to the laser beam; all other conditions the same as in (A). CV = standard deviation x 100/mean, assuming a normal distribution. Redrawn from Pinkel, et al.[47]

EPIDEMIOLOGICAL STUDIES FOR DETECTION OF TERRATOGENS

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INTRODUCTION

It is estimated that some 5% of all the newborn have an abnormal condition which can be traced back to disturbances in intrauterine development. This estimate, of course, does not include the number of embryos aborted during early pregnancy, but these cases provide the most adequate material available for epidemiological studies for detection of causative factors. Compared to epidemiological studies in many other fields of medicine, teratologic studies have one great advantage: the time interval between the exposure and the recording of its effect is very short, in the order of 7 to 8 months. Yet, as recently emphasized by Miller, not a single teratogen has been detected by epidemiological methods.[1] At the same time, some 70% of all congenital defects are still without a causal explanation.[2] There are three possible explanations for this present situation:[3]

- Environmental factors play no major role in human teratogenesis because the majority of defects are genetically determined, as suggested by Roberts and Powell.[4]

- Environmental teratogens act mostly additively to other exogeneous and genetic factors, and the study situation becomes confounded by too many unknowns for decisive epidemiological studies.

- Present methods are inadequate and their power to detect causative factors with low expressivity is insufficient.

In this presentation I will focus on the second and third alternatives, and discuss the possibilities and limitations of epidemiological studies to detect teratogens. While doing so, I will give several examples, most of which are drawn from our own studies of the material collected by the Finnish Register of Congenital Malformations. Three approaches have been used: surveillance, cohort studies, and case-control investigations.

POPULATION SURVEILLANCE

A major function of medical registers is continuous monitoring of the population to detect significant changes in the incidence of the disease under surveillance, and subsequently, to search for genetic or environmental clues to the causes of these changes. In teratology, such secular and seasonal variations have been reported frequently and their background factors discussed, but no conclusive associations with causative factors have been detected (e.g., 5). Several retrospective studies on the epidemiology of the thalidomide embryopathy are exceptions, and show a clear temporal

association between the typical defect and the sale of thalidomide. These studies show that surveillance could lead to the detection of new teratogens when:

- A previously rare defect increases rapidly,
- The effect of the teratogen is specific and leads to easily recognizable defects,
- The teratogen is rapidly spread to a large population and its teratogenicity is high,
- Information on the exposure is available and reliable.

In practice, this ideal situation must be extremely rare. In fact, when searching for exogeneous teratogens, our study situation is almost the opposite because:

- Most teratogens seem to have a variety of effects depending on the time and dose of exposure, and the foetal genotype,
- New teratogens are spread slowly, and usually only a small and scattered population is exposed,
- Reliable exposure data are rare,
- The study situation is confounded by other factors.

The following is an example of the difficulties in surveillance studies.

Influenza Epidemic and Congenital Defects

After the severe influenza epidemic in 1957, with an estimated attack rate of 30% in Finland, a cohort of 6,000 newborn children was followed up to the age of 10 and all defects recorded. The children were grouped according to their stage of intrauterine development during the short epidemic in October 1957, but only those who were in the stage of organogenesis ("sensitive period") were considered study cases. These were compared to the rest of the material. A significant association between defects of the CNS and the influenza epidemic was detected.[6] However, since it was assumed that the study situation was confounded, information on drug consumption during the epidemic was collected and compared to the sales figures of the pharmacies in the study area (Helsinki) for four consecutive years. The comparison showed a clear association between the epidemic and an increased sale (consumption) of drugs. Thus, the study situation was confounded, and did not justify final conclusions until additional results were available.

CASE-CONTROL STUDIES

A properly designed and functioning register can collect valuable material for case-control studies. This has become the main function of the Finnish Register of Congenital Malformations established in 1963.

The Finnish Register of Congenital Malformations

The organization and the material of the Register have been described in detail elsewhere,[7] and only some features are given here.

Since 1963, notification of the National Board of Health of all malformations detected in newborns has been compulsory in Finland. Toward the end of 1977, 13,559 malformed cases had been reported from the 1,049,189 births. This gives a crude incidence of 1.29%, which, however, suffers from several defects when reporting and detecting malformations.

Of the reported malformations, certain groups have been chosen to serve as "indicator defects" for special studies. Defects of the CNS, structural malformations of the skeleton, and oral clefts have been exposed to a detailed analysis, and for each case a pair of controls has been selected. The pair is matched according to maternal domicile (same antenatal clinic), and time of birth (the child born immediately before). Information on the study and control cases is collected from the following sources (FIGURE 1):

Primary notification card,
Records of the antenatal clinics,
Records of the obstetric and paediatric wards,
Death certificates,
Specially designed questionnaires filled during a personal interview after delivery,
Blood samples of the mother.

A total of 4,619 indicator defects have so far been selected from the material and exposed to detailed analysis. Of these 97.6% have been accepted into the final material after complete information from the matched pair control has been collected. Several analytic studies have been performed on this material, some of which are described here as examples of the possibilities, but also the limitations and pitfalls of such epidemiological studies.

Association Between Defects and Maternal Factors

As expected, many associations between maternal histories and various defects have been detected in these studies (TABLE I). For example, a detailed study of 710 mothers of children with CNS defects has been carried out. Again, when examining maternal diseases and consumption of drugs during pregnancy, Granroth observed several significant associations[8] (TABLE II)

Evaluation of Detected Associations

The associations listed in TABLES I and II, like most results in epidemiological studies, are affected by many limitations and pitfalls, and should be evaluated critically before any conclusions on their biologic significance are made. The main limiting factors to be considered are the following:[3]

- Loss and inadequate definition of defects,
- Chance correlations,
- Maternal memory bias,
- Confounding bias.

These major drawbacks and their handling are briefly discussed below, and examples of efforts to eliminate them are given.

Detection and Classification of Cases

The Finnish Register of Congenital Malformations, like many similar surveillance systems, suffers from both the underreporting of cases and the low detection rate of malformations. We have estimated that only some 70% of malformations detected during the perinatal period are notified and, especially, minor defects remain unreported. In addition, even in the best obstetric wards with specialized personnel, one-third of malformations remain undetected at birth and immediately thereafter.[9,10] Thus, a Register like the Finnish one will in fact record only some 50% of all congenital defects. Evidently, overall incidence figures based on such surveillance systems should be treated with great caution. For example, minor variations in time and place may merely reflect differences in the reporting rate, and in the training and density of the medical personnel.

The use of indicator defects may somewhat lessen the drawbacks caused by low reporting and detection rates. Such indicator defects should be clearly defined, be easily detectable at birth and be severe enough to focus the clinicians' attention.

Proper selection of study cases for correlative epidemiology is important for another reason. Inclusion of ill-defined conditions with a doubtful prenatal origin, and of those apparently developed during late intrauterine life, can "dilute" true associations between malformations and risk indicators. This was recently shown by Klemetti in a prospective study of 3,700 mother-child pairs followed up for 7 years.[11] The abnormalities gradually detected in this cohort were classified into three categories:

1. Structural malformations reflecting impaired organogenesis.
2. Group 1 plus "congenital defects" with doubtful origin from early development (e.g., pyloric stenosis, malposition of the feet, etc.).

3. Groups 1 and 2 plus all other congenital aberrations.

The associations of these three groups with certain risk indicators (TABLE III) show a clear "dilution effect."

Chance Correlations

Chance or nonsense correlations are unavoidable in studies where a great number of variables is analyzed without an hypothesis. There are basically two ways of recognizing such false associations. One is by repeating the study on independent material, and considering the biological meaning of the associations detected. For example, an association between any potential teratogen during the second trimester and cleft palate is not meaningful since the palatal shelves fused much earlier. This possibility of an "internal control" is seen from TABLE IV, giving the overall drug intake of mothers of children with cleft palate. The excess consumption still present during the second trimester is mainly due to mothers who have continued their medication after the critical first trimester. However, maternal memory bias should also be considered.

Maternal Memory Bias

Whenever use is made of retrospective data based on maternal interviews after delivery, memory bias should be seriously taken into account, as the mother, and frequently also the interviewer, are aware of the pregnancy outcome. Mothers of defective children may more effectively search for "causative" factors during their early pregnancy, than mothers of healthy babies who have usually lost their interest in the pregnancy. This bias has never been conclusively shown, but using repeated interviews we have demonstrated that maternal histories do change during pregnancy. Whether this is selective or not could not be evaluated in this study, because it was based on a rather small number of cases.[12]

The only way to completely eliminate the possibility of maternal memory bias is by a prospective study--prospective in the sense that all information is collected before the pregnancy outcome is known. Since the best and most extensive of such studies has recently been published in part,[13] I will comment on this type of study very briefly.

Collection of enough defective cases for a meaningful analysis requires an enormous study group, and this means that data must be collected from many hospitals and by a great number of people. It is not easy to standardize the collection of all this data. Moreover, such studies are slow, laborious, and expensive, which makes undertaking them unrealistic in many countries. For testing some relatively simple hypotheses, prospective studies will certainly prove useful.

Despite the retrospective design of a study, as in our surveillance program, much prospectively collected data is available. Practically every mother in Finland regularly visits an antenatal clinic during her pregnancy, the mean number of visits being 18. These clinics routinely record data on

maternal conditions, her diseases, all prescribed drugs, and all physical and laboratory examinations performed. Minor complications and illnesses, as well as nonprescribed drugs, are incompletely recorded, and here the information is mostly based on retrospective interviews, and hence is exposed to maternal memory bias.

One way to deal with this problem is to select the controls from mothers with similarly defective children. To investigate the relative significance of maternal influenza and consumption of drugs in teratogenesis, a special study was devised. From the files of our Register, we extracted data for 80 mothers of defective children with a positive history of influenza during the first trimester of pregnancy. The controls were selected from mothers of children with similar CNS-defects but a negative history of influenza. Risk ratios were calculated by comparison with their matched pair controls (TABLE V). There was a clear trend for the risk ratios to be considerably higher in the influenza group, suggesting that this disease acts either like a teratogen, or an additive factor, to drug-induced maldevelopment.

Another type of control material was recently used by Granroth.[8] Presuming that polydactyly was a defect with a predominantly hereditary background, and showing that it was less affected by exogeneous factors, he considered this an adequate control for the group with CNS-defects. Comparison of the risk ratios of the factors, listed in TABLE II, in the two groups of defects, showed that many of these were also associated to polydactyly. Whether this can be explained entirely by maternal memory bias, or whether these factors may, in fact, be associated to polydactyly cannot be decided, but the first explanation seems more plausible. When the factors associated with both types of defects were eliminated from the list (TABLE II), the number of risk indicators for CNS-defects was reduced to five: influenza during the first trimester, depressive state in that period, toxaemia, maternal diabetes, and intake of cough medicine during early pregnancy. Interestingly enough, the much discussed salicylate showed very much the same risk ratios in both groups.

Confounding Bias

In a nonexperimental study situation, the most serious limitation results from confounding factors, i.e., factors linked to the actual causative agents, and hence also to the defects. FIGURE 2 illustrates the main types of such linkages. By combining such different types of linkages, and adding several confounders to the hypothetical situation, one can build any kind of scheme for the study situation (FIGURE 3). In this particular "pedigree," the defect is associated with only one "single" teratogen. But a proper epidemiological analysis would, in addition, reveal associations with four potential teratogens, and three completely harmless confounders.

Two examples of such, perhaps somewhat less complicated, study situations are as follows:

Our register data showed that both cleft palate and CNS-defects were associated with maternal age, and were most frequent in the group of

mothers over 40 years of age. This result was obtained by comparing the age distribution of these mothers to that for the whole country, but it could not be shown by comparison to the matched pairs. This was because the older age groups were also overrepresented in the time-area matched controls. The answer came from the observation that both defects were most frequent in a certain district of the country, the province of North Karelia, where the average age of the mothers was greater than in the rest of the country and the oldest age group was especially large (TABLE VI). This example also suggests the dangers of overmatching.

If, as is only rarely the case, confounders cannot be eliminated by the design of the study, the correct way to deal with the problem is a multivariant analysis.[13] The many associations between risk indicators and CNS-defects (TABLE II) have recently been exposed to such an analysis.[14] A complicated network of associations was detected between various indicators, whereafter multivariant analysis was performed. The work is still in progress, and only one example of the results (TABLE VII) dealing with maternal influenza and its confounders is given. The results suggest that influenza "explains" the associations between CNS-defects and many drugs, and should itself be considered a potential teratogen. Salicylates show an additive effect.

PRACTICAL CONSEQUENCES

Results of epidemiological studies, such as those described here, can lead to two types of practical consequences: on rare occasions a single teratogen may be found and consequently eliminated. Especially drugs, and also many chemicals, may easily be withdrawn from the market or a warning launched, but this should be done only after thorough consideration. For instance, there are good reasons to believe that certain untreated diseases are more deleterious to the developing fetus than adequate medication.

Another way to proceed from epidemiological data to practical measures might, in the future, be by delineation of risk groups, i.e., to define and characterize those mothers who have an increased risk of delivering a defective child. In these cases, various diagnostic and preventive measures already available could be considered, and ultimately the pregnancy terminated. An interesting theoretical possibility would be to use some confounding factors in the definition of these risk groups. If a factor is significantly associated with congenital defects, and is easily detected and recorded during early pregnancy, it may become a valuable risk indicator regardless of whether it is a causative factor or a harmless confounder.

COMMENTS ON INDUSTRIAL TERATOLOGY

Since this meeting is called to discuss problems related to industrial toxicology, let me close with some comments on the possibilities and limitations of epidemiological methods in this particular field. Acting as a consultant to two ongoing projects related to industrial teratology in Finland, I am beginning to see some advantages, but also some obvious difficulties, in such epidemiological studies designed to detect industrial teratogens.

Let me stress that our experience is still limited, and my list of the advantages and difficulties should be considered merely as a personal view.

The advantages are:

- Relatively good information about the environmental chemicals used,
- Relatively good and exact information of the time of exposure when known factors are studied,
- Advanced medical care and recording in many industrial facilities,
- The possibility of finding time-area-age matched pair controls from the same, or nearby, facility with different working conditions.

The main difficulties are:

- The great number of compounds to be examined as potential teratogens,
- Confounding by other compounds and conditions,
- In most cases the relatively small number of females exposed to the suspected or unknown factors.

Based on the views presented in this review, there seems to be two major avenues to be followed in the search for industrial teratogens by epidemiologic methods. A retrospective approach starts from the cases of any registration system for congenital defects, selects adequate indicator defects, and thereafter defines the mothers whose working conditions should be reviewed. In case of industrial exposure, the information can largely be based on objective data collected directly from the working environment and the files of the employer, without having to rely on retrospectively collected maternal information. The advantage of this approach is that large materials can be collected relatively rapidly, but its obvious drawback is the retrospective nature of data collection.

Another way to approach the problem would be a prospective cohort study where every female employee of child-bearing age is under constant surveillance, and, where known, both the possible exposures and the outcome of her pregnancy are recorded. This "method," which eventually will become routine in industry dealing with potential teratogens, is slow and not free of confounding factors. Since such surveillance would only occasionally lead to collection of materials large enough for epidemiological analysis, its main function might be in developing hypotheses. A few cases of detected associations should lead to a working hypothesis, which can then be tested by the retrospective method.

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TABLE I

SOME SIGNIFICANT ASSOCIATIONS BETWEEN MATERNAL HISTORIES AND THREE GROUPS OF CONGENITAL DEFECTS

	CNS Defects	Skeletal Malformations	Oral Clefts
N	(684)	(766)	(791)
Maternal age >40 years	+++	++	++
Unmarried mother	++		++
Previous abortions	+		
Previous stillbirths	+++		
Previous defects in children	++	++	++
Threatened abortion	+++	++	+++
Birth weight <2.500 g.	+++	+++	+++
Maternal influenza during I trimester	+++	+++	+++
Maternal intake of drugs, I trimester:			
Overall	+++	++	+++
Analgesics	+++	+++	+++
Penicillin			+++

SOURCE: References #15, 7

TABLE II

MATERNAL EXPOSURES (DURING FIRST TRIMESTER OF PREGNANCY) SIGNIFICANTLY ASSOCIATED WITH DEFECTS OF THE CNC

Diseases and conditions
Influenza
Threatened abortion
Depressive state
Toexemia
Diabetes
Drugs
Analgesics
Sympathomimetics
Psychopharmaca
Cough medicine
Insulin

SOURCE: Reference #8

TABLE III

PERCENTAGE OF CERTAIN RISK INDICATORS IN THREE DIFFERENT CATEGORIES OF CONGENITAL DEFECTS

N =	I (93)	II (197)	III (334)	Controls (2,579)
Unmarried mother	4.3[x]	3.1	2.4	1.4
Low placental weight	13.5[xxx]	8.9[xx]	6.0	3.5
Low birth weight	16.1[xxx]	9.7[xxx]	8.4[xxx]	3.3
Maternal influenza	11.8[x]	10.7	8.7	6.0
Intake of sedatives	6.5[x]	4.6	3.3	1.8

x p <0.05

xx p <0.01

xxx p <0.001

I. Structural malformations

II. All congenital defects

III. All congenital (?) disorders

SOURCE: Reference #10

TABLE IV

OVERALL INTAKE OF DRUGS (OTHER THAN VITAMINS)
BY MOTHERS OF CHILDREN WITH ORAL CLEFTS
AND BY THEIR MATCHED-PAIR CONTROLS

	Case (791) %	Control (782) %
First trimester	45.4[xxx]	27.2
Second trimester	35.9[x]	30.4
Third trimester	43.9	41.7

xxx p <0.001

x p <0.05

SOURCE: Reference #15

TABLE V

INTAKE OF DRUGS DURING THE FIRST TRIMESTER OF PREGNANCY IN TWO GROUPS OF MOTHERS OF CHILDREN WITH CNS-DEFECTS AS COMPARED TO MATCHED-PAIR CONTROLS OF HEALTHY INFANTS (N = 80)

	Relative Risk	
	Defective Child, Maternal Influenza	Defective Child, No Maternal Influenza
Salicylates	13.5	2.5
Undefined analgesics	7.0	2.0
Sedatives	2.5	1.0
Sulfonamides	7.0	8.0
Antibiotics	4.8	3.0

SOURCE: Reference #17

TABLE VI

INCIDENCE OF ANENCEPHALY AND CLEFT PALATE IN THE PROVINCE NORTHERN CARELIA AND IN THE REST OF FINLAND AS COMPARED TO THE MATERNAL AGE STRUCTURE

	Northern Carelia	Rest of Finland	P
Incidence of anencephaly	0.61 o/oo	0.31 o/oo	<0.05
Incidence of cleft palate	1.58 o/oo	0.84 o/oo	<0.01
Mothers over 40 years	4.2%	2.7%	<0.001

SOURCE: Reference #18, 19

TABLE VII

INFLUENCE OF CONFOUNDERS ON ASSOCIATIONS OF MATERNAL INFLUENZA AND SALICYLATE CONSUMPTION WITH DEFECTS OF THE CNS IN THE LINEAR LOGISTIC REGRESSION MODEL

Exposure Factor	Number of Discordant Pairs	Odds Ratio No Corrections	Odds Ratio, After Analysis with the Following Confounders	
Influenza	227	1.9^{xxx}	1.5^{xx}	Salicylates Penicillins Cough medicines Sulfonamides Psychopharmaca
Influenza, I trimester	39	2.5^{xxx}	1.7	Salicylates
Salicylates	307	2.0^{xxx}	2.0^{xxx}	Influenza Sympathomimetics Antineurotics Pyrazolones Antibiotics Maternal age over 34
Salicylates, I trimester	60	4.3^{xxx}	4.0^{xxx}	Influenza
Influenza and salicylates	152	2.7^{xxx}	2.7^{xxx}	Sympathomimetics Cough medicines Antibiotics Pyrazolones

xx $p < 0.01$

xxx $p < 0.001$

SOURCE: Reference #14

FIGURE 1

Scheme of the surveillance system of the Finnish Register of Congenital Malformations

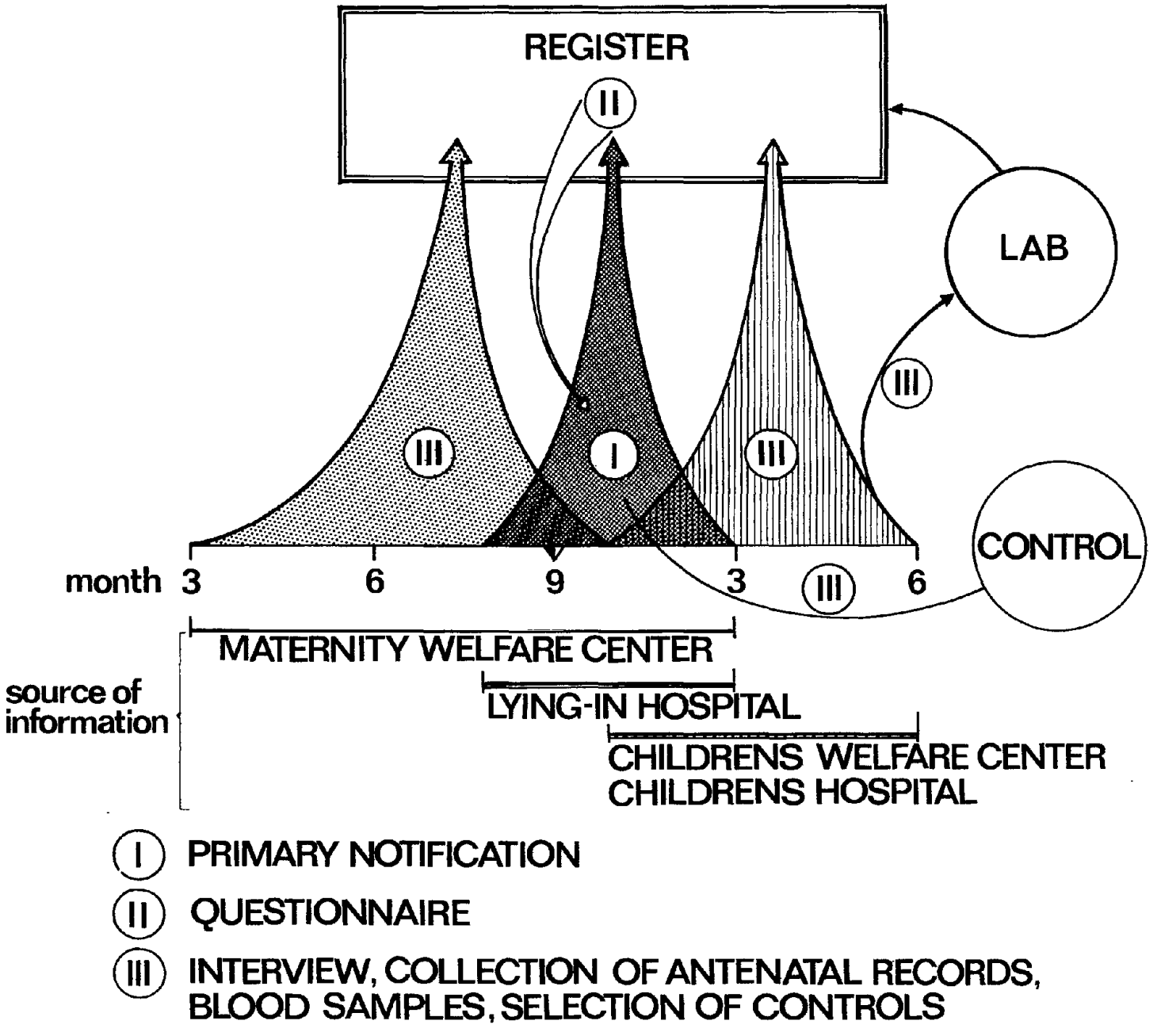


FIGURE 2

Scheme of various types of linkages between teratogens and confounders:

A: A single teratogen leads to a defect.

B: A single teratogen is linked to a harmless confounder.

C: A potention teratogen leads to a teratogenic condition.

D: A defect is linked to a nonteratogenic indicator.

E: Two potential teratogens act additively and simultaneously.

F: Two potential teratogens act consecutively and cumulatively.

G: A nonteratogen prevents the action of a teratogen.

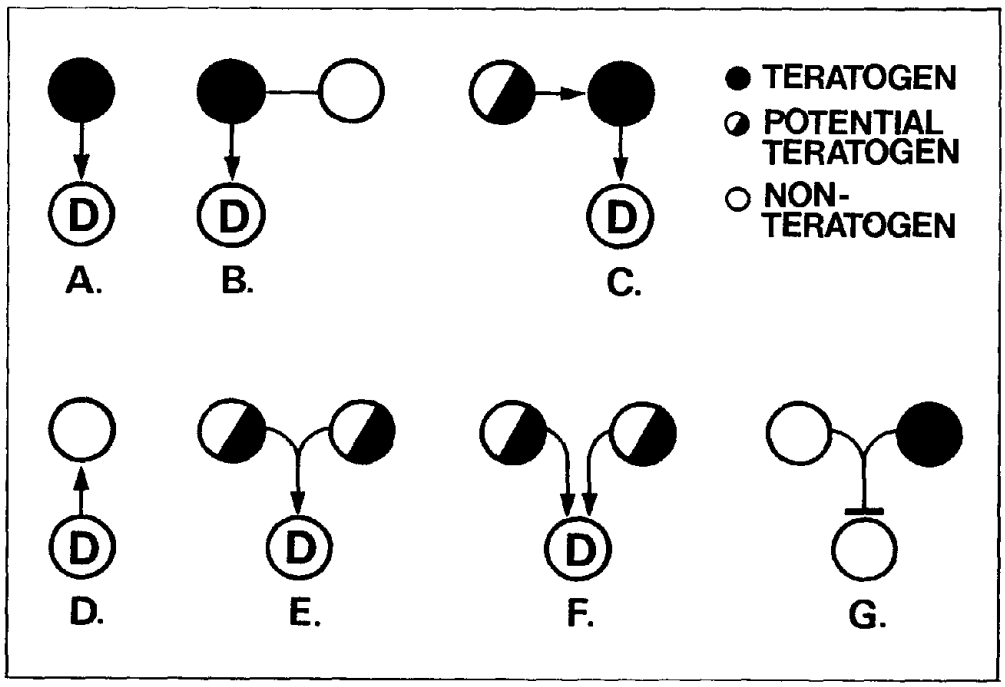
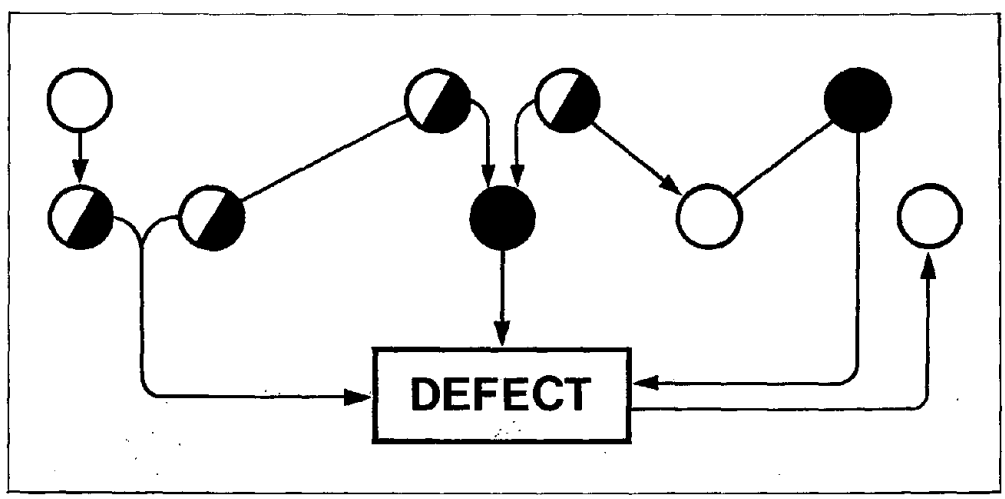


FIGURE 3

A hypothetical "pedigree" of a defect with various types of linkages and confounders



OCCUPATIONAL HAZARDS TO PREGNANT WOMEN IN THE COLLABORATIVE PERINATAL PROJECT

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INTRODUCTION

Large populations appropriate and available for longitudinal epidemiologic studies are scarce. Data collection over a period of time necessary for profitable analysis frequently suffers from lack of funding and professional commitment.[1] Therefore the investment of time and money in good studies demands the best use of data already collected even though the original aim of the study may not closely match questions which have since arisen.

In particular the question of reproductive effects of occupational exposure to hazardous conditions has not been of scientific and public concern since early in the century,[2] so that there has not been a consistent development of information comparable to occupationally related effects, such as cancer and chronic respiratory disease.

This report describes an exploration of a prospective longitudinal study, the Collaborative Perinatal Project--well designed and maintained for 20 years by the National Institute of Neurological Diseases and Blindness (now the National Institute of Neurological and Communicative Disorders and Stroke).

The specific aims of the exploration were to identify those in the total study of the Collaborative Perinatal Project who reported exposure to occupational hazards and to establish appropriate classifications for the analysis of variables pertaining to pregnancy history, placental characteristics, and the growth and development of the children who were born; to examine the occupational exposure patterns of women with a work history; and to examine, in more detail, occupational conditions which appear to show associations with adverse pregnancy outcome.

DESCRIPTION OF TOTAL COLLABORATIVE PERINATAL POPULATION

In 1955, the protocol for a perinatal collaborative study was drawn up by the National Institute of Neurological Diseases and Blindness (NINDB) with the following objectives:

(1) "to make a more precise determination of fetal, environmental, and medical factors leading to the various forms of cerebral palsy.

(2) to link the symptoms of this group of disorders to the causative brain damage."

The inadequacy of hospital records was recognized early in the planning of the collaborative clinical-pathological study of pregnancy and its outcome. To offset the retrospective information gathered about pregnancy and perinatal events from parents of children with defects, the prospective approach was chosen for the Collaborative Perinatal Project (CPP). Data were collected on pregnancy and perinatal events as they occurred to eliminate biases of known pregnancy outcome. The aim of the study was to examine a very large number of cases in minute detail, so that the effects of perinatal factors on the health of the individual child could be evaluated. Twelve hospitals throughout the United States met the study's objective of enrolling a total of 50,000 to 60,000 pregnant women in less than a decade. The final enrollment total was 58,760. The study was continued up to the 7th birthday in 73% of the study children to permit the evaluation, not just of the immediate pregnancy outcome of perinatal death or survival, but also of any disorders or abnormalities that might only become manifest in later childhood.

In 1958, there was a pretest period when considerable time and effort were spent recruiting and training staff for the collaborating centers and the NINDB. To assure uniform data collection, great detail was given to the writing of procedures for the selection of patients, the development of forms and manuals for data recording, workshops, meetings, and pretests. The creation, printing, and pretesting were extremely time consuming and the revisions of forms continued through 1959. The development of forms and manuals for the later examinations of study children continued for several additional years.

Protocols were developed for obtaining neuropathological specimens from stillbirths and neonatal deaths, and for follow-up examinations of survivors beyond the newborn period. The limitations of newborn appraisals in identifying congenital malformations had been demonstrated by McIntosh et al. who, in a follow-up of 5,739 children from birth to 1 year of age, found over twice as many malformations at 1 year as were identified at birth.[3]

In 1959, it was realized that the ability of the 12 centers to follow their study children would greatly determine the success of the total study, and each hospital had unique difficulties in its ability to follow study children. By the very nature of their populations, some hospitals were more successful than others. Four collaborating hospitals dropped out of the study for various administrative reasons. Children's Hospital in San Francisco withdrew after the pretest phase in 1959. Yale Grace-New Haven Hospital dropped out of active participation in 1961, after registering 900 pregnancies. In 1963, Columbia-Presbyterian Medical Center, New York, halted its obstetrical intake, but continued to follow the children through 8 years of age. New York Medical College, having completed the examination of children at age 4, terminated in 1970 because of insurmountable difficulties in achieving an adequate retention rate for children at age 7.

While the other study institutions had a return rate of over 70%, the rate at New York Medical College was only 45%-55%, with little hope of improvement. This was attributed to the mobility of the Puerto Rican population that comprised a large portion of the sample.

Early development evaluation was performed at critical time periods of the study child's life. A general pediatric exam was scheduled at 4 months, an assessment of mental and motor development at 8 months, a neurological examination at 1 year, and a psychological evaluation at 4 years. A final evaluation, including neurological and psychological appraisal, was made around 7 years of age.

The registration continued for 7 years, beginning January 1, 1959, and ending December 1965.

SELECTION OF PARTICIPANTS

In all of the hospitals, certain constraints were imposed upon the participants selected from the general obstetric patient population. Common disqualifications were a woman's intention to move from the area; certain geographic areas of residence; and so-called "walk-in" cases--delivery on the same day as first registration to the study.

In two of the hospitals, all presenting gravidas in the sampling frame were entered into the study. In nine hospitals, systematic selection was based upon the last digit of the patient number, or the woman's day of birth, or some similar device. In one hospital, random sampling was used. In each of the participating institutions, the sample enrolled in the study did not differ with regard to age, ethnic group, marital status, or weeks of gestation at admission, as compared with the total group of women who initially formed the sampling frame. A woman could be represented in the CPP more than once if she had a subsequent pregnancy which was registered in a collaborating clinic.

DATA COLLECTION

On entry to the study, during visits to the collaborating perinatal clinic, the mother provided specially trained interviewers with her medical history, socioeconomic and genetic information about herself and her family, the baby's father, and his family. A medication history covering the time from the last menstrual period was also obtained. Detailed, structured forms were used, and the data collection procedure was guided by the use of extensive manuals. Prenatal clinic visits were scheduled every month during the first 7 months of pregnancy, every 2 weeks during the 8th month, and every week thereafter. Obstetricians recorded the results of physical examinations, histories, and laboratory tests. The assembled material was sent to a central facility where it was processed into computer files. Specific computer procedures were routinely employed to identify and correct mistakes in the original computer files.

When the mother was admitted for delivery her physical status was re-evaluated. Her labor and delivery were recorded by a trained observer. The attending obstetrician completed a summary of the labor and delivery. Pathologists specially trained as CPP staff pathologists examined the placenta and conducted post mortem examination of stillbirths and neonatal deaths.

The neonate was first observed in the delivery room; subsequently Apgar tests were administered at 5, 10, 15, and 20 minute intervals. A pediatrician then examined the child at 24-hour intervals in the newborn nursery. At 2 days of age, a neurological exam was performed. Other information from the nursery period was collected from laboratory tests and nurses' observations.

After the neonatal stage, an "interval history" was taken of both the mother and child, with different physical and psychological emphases at each examination from 2 to 4 years, and from 5 to 8 years. Family and social history was updated by the mother at the 7-year exams. Diagnostic summaries were prepared by physicians at 1 and 7 years.

DEMOGRAPHIC CHARACTERISTICS

The pregnancies in the CPP were classified into categories of the ethnic group of the gravida: Black, White, Puerto Rican, Oriental,* and "Other." A socioeconomic index score based on the methods used by the U.S. Bureau of the Census, and described in detail by Myrianthopoulos et al., was prescribed for each study woman.[4,5] The CPP index, like that of the Census Bureau, combined scores for education, family income, and occupation into a single score. Occupation and educational achievement were determined for the heads of households, and each of the three scores was ranked as a percentile. The socioeconomic index is the mean of the three scores and has a range of 0 to 95.

Using the USBC scores for the first 36,110 cases of the study and 1960 census records, Myrianthopoulos and French showed that the distribution of the socioeconomic index scores in the study population was displaced toward the low end of the scale relative to the U.S. population.[5] The median SEI scores were 42 and 57 respectively. However, most of the CPP scores fell between 20 and 49, and a few scored 60 or above.

The median SEI score for black registrants was 37, higher than for the black population in the United States--33. Most of the U.S. black population had scores under 20, while the CPP population had scores between 20 and 59. When the Whites in the study were compared with the U.S. white population, the SEI scores overlapped more. The CPP white population median SEI score was 51, while the SEI score for the U.S. population was 59. The CPP white population was a closer representation of the SEI scores in the general population.

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*Racial designations used in the CPP.

Buffalo, the only institution in the study to register all private patients, had the CPP population with the highest mean SEI score, 78. Boston and Minnesota followed with 61 and 60 respectively. These three hospitals contributed 33% of the study population; 68% of the Whites, and 5% of the Blacks, 1% of the Puerto Ricans and 64% of the "Other" ethnic groups. Charity Hospital at New Orleans, the University of Tennessee College of Medicine, and the Medical College of Virginia comprised the study populations with the lowest mean SEI scores, 30, 32, and 33 respectively. Together these institutions contributed 18% of the total population, 34% of the Blacks, and 3% of the Whites.

The median maternal age was 23.6. More than one-third of the women were between 15 and 19 years, and one-fifth between 25 and 29 years. Black registrants' median age was 23, while that of the white registrants was 24 years.

WOMEN LOST TO THE STUDY

A case was termed "lost to the study" if the gravida refused to participate after registering, or if she moved away without a trace. These "lost to study" cases were women who dropped out of the study before completion of their pregnancy. They comprised 4.1% of the study registrants.

In order to evaluate any possible bias to the study resulting in the women "lost to study," certain characteristics of these women were examined for comparisons with those who were followed. The more highly educated mothers of both racial groups were more frequently lost to the study than those of the lower educational group. Also, fewer of the very young women, white or black, dropped out than expected. Neither of the above two observations was consistent by collaborating center. Nor was there consistency among the collaborating centers concerning the marital status of the two groups of gravida.

A continuous effort was made to prevent attrition of the study population during the entire study. A small number of infants had to be delivered at hospitals other than the intended collaborating clinic, and so were not present for the study nursery exam. In all, 85% of the study children were successfully followed to receive the 1-year neurologic examination within the prescribed brief time period. Although the rate was not consistent by institution, there was a 73% rate of successful follow-up providing clinical evaluation up to the 7th year of the study child's age.[6]

LIMITATIONS OF STUDY OF EXPOSURE TO OCCUPATIONAL HAZARDS

Access to the voluminous data base of the CPP is not a straightforward process. The accumulation and coding of data over the period from first registrations to the present by the National Institute for Neurological and Communicative Disorders and Stroke (NINCDS) have progressed through a series of stages as particular emphases developed for detailed study. The series of questions dealing with work experience and exposure to hazardous

conditions was not part of earlier studies. It was only during the latter part of the enrollment period, 1962 to 1965, that the recorded answers concerning occupation were coded.

Several critical data points are difficult, or impossible, to retrieve. There was no linkage question to establish when exposure to a hazardous condition occurred in relation to the pregnancy under study. The question was phrased, "Have you ever worked with . . ." followed by "Describe work situation" and "Approximately how long? (in months)." It was therefore impossible to establish from the coded data whether exposure had occurred many years before and terminated, or over the period of time reported up to, and including, the current pregnancy. Detailed examination of the original records in some cases could provide direct, or indirect, evidence about the time of the woman's exposure. We found the reliability of the conclusions which could be reached indirectly was far from uniform as we examined individual original records for this particular item of information. We decided that only one basic assumption could be made, namely that exposure to hazardous work conditions had occurred before the date of interview. It was also assumed to be unlikely that many such exposures were related only to the few prenatal months, without exposure preconception. Some women could have experienced an occupational exposure to hazardous conditions only during the postconception period, but we judged these to be very few and virtually unidentifiable from the responses recorded. This observation led us to the realization that occupational exposure to hazardous conditions for the population being examined was most likely to have been a preconception experience. We were therefore looking at the work/reproduction relationship in a far broader context than the limited view of the immediate experience of a pregnant woman. The categories of occupational exposure must then be viewed in terms of an overall potential influence on the reproductive system. It is scarcely appropriate to view pesticide intake and resulting residue accumulation in the human body as a single event or a short-term influence on a particular pregnancy. The exposure experience reported by virtually all the respondents who were identified as having worked with tobacco or cotton quite clearly reflected a long-term environmental and occupational exposure. Exposure to chemicals with accumulation of a body burden for a particular chemical, or its metabolites, could be viewed similarly.

In contrast, effects of exposure to heat and external ionizing radiation preconception, although little explored in the former, and only partially understood in the latter, could not be expected to result in a body burden with continuous influence. However, long-term physiologic or pathologic changes in reproductive function affected by these physical agents need to be examined.

A working environment which involved lifting weights presents a physiologic environment for some women which allows them to improve their physical conditioning. For others, the same conditions may constitute a physical stress. It is difficult, therefore, to know whether the self-reporting of lifting weights represents more women for whom heavy lifting was stressful, with the likely exclusion of physically stronger women who did not consider the same level of lifting as "heavy" work. The heavy lifting category in this study is the most difficult to define in terms of hazardous exposure,

and may be more of a descriptor of the population than a measure of stress imposed, in contrast to other exposure categories.

An estimation of "dose" can be made only directly from the response given to the question "Approximately how long?" describing the exposure to an occupational hazard. We coded the response into 1 to 6 months, 7 to 18 months, and more than 18 months.

Gestation at registration in the CPP varied markedly, further complicating any estimate of postconception occupational exposure to a hazardous condition. It was therefore included as a demographic variable in the analysis.

The choice of variables carried with it the likelihood that the number of missing values would differ, one from the other. The quality of recording and coding was high in the CPP, with repeated checks throughout the entire period of enrollment, and subsequent coding to establish accurate entry into the data base. The recall of patients for subsequent examination could not be as closely controlled. Therefore there are few missing values relating to pregnancy variables, when compared with those for the children at later examinations. There were only minor differences in the percentages of missing values between the exposure groups, for those variables associated with each child examination at 4 months, 8 months, 1 year, 4 years, and 7 years. Otherwise missing values were usually only 2% to 3%, with a few at 5%. There was no marked difference in the proportion of missing values between exposed and unexposed groups.

Information obtained at the first interview concerning employment and occupational exposure to hazardous work conditions was recorded, but not often coded, in the early years of the CPP. Our study was confined to the 23,961 first pregnancies to the study for which answers to the questions concerning exposure to hazardous work conditions were recorded and coded. Less than 1% of the pregnancies we examined were registered from 1958 to 1961 (inclusive), so that almost all of the pregnancies with coded information on occupational hazards occurred in the latter half of the CPP enrollment period.

Subsequent pregnancies and progeny were excluded from our study. The first recorded twin in a first pregnancy to the study was considered to be the first child for which occupational information of the mother was available. There were 50 such infants.

OCCUPATIONAL EXPOSURE TO HAZARDS

TABLE I shows the format of the Socioeconomic Questionnaire which provided the basic occupational exposure information we used. These questions were usually asked at the first interview at registration. For 4,575 of the 23,961 pregnancies, women reported that they had been exposed to X-rays or fluoroscope equipment, radioactive elements or isotopes, tobacco dust or leaf, steam or very high heat, chemicals and their dusts, gases or fumes, animals or birds, and had lifted weights at some time in the past.

There was considerable variability among different hospitals, with Tennessee and New York Medical showing the lowest percentage of pregnancies with reported occupational exposures (10.7% and 5.8% respectively), and Minnesota and Providence with the highest (34.2% and 33.2% respectively) (TABLE II). The proportion of pregnancies with reported exposures remained quite consistent through the enrollment period from 1962 to 1965 ranging from 25.7% to 28.2% for Whites, 18.0% to 24.6% for Blacks, and from 5.5% to 8.3% for other races (TABLE III). Comparison by race showed a higher proportion of Blacks in the exposed group than in the unexposed group and a lower proportion of Whites in the exposed group when compared with the unexposed group (TABLE IV). The distribution of socioeconomic status was more consistent, with 60.2% in the mid-socioeconomic category (socioeconomic score 40 to 59) for the unexposed group and 58.0% in the exposed group. The maternal age distribution was weighted more heavily to the younger 10-19 year age group in the unexposed (26.3%), when compared with the exposed group (13.3%), with fewer in the 30-39 year age group in the unexposed group (15.4%) when compared with the exposed (21.0%). The number of prior pregnancies was more comparable particularly for those with one to three prior pregnancies (47.3% unexposed, 46.6% exposed), with no prior pregnancies being more prevalent in the unexposed (30.1%) than the exposed group (25.0%). The level of education was comparable for high school graduation, 30.3% for unexposed versus 29.5% for exposed. Among the unexposed were more with less than 7 years' education, and fewer with more than 12 years' education (10.2% and 12.1% respectively), when compared with the exposed (7.6% and 18.9% respectively).

CHOICE OF VARIABLES AND STATISTICAL DESIGN

The use of contingency tables, including two-factor and three-factor analysis, encourages a judicious choice of variables to constrain computer costs and print-out examination. The final choice of 44 variables developed from considerations of possible associations with hazardous work conditions and included those which described previous reproductive experience, outcome of pregnancy under study, prenatal symptoms, placental characteristics, newborn status, 8-month, 1-year, 4-year, and 7-year growth and development, and demographic characteristics (TABLE V). Earlier studies of CPP data were also useful in making judgments on choice of variables.[7,8]

Two-factor analysis was run for exposure to each of the hazards--ionizing radiation, animals, heat, heavy lifting, chemicals, and pesticides with each of the 44 variables. Then three-factor analysis was run for exposure to each of the hazards with every possible pair from the 44 variables.

We did not retain those with a confidence value of $p > 0.05$, and interpretation of three-factor interactions was dependent on finding a two-factor interaction between exposure to a hazard, and one of the pair of variables in the three-factor analysis.

The statistical approach involved log-linear model analysis of cross-classified categorical data.

When data are recorded in categories instead of on a continuous scale, they are usually cross-classified into tables of counts, referred to as contingency tables. For example, in this study the relationship (if any) between amount of chemical exposure (none, low, medium, high), socioeconomic status (low, medium, high), and uterine bleeding (present or not), was examined. A frequency count of women falling in the various categories results in a three-way contingency table (TABLE VI). Here, for example, X_{111} denotes the number of women with uterine bleeding who had no chemical exposure and who were in the "low" socioeconomic status level; n_{11} denotes the total number of women with no chemical exposure in the "low" socioeconomic level.

In this example, we treat amount of chemical exposure (A) and socioeconomic status (B) as factors, and presence, or not, of uterine bleeding (C) as a response variable. For each category of A and B we can calculate the observed proportion of women having uterine bleeding, to obtain the following table of observed probabilities (TABLE VII).

Here, $p_{ij} = X_{ij1}/n_{ij} = X_{ij1}/(X_{ij1} + X_{ij2})$.

From TABLE VII we can also form three 2-way (marginal) tables: TABLE VIIIa describes the sampled population of women by amount of exposure to chemical and socioeconomic status, while TABLES VIIIb and VIIIc reflect the incidence of uterine bleeding in women according to socioeconomic status VIIIb, and by amount of chemical exposure VIIIc.

In analyzing the effect of the two factors (A and B) on incidence of uterine bleeding (C), we might initially examine the effects of A and B separately. Taking this approach, we would begin by analyzing TABLES VIIIb and VIIIc. In TABLE VIIIb, we would test the hypothesis that the proportion of women having uterine bleeding is the same for each socioeconomic level while in TABLE VIIIc, the hypothesis to be tested is that the proportion of women having uterine bleeding is the same irrespective of the amount of chemical exposure. These hypotheses are tested using the usual X^2 statistic

$$X^2 = \Sigma \text{ (observed - expected)}^2/\text{expected}.$$

For TABLE VIIIb, if the population proportions are the same we would then estimate the common proportion by $\hat{p}_{..1} = X_{..1}/n$ and calculate the expected frequencies by multiplying $\hat{p}_{..1}$ by $n_{.1}$, $n_{.2}$, and $n_{.3}$, respectively. Thus, the table of expected frequencies is as follows: (TABLES IX and X)

and $X^2 = \sum_{j=1}^{3} \sum_{k=1}^{2} (X_{.jk} - n_{.j}X_{..k}/n)^2/(n_{.j}X_{..}/n)$. We reject the hypothesis of equal proportions if X^2 exceeds the upper α% point of X^2 with 2 degrees of freedom.

If we take the natural logarithm of the estimated expected cell frequencies, denoted by $\hat{m}_{jk}$, we get

$$\ell n\, \hat{m}_{jk} = \ell n(n_{.j}X_{..k}/n) = \ell n\, n_{.j} + \ell n X_{..k} - \ell n\, n.$$

This model can be expressed in a form similar to analysis of variance notation:

$$\hat{\ell}_{jk} = \ell n\, \hat{m}_{jk} = \hat{u} + \hat{u}_{1(j)} + \hat{u}_{2(k)}.$$

The model contains a grand mean (u) and terms $u_{2(k)}$ for the response variable and $u_{1(j)}$ for the socioeconomic variable. If the proportions are not equal, the model would also have an interaction term $u_{12(jk)}$. In general, the log-linear model for the expected cell frequencies is specified by

$$\ell_{ij} = \ell n\, m_{jk} + u + u_{1(j)} + u_{2(k)} + u_{12(jk)}.$$

The model parameters have the following interpretations:

(a) if $u_{12(jk)} = 0$, the proportions are equal.

(b) if $u_{2(k)} = u_{12(jk)} = 0$, the overall proportion of women with uterine bleeding is 0.5.

(c) if $u_{1(j)} = u_{12(jk)} = 0$, the proportion of women in each socioeconomic status is the same, namely, 1/3.

Methods for analyzing contingency tables with more than two dimensions have, until recently, been limited. The log-linear model approach provides a general method that can be used for any number of dimensions. In particular, for a three-dimensional table, the full (saturated) log-linear model is given by

$$\ell_{ijk} = u + u_{1(i)} = u_{2(j)} = u_{3(k)} + u_{12(ij)} + u_{13(ik)} + u_{23(jk)} + u_{123(ijk)}.$$

By setting various terms in this model equal to zero, we obtain submodels which are directly interpretable. For example, if we set $u_{123(ijk)} = 0$, we get the zero three-way interaction model. It is most easily interpreted in terms of cross-product ratios which measure association between categories. For the three-way table involving (a) amount of chemical exposure; (b) socioeconomic status; and (c) incidence of uterine bleeding, the zero three-way interaction model says that the odds ratios

$$P_{11}/P_{21}, P_{12}/P_{22} \text{ and } P_{13}/P_{23}$$

are equal, i.e., they do not depend on socioeconomic status. Similarly, the ratios

$$P_{11}/P_{12},\ P_{21}/P_{22},\ P_{31}/P_{32},\ \text{and}\ P_{41}/P_{42}$$

are equal, i.e., they do not depend on the amount of chemical exposure. Thus, we can interpret the zero three-way interaction model by saying that chemical exposure may affect incidence of uterine bleeding, but if it does so, it affects it independently of socioeconomic status. Alternatively, one could have an association between socioeconomic status and uterine bleeding, but this association does not depend on the amount of chemical exposure.

When there is a response variable C at two levels (as is the case with uterine bleeding) and factors A and B (as in the example used here), it is convenient to write a linear logistic model for the response variable. This model is given by

$$\lambda_{ij} = \ell n\, P_{ij}/1 - P_{ij} = \ell_{ijl} - \ell_{ij2} = W + W_{1(i)} = W_{2(j)} + W_{12(ij)}.$$

Here, $W_{12(ij)} = u_{123(ijl)} - u_{123(ij2)}$, $W_{1(i)} = u_{13(il)} - u_{13(i2)}$,

$(a)_{2(j)} = u_{23(jl)} - u_{231,2}$ and $W = u_{3(1)} - u_{3(2)}$.

Hence, if $u_{123(ijk)} = 0$, then $W_{12(ij)} = 0$. In this case, the logistic model asserts that factors A and B may affect the ratio $P_{ij}/1 - P_{ij}$, but if they do, they do so independently. If $W_{1(i)} \neq 0$ (or $u_{1e(ik)} \neq 0$), then factor A (amount of chemical exposure) affects uterine bleeding, while if $W_{2(j)} \neq 0$ (or $u_{23(jk)} \neq 0$), we would conclude that socioeconomic status affects uterine bleeding (or that the odds of incidence of uterine bleeding differ in the various socioeconomic strata).

In the example presented here to describe three-way analysis using a log-linear model, exposure to chemicals was found to be associated more with uterine bleeding during the 2nd trimester among women of low socioeconomic status than would have been expected (TABLE VI). The three separate two-way analyses had shown that for the total population of women (exposed and not exposed to chemicals), there were considerably fewer women than expected of lower socioeconomic status who experienced 2nd trimester bleeding (TABLES VIIIb and IX). Also there were far more women of higher socioeconomic status than expected who were exposed to chemicals (p value < 0.0005) in the total population (TABLE VIIIa). Thirdly there were more women of the total population than expected who experienced both chemical exposure and uterine bleeding in the 2nd trimester (TABLES VIIIc and X). Both 1st and 3rd trimester uterine bleeding had also shown a statistically significant association with chemical exposure in the two-way analyses (both with a p value < 0.0005). In contrast to the finding for 2nd trimester uterine bleeding the three-way analyses for 1st and 3rd trimester uterine bleeding which included socioeconomic status did not show any statistically significant association with chemical exposure, indicating that uterine bleeding in the 1st and 3rd trimester was probably associated with all three socioeconomic groups among those who were exposed to chemicals.

It could be concluded that chemical exposure was associated with uterine bleeding throughout pregnancy for those of low socioeconomic status, whereas those who enjoyed a higher standard of living retained the usual uterine stability of the 2nd trimester. Women of all socioeconomic groups exposed to chemicals were similarly at risk in their 1st and 3rd trimesters to uterine bleeding.

DISCUSSION

Work experience brings with it exposure to physical and chemical agents over a period of time. Each childbearing experience involves 9 months and

may occur before, during, and/or after these exposures. There are likely to be few opportunities allowing examination of pregnancy experience which is coterminous with an exposure which has not occurred before conception. We were unable to clearly identify women with such well-defined characteristics, and concluded that in almost all cases of reported exposure to hazardous conditions, at least part of the woman's exposure experience was most likely to have occurred during an undefinable period preconception. Therefore, the expectation that it is possible to examine a direct relationship between occupational exposure and a particular pregnancy experienced at the time is, in our view, unrealistic.

Gestation at registration in the CPP varied markedly, further complicating any estimate of postconception occupational exposure to a hazardous condition. This variable was therefore included as a demographic variable in the analysis.

An effort was made to retrieve information on the father's occupation and work experience. Paternal occupation was recorded with far less accuracy than was maternal occupation, and many women appeared to have little understanding of their partner's work. The reports provided by the wives were clearly unreliable, with insufficient description to provide clues for any occupational exposure to hazardous conditions.

The identification of women in the CPP exposed to hazardous work conditions can now lead to a better examination of rare events, using other statistical methods. The problem of exposure to multiple hazards within each primary exposure group needs further analysis. One approach, using the same contingency table analysis would be to include each additional hazard as another demographic variable. Parity and birth order should also be further evaluated.

An estimation of "dose" from an occupational hazard could only be made indirectly from the response given to the question "Approximately how long?," describing the exposure to an occupational hazard. We coded the response into 1 to 6 months, 7 to 18 months, and more than 18 months. The results, however, are being presented in terms of exposure or no exposure, with the expectation that future, more detailed, analyses can be done in terms of a dose response relationship.

Prematurity was identified as less than 35 weeks gestation, and was found to be associated with exposure to pesticides and not with any of the other exposure groups. We also included a category for gestation of 40 weeks or more, and found those exposed to ionizing radiation, heavy lifting, and animals, were associated with longer gestation. Although we recorded this observation as a pregnancy outcome variable not usually considered to be adverse to the mother or child, more detailed examination is suggested. Ouellette et al., in their study of alcohol abuse during pregnancy, noted an increase in the proportion of postmature infants from 8% and 9% for abstinent and moderate drinkers to 20% for heavy drinkers, although the difference did not meet their statistical criterion of a p value less than 0.01.[9]

An examination of outcome variables and statistical power was not possible during the period of this study. However an extrapolation from calculations made by Kline et al. provides some information.[10] They estimated the sample size and relative risk necessary to detect a change in the frequency of spontaneous abortion in cohorts of pregnant women with a power of 0.80.

	Frequency in exposed	Frequency in unexposed	Relative Risk	Sample size needed for each exposed and unexposed group
Incidence of spontaneous abortion	0.20	0.15	1.3	901

Further examination of Type 2 error does seem feasible for many of the outcome variables, particularly for the groups exposed to chemicals and heavy weights.

The identification within the CPP population of an unexposed control sample with no evidence of adverse pregnancy characteristics is possible. The comparisons with groups of smaller sample size exposed to single hazards may then become clearer. Other refinements of the data are possible, for example, testing for the reliability of a woman's response to questions on her previous pregnancy experience in consecutive pregnancies in the CPP.

SUMMARY

The Collaborative Perinatal Project provides data on a well described population of women pertaining to occupational exposure to hazardous conditions. We examined only 23,961 of the 58,760 pregnancies registered and recoding would be necessary to identify those among the other 35,000 who were also exposed to hazardous work conditions. It is possible, then, to replicate the study reported here or to expand it to a larger sample size. Exposure to one or more hazardous work conditions was reported by 4,575 women of the total 23,961 women for whom this response was coded.

The statistical analysis involved log-linear model analysis of cross-classified categorical data. The influence of demographic variables was identified by testing for three-factor interaction with occupational exposure categories and pregnancy outcome variables. The choice of variables pertaining to pregnancy history, placental characteristics, and the growth and development of the children could be extended to some behavioral characteristics, for which data are available from the CPP. Several variables, e.g., number of umbilical arteries, some placental characteristics, ataxia, seizure states, failure to thrive at 1 year, explored in this study, would not need to be included, in that they do not appear to be sensitive indicators for occupational exposure effects.

Women with a work experience of pesticide exposure had the most adverse reproductive history, observed as more fetal deaths and stillbirths,

premature low weight babies with low 5 minute Apgar scores, suspected neurological abnormalities at 1 year and low I.Q. at 4 years. No statistically significant interaction with demographic variables was found, indicating that all socioeconomic and racial groups were affected comparably.[11]

In addition, occupational exposure to chemicals, heat, and heavy lifting was associated with adverse pregnancy and pregnancy outcome experience. Occupational exposure to ionizing radiation and animals did not show comparable effects.[12]

The occupational experience reported in this study was not coterminous with the pregnancies studied, but reflected the integrated workforce participation and exposure to hazardous conditions for each mother up to and, in some cases, during the pregnancy studies.

Exploration of the CPP data has identified occupational exposure to hazardous conditions for 19% of the women studied. Association of some of these exposures with adverse pregnancy outcome should now lead to more detailed evaluation of birth order, work experience during pregnancy, and total period of occupational exposure to hazards. The effects of a single hazard versus multiple hazard exposures should also be examined further. Expansion of the study population to include women enrolled in the CPP before 1962 should markedly increase the sample size within each exposure group, and a new choice of pregnancy outcome variables can be made to replace those found not to be associated with exposure to hazardous work conditions.

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TABLE I

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4-63
661

SOCIO-ECONOMIC INTERVIEW

25. PATIENT IDENTIFICATION

SECTION D

GRAVIDA'S WORK HISTORY

Now I'd like to talk to you about the kinds of jobs you've had.

REPEAT PREGNANCY — ABRIDGED FORM

R-3. HAVE YOU CHANGED JOBS OR STARTED TO WORK SINCE _____ (DATE from ITEM 8A)

☐ NO (skip to Item 31) ☐ YES (do Section D)

26. DO YOU HAVE A JOB NOW? ☐ YES (complete 26, omit 27) ☐ NO (omit rest of 26, ask 27)

A. HOW MANY HOURS PER WEEK DO YOU WORK? _____ ☐☐ HRS/WK

B. HOW LONG HAVE YOU HAD THIS JOB? _____ ☐☐ YRS ☐☐ MOS ☐ WKS

C. WHAT KIND OF WORK DO YOU DO ON THIS JOB? _____ ☐☐

27. HAVE YOU EVER HAD A JOB? ☐ YES (complete rest of 27) ☐ NO (skip to 28)

A. HOW MANY HOURS PER WEEK DID YOU WORK ON YOUR LAST JOB? _____ ☐☐ HRS/WK

B. HOW LONG DID YOU HAVE THIS JOB? _____ ☐☐ YRS ☐☐ MOS ☐ WKS

C. WHAT KIND OF WORK DID YOU DO ON THIS JOB? _____ ☐☐

D. WHEN DID YOU STOP WORKING? _____ ☐☐ MO. ☐☐ YEAR

28. HOW MANY OTHER KINDS OF WORK HAVE YOU DONE? ☐

☐ ONE OR MORE (specify number in box. Omit A.) ☐ NONE (omit A.)

A. HOW MANY JOBS HAVE YOU HAD? ☐ ONE (omit Item 30) ☐ SEVERAL (omit Item 29 B)

29. A. WHAT KIND OF WORK DID YOU DO FOR THE LONGEST PERIOD OF TIME? _____ ☐☐

B. FOR HOW LONG DID YOU DO THIS KIND OF WORK? _____ ☐☐ YRS ☐☐ MOS ☐ WKS

30. OCCUPATIONAL HAZARDS

QUESTION	NO (0)	YES (1)	DESCRIBE WORK SITUATION	APPROXIMATELY HOW LONG? (In Months)
A. HAVE YOU EVER WORKED WITH X-RAY OR FLUOROSCOPE EQUIPMENT OR BEEN EXPOSED TO X-RAYS IN YOUR WORK?				
B. HAVE YOU EVER WORKED WITH OTHER RADIATION SUCH AS RADIOACTIVE ELEMENTS OR ISOTOPES?				
C. HAVE YOU EVER WORKED WITH TOBACCO DUST OR LEAF OR HANDLED TOBACCO?				
D. HAVE YOU EVER WORKED WITH STEAM OR VERY HIGH HEAT WHERE SPECIAL CARE HAD TO BE TAKEN?				
E. HAVE YOU EVER WORKED WITH CHEMICALS, THEIR DUSTS, GASES OR FUMES IN A JOB SITUATION?				
F. HAVE YOU EVER HAD TO LIFT HEAVY WEIGHTS ON ANY OF YOUR JOBS?				
G. HAVE YOU EVER HAD TO HANDLE ANY LIVE OR DEAD ANIMALS OR BIRDS ON ANY OF YOUR JOBS?				

31. HAVE YOU EVER CARED FOR, HANDLED, PLAYED WITH OR WORKED AROUND FARM ANIMALS, SUCH AS CHICKENS, DUCKS, GEESE, COWS, PIGS, HORSES, DONKEYS, MULES, SHEEP OR GOATS, ETC.? (circle answer)

A. YES NO (omit B and C)

B. HOW OFTEN? _____ INCIDENTALLY (1) FREQUENTLY (2) OTHER (3)

C. WHEN WAS THE LAST TIME THAT YOU DID THIS? _____ ☐☐☐☐ MO. YR.

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PERINATAL RESEARCH BRANCH, NINDB, NIH
BETHESDA 14, MD.

TABLE II

EXPOSURE EXPERIENCE BY HOSPITAL OF REGISTRATION

Hospital	% Exposed
Boston Hospital for Women Lying-in Division	22.6%
Children's Hospital, Buffalo	31.5%
Charity Hospital New Orleans	15.8%
Columbia-Presbyterian Medical Center New York	19.2%
The Johns Hopkins Hospital, Baltimore	30.3%
Medical College of Virginia Richmond	30.5%
University of Minnesota Hospitals, Minneapolis	34.2%
Metropolitan Hospital, N.Y. Medical College	5.8%
University of Oregon Medical School, Portland	21.2%
Pennsylvania Hospital & the Children's Hospital of Philadelphia	19.1%
Child Study Center Providence, RI	33.2%
Gailor Hospital, Memphis Tennessee	10.7%
TOTAL	22.6%

TABLE III

ENROLLMENT IN CPP (1962-1965)-OCCUPATIONAL EXPOSURE

Year	White % Exposed	Black % Exposed	Other % Exposed
1962	28.2%	24.6%	8.3%
1963	26.7%	22.1%	5.5%
1964	26.4%	20.1%	6.9%
1965	25.7%	18.0%	6.7%
TOTAL	26.5%	21.5%	6.4%

TABLE IV

DEMOGRAPHIC DESCRIPTION
OCCUPATIONAL EXPOSURE TO HAZARDOUS CONDITIONS

Race	Unexposed	Exposed
White	47.2%	43.0%
Black	44.8%	55.1%
Other	8.0%	1.9%
Socio Economic Status		
SES 1	8.7%	5.0%
SES 2	60.2%	58.0%
SES 3	31.1%	37.0%
Maternal Age		
10-19 Years	26.3%	13.3%
20-29 Years	56.8%	63.5%
30-39 Years	15.4%	21.0%
40+ Years	1.4%	3.3%
Prior Pregnancies		
0	30.1%	25.0%
1 - 3	47.3%	46.6%
4 - 6	16.5%	19.8%
> 6	6.1%	8.6%
Education		
7 yrs. or less	10.2%	7.6%
8 - 11 years	47.4%	43.9%
12 years	30.3%	29.5%
>12 years	12.1%	18.9%

TABLE V

DEMOGRAPHIC AND PREGNANCY OUTCOME VARIABLES

Demographic	
Race	White, Black, Other
Socio-Economic Index	0-39, 40-59, 60-95
Age of Gravida	<20, 20-29, 30-39, 40+ years
Gestation at Registration	<20, 20-29, 30+ weeks
Smoking during Pregnancy	Yes, No
Abdominal X-ray during Pregnancy	Yes, No
Pregnancy Experience	
Prior Premature Births	None, 1, 2, 3 or more
Prior Fetal Deaths	None, 1-2, 3 or more
Prior Stillbirths	None, 1-3, 4 or more
Current Pregnancy	Liveborn, still living Fetal Death Neonatal Death Death at less than one year Death after one year - yes, no
During Pregnancy	
Uterine Bleeding	1st, 2nd, and/or 3rd Trimester
Anemia	Yes, No
Convulsive Disorder	Yes, No
Kidney, urinary bladder infection	Yes, No
Hydramnios	Yes, No
Placental Characteristics	
Weight	0-300, 301-500, 501-2000 grams
Infarcts	None, all less than 3 cms, at least one greater than 3 cms
Abruptio placenta	No, partial, complete

TABLE V (cont'd)

Placental Characteristics (Cont'd.)		
	Placenta previa	None, total or marginal, marginal or low implantation
	Single umbilical artery	No, Yes
Neonatal Characteristics		
	Gestation at Delivery	Less than 20 weeks, 21-34 weeks, 35-39 weeks, 40 or more weeks
	Birthweight	0-1500, 1501-2500, 2501-4100, 4101+ grams
	5 Minute Apgar Score	0-3, 4-6, 7-10
	Hematocrit (neonatal)	0-49, 50-69, 70+
Growth and Development		
	8 month Mental Score	Abnormal, Suspect, Normal
	8 month Motor Score	Abnormal, Suspect, Normal
1-Year	Ataxia	Yes, No
	Delayed Motor Development	Yes, No
	Seizure States	Yes, No
	Failure to Thrive	Yes, No
	Cleft Palate	Yes, No
	Neurological Abnormality	None, suspicious, abnormal
4-Year	I.Q.	25-79, 80-109, 110-175
7-Year	Neurologic Abnormality	None, Suspicious, Definite
Head Circumference		
	Neonatal	≤30, 31-34, 35-36, 37-38, 39+ cms
	4 months	≤36, 37-40, 41-44, 45+ cms
	8 months	≤40, 41-43, 44-46, 47+ cms
	4 years	≤44, 45-47, 48-50, 51-52, 53+ cms
	7 years	≤48, 49-51, 52-53, 54-56, 57+ cms

TABLE VI

THREE-WAY CONTINGENCY TABLE OF WOMEN OF LOW, MEDIUM, OR HIGH SOCIOECONOMIC STATUS, WITH OR WITHOUT 2ND TRIMESTER UTERINE BLEEDING WHO HAD OCCUPATIONAL CHEMICAL EXPOSURE OF LESS THAN 6 MONTHS, 6 TO 18 MONTHS, MORE THAN 18 MONTHS, OR NONE

Chi-square 15.57, 6 degrees of freedom, p value = 0.016

B: Socioeconomic Status

C: 2nd Trimester Uterine Bleeding		Low			Medium			High		
		Yes	No	Total	Yes	No	Total	Yes	No	Total
A: Amount of Chemical Exposure	None	X_{111}	X_{112}	n_{11}	X_{111}	X_{112}	n_{12}	X_{131}	X_{132}	n_{13}
	Low	X_{211}	X_{212}	n_{21}	X_{221}	X_{222}	n_{22}	X_{231}	X_{232}	n_{23}
	Med.	X_{311}	X_{312}	n_{31}	X_{321}	X_{322}	n_{32}	X_{331}	X_{332}	n_{33}
	High	X_{411}	X_{412}	n_{41}	X_{421}	X_{422}	n_{42}	X_{431}	X_{432}	n_{43}
		$X_{.11}$	$X_{.12}$	$n_{.1}$	$X_{.21}$	$X_{.22}$	$n_{.2}$	$X_{.31}$	$X_{.32}$	$n_{.3}$

C: 2nd Trimester Uterine Bleeding		Low			Medium			High			
		Yes	No	Total	Yes	No	Total	Yes	No	Total	
A: Amount of Chemical Exposure	None	692	7873	8565	622	5905	6527	582	6355	6937	22029
	Low	18	100	118	17	133	150	17	141	158	426
	Med.	14	55	69	5	93	98	12	166	178	345
	High	16	129	145	22	146	168	28	284	312	625
		740	8157	8897	666	6277	6943	639	6946	7585	2342

TABLE VII

OBSERVED PROBABILITIES OF WOMEN OF LOW, MEDIUM, OR HIGH SOCIOECONOMIC STATUS, WITH NO CHEMICAL EXPOSURE, EXPOSURE OF LESS THAN 6 MONTHS, 6 TO 18 MONTHS, OR MORE THAN 18 MONTHS

		Socioeconomic Status		
		Low	Medium	High
Amount of Chemical Exposure	None	p_{11}	p_{12}	p_{13}
	Low	p_{21}	p_{22}	p_{23}
	Med.	p_{31}	p_{32}	p_{33}
	High	p_{41}	p_{42}	p_{43}

		Socioeconomic Status		
		Low	Medium	High
Amount of Chemical Exposure	None	.081	.095	.084
	Low	.153	.113	.108
	Med.	.203	.051	.067
	High	.110	.131	.090

TABLE VIIIa

SAMPLED POPULATION OF WOMEN
SOCIOECONOMIC STATUS BY AMOUNT OF CHEMICAL EXPOSURE

Chi-square 191.09, 6 degrees of freedom, p value < 0.0005

		Socioeconomic Status			
		Low	Medium	High	
Amount of Chemical Exposure	None	n_{11}	n_{12}	n_{13}	$n_{1.}$
	Low	n_{21}	n_{22}	n_{23}	$n_{2.}$
	Med.	n_{31}	n_{32}	n_{33}	$n_{3.}$
	High	n_{41}	n_{42}	n_{43}	$n_{4.}$
		$n_{.1}$	$n_{.2}$	$n_{.3}$	n

		Low	Medium	High	
Amount of Chemical Exposure	None	8565	6527	6937	22029
	Low	118	150	158	426
	Med.	69	98	178	345
	High	145	168	312	625
		8897	6943	.7585	23425

TABLE VIIIb

SAMPLED POPULATION OF WOMEN
2ND TRIMESTER UTERINE BLEEDING BY SOCIOECONOMIC STATUS

Chi-square 9.27, 2 degrees of freedom, p value < 0.01

		2nd Trimester C: Uterine Bleeding		
		Yes	No	
Socioeconomic Status	Low	$X_{.11}$	$X_{.12}$	$n_{.1}$
	Med.	$X_{.21}$	$X_{.22}$	$n_{.2}$
	High	$X_{.31}$	$X_{.32}$	$n_{.3}$

		Yes	No	
Socioeconomic Status	Low	740	8157	8897
	Med.	666	6277	6943
	High	639	6946	7585
		2045	21381	23425

TABLE VIIIc

SAMPLED POPULATION OF WOMEN
CHEMICAL EXPOSURE BY 2ND TRIMESTER UTERINE BLEEDING

Chi-square 9.93, 3 degrees of freedom, p value = 0.019

		2nd Trimester C: Uterine Bleeding		
		Yes	No	
A: Amount of Chemical Exposure	None	$X_{1.1}$	$X_{1.2}$	$n_{1.}$
	Low	$X_{2.1}$	$X_{2.2}$	$n_{2.}$
	Med.	$X_{3.1}$	$X_{3.2}$	$n_{3.}$
	High	$X_{4.1}$	$X_{4.2}$	$n_{4.}$
		$X_{..1}$	$X_{..2}$	n

		Yes	No	
A: Amount of Chemical Exposure	None	1896	20133	22029
	Low	52	374	426
	Med.	31	314	345
	High	66	559	625
		2045	21380	23425

TABLE IX

EXPECTED FREQUENCIES OF 2ND TRIMESTER UTERINE BLEEDING BY SOCIOECONOMIC STATUS

Chi-square 9.27, 2 degrees of freedom, p value < 0.01

		2nd Trimester Uterine Bleeding		
		Yes	No	Total
Socio-economic Status	Low	$n_{.1} X_{..1}/n$	$n_{.1} X_{..2}/n$	$n_{.1}$
	Medium	$n_{.2} X_{..1}/n$	$n_{.2} X_{..2}/n$	$n_{.2}$
	High	$n_{.3} X_{..1}/n$	$n_{.3} X_{..2}/n$	$n_{.3}$
		$X_{..1}$	$X_{..2}$	n

		Yes	No	Total
Socio-economic Status	Low	776.71	8120.29	8897
	Medium	606.12	6336.88	6943
	High	662.17	6922.83	7585
		2045	21380	23425

TABLE X

EXPECTED FREQUENCIES OF 2ND TRIMESTER UTERINE BLEEDING BY CHEMICAL EXPOSURE

Chi-square 9.54, 3 degrees of freedom, p value < 0.025

		2nd Trimester Uterine Bleeding		
		Yes	No	Total
Chemical Exposure	None	1923.13	20105.87	22029
	Low	37.19	388.81	426
	Medium	30.12	314.88	345
	High	54.56	570.44	625
		2045	21380	23425

THE OXFORD SURVEY OF CHILDHOOD CANCERS

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INTRODUCTION

The Oxford Survey has covered children's cancer and leukaemia in England, Wales, and Scotland since 1953. It is considered important in being a national survey, first, because it is unique in the world and second, since childhood neoplasia is a rare disease, it is difficult to accumulate a sufficient number of cases for epidemiologic study of cancer causation.

On magnetic tape, there are now complete details on 26,325 index children, and nearly as many controls. Approximately 300 variables are recorded for each child. The advantage of the Oxford Survey over other registries is that the coverage is more complete as both cancer registrations and death certificates are available. Through the sustained cooperation of personnel of the Area Health Authorities, from Government funds, parental interviews are obtained on approximately 80% of the children, producing facts for epidemiological study which are not available so cheaply by other methods.

The present position is that there is a large quantity of basic data now available for study; much of this has already been analyzed and published, as detailed later. Other studies are in progress. In 1973, there was a general review of the material collected, under Professor Doll's supervision, and since that time the questionnaire has been altered, and some details previously collected have been dropped--e.g., parents' childhood illnesses and blood groups, information on grandparents and other close relatives. The present questionnaire concentrates on the relevant maternal antenatal history; details of siblings, including miscarriages and stillbirths, congenital defects and deaths; and parental occupations, environmental hazards, smoking, and contraceptive habits.

The future strategy can be adapted to fit in with special projects and with current needs. Obviously it is desirable to continue descriptive and case/control studies on data already collected, but at the same time this store of data could be used not only as a retrospective survey, but as the end point of prospective studies in collaboration with other workers, who could use the material in conjunction with their own work.

BACKGROUND

This Survey of Childhood Cancers was started in Oxford, in 1955, by Dr. Alice Stewart, and has continued since then as a retrospective case/control study of all cases of children reported to us as suffering from neoplasm (leukaemia or cancer) by death certificates or cancer registrations, in England, Wales, and Scotland. Epidemiological facts are

ascertained by parental interviews, and antenatal and medical information is obtained by postal application to general practitioners, antenatal clinics, and hospitals. It should be emphasized that most of the parental interviews are undertaken by the Area Health Authorities, and that this unpaid assistance was incorporated into the Survey from its earliest days, and has successfully survived the 1974 reorganization of the National Health Service.

Close collaboration is maintained between this Unit (which holds all original records and does all data collection) and the Units in Birmingham and Oxford. The whole Survey was divided subsequent to Dr. Alice Stewart's retirement from Oxford in 1974. Dr. Stewart and her statistician, Mr. George Kneale, have continued to work in Birmingham University on past and recent data supplied to them by my Unit. The Unit left in Oxford is, at present, The Childhood Cancer Research Group (Director, Dr. G. Draper, in Professor Sir Richard Doll's Department of Medicine), and is a national childhood cancer registry which is not directly concerned with epidemiology.

PLAN OF PROJECT, METHODS, AND TECHNIQUES

1. Procedures Currently Completed in Oxford

Photocopies of cancer registration abstracts and death certificates (for deaths coded to i.c.d. numbers 140-239) are sent to Oxford from the Office of Population Censuses and Surveys, and from the Scottish Home and Health Department. Here they are checked, and abstracts are transferred to magnetic tape. At this point, copies of the cancer registrations and death certificates are sent to Oxted. From here on, my Unit is entirely responsible for collecting further data, for processing it into suitable coded form, and for transferring it to the Birmingham and Oxford University computers, by using on-line console.

2. Procedures Currently Completed in Oxted

(i) Family and Epidemiological Data

The incoming death certificates are carefully scanned and sorted into areas. Every year, a list of children who died during the previous 12 months is sent out to each of the Area Health Authorities' Medical Officers. There are 88 of these areas in England, 8 in Wales, and 15 in Scotland. Interview papers, together with letters of approach to the parents of the dead children, are enclosed together with all the latest information from the Survey. The Area Health Authorities then undertake to make contact with the parents, having first asked permission and advice from the general practitioners, and conduct the interviews for us.

This work is undertaken voluntarily, and no fee is charged. Eleven out of the 111 Authorities are unable to cooperate with this, and the Unit undertakes to find and fund adequate alternative personnel to carry out the interviews. The Area Health Authorities also undertake to find healthy control children from their own birth registers. Each case child within the

area is matched with six other children according to sex, date of birth, and locality. From this list of six children, the interviewer then finds one control child, whose parents are asked to give exactly the same information as that required from the parents of the deceased children. The Area Health Authorities have continually given the Survey their interest and support, and a close liaison now exists which was able to survive the recent reorganization of the whole National Health Service in April 1974. There has been a steady interview success rate of 78% during the whole period of the Survey; 9% are parental refusals, 8% lost addresses, and 5% other causes, such as G.P. advice. When the interview forms are completed, they are returned to the Unit and information is abstracted relevant to the family history, the health record of the child, and the previous medical history of the mother and father. Special attention is given to the siblings with reference to congenital defects or other malignancies, and to the mother's antenatal history. All this information is then coded, ledgered, and transferred to the computer through the on-line console in the Unit's department.

(ii) Antenatal Data

The interview forms contain information as to where the mother had her antenatal care and a request for her consent for further follow-up. Forms are subsequently sent to the G.P., antenatal clinic, or hospital, requesting information on the case and control mother's health during the relevant pregnancy, details of any illnesses, or of drugs administered, incidence of any X-rays taken, or of the use of ultrasonic techniques, details of the birth, and puerperium. If abdominal X-rays or ultrasound tests have been used, then further information is requested on number of exposures, interval before birth, reason for the X-ray or ultrasound, and the findings. These are independently collected data which confirm or deny the mother's statement, and therefore exclude the natural bias entailed in interviewing bereaved mothers. We consider this part of the work to be of great importance in the light of present hypotheses on the effect of antenatal events upon the foetus. It is desirable for the work to continue so that the most recently included questions as to the effects of ultrasound, previous parental occupational hazards, smoking, and the use of drugs and hormones in pregnancy may build up into a store of necessary and useful information.

(iii) Medical Data of Child

General Practitioners' records are obtained by post at no charge from Family Practitioner Committees. From these Survey abstracts, the name, sex, date of birth, region, general practitioner's name, hospitals attended with dates and hospital numbers, date of onset of the relevant illness, and a brief history of the illness are obtained. If these records prove to be incomplete, the specified hospitals are written to, and are given the choice of either loaning their notes by post for a few days, or of filling out one of the Survey forms. The medical data thus collected are coded and ledgered preparatory to transfer to the computer. Full and adequate medical data are collected in 98% of all cases.

(iv) Surviving Children

The procedure for surviving children is slightly different, as we consider it unethical for strangers to conduct interviews with parents without prior knowledge of the current health of the child. The names of surviving children are obtained by us from regional cancer registries, then the name and address of the general practitioner is found by approaching the hospital of treatment or the Family Practitioner Committee. Each child's own practitioner is then written to, and asked to send information regarding the child's illness, the mother's antenatal history and details of the family, sibling deaths, or congenital defects, etc. It is left entirely to his discretion whether he interviews the mother, or completes the questionnaire from his own notes and knowledge of the case. Thereafter, the medical and antenatal data are completed by the same method as stated above for the deceased children. It must be stressed that these cases are not matched for controls, due to the difficulties and expense involved in asking busy general practitioners to spend further time in finding adequate control children.

The material gained from the study of surviving children is mainly used in survival analyses, and in comparison of treatment with different hospital centres and trials, and in the study of familial diseases or congenital defects between the dead and the survivors' series. The surviving children's series cannot be compared with the retrospective case/control study of the deceased children. For the past 10 years the epidemiology of survivors has been obtained in this way. There is a necessary time lag as we do not approach the parents of surviving children until they have survived at least 3 years.

PRESENT EPIDEMIOLOGY STUDIES

1. Congenital Defects Associated with Cancer Purpose

There are many studies on the relationship between some children's cancer and congenital defects, and many anecdotal papers on this subject. It has been thought worthwhile to analyze our material to try to clarify the position by using our large numbers of case and control children. A large-scale study has therefore already been started on congenital defects in index children, and among their siblings, and on congenital defects among the control children, and their siblings. Also, all consanguineous marriages have been identified, and any increased incidence of abnormalities and/or cancer will be described.

Background

This work was originally proposed in collaboration with Dr. J. F. Bithell (late of the Childhood Cancer Research Group), Oxford University. The abstract of a collaborative paper given in 1975 at the 2nd Joint Scientific Meeting of Epidemiology and Social Medicine was as follows:

The Marie Curie/Oxford Survey of Childhood Cancers collected data on some 12,000 children dying under 16 years of age with malignant disease

between 1953 and 1967. Interviews were obtained (usually with the mothers) in about 75% of cases and of these, 950 children were recorded as having a coexisting congenital defect.

The paper analyzes the defects found, comparing the observed frequencies with national rates, where known, and discusses the possible genetic significance of the associations with malignancy. Certain associations are already well-known; thus of 117 children with Down's Syndrome in the Survey, for example, 114 had leukaemia, an excess indicating a crude risk of leukaemia to mongols some 13 times the population risk. Adjusting for the attenuation of the mongol population owing to deaths from other causes gives estimated risks to survivors at specified ages; the relative risk exceeds 90 in the second year of life.

Again, the known association between Wilms' tumour and growth defects stands out clearly; thus 10 cases of Wilms' tumour also affected by hemihypertrophy were observed where only 1 was expected: similarly with aniridia and genitourinary anomalies. Inevitably there are certain previously unreported associations apparent which may be due to the small numbers involved. Among children with bone cancers there are not only the known associations with bone abnormalities, but also with mental retardation. In most cases, associations recorded for the index children are not reflected as associations between a tumour and a congenital abnormality in the sibs of propositi with that tumour. An analysis of the sibs does, however, yield some interesting families with matching defects. Information on consanguinity is also available for some of the cases. Although the numbers of families involved are very small, there is evidence of an increased risk both of cancers and congenital abnormalities in general.

Plan of Project, Methods, and Techniques

So far, the years under study have been extended from 1967 to 1971. All the necessary information has been abstracted from the original documents and transferred to computer cards. Much of the analysis has already been done and tables have been computed. It now remains to study these and to write up the paper in detail.

2. Influence, if any, of Antenatal Drugs or Hormone-Administration; Parental Occupations (Re: Contact with Chemicals, Radioactive Substances, etc.), Ultrasonic Tests, Smoking in Pregnancy, and the Use of Contraceptive Pills

Purpose

Since 1971, direct questions relating to the above mentioned agents have been included in the interview forms. Since the numbers of cases are still too small, it is too early to analyze any effect on case children as compared with controls. It is therefore proposed to continue data acquisition for these studies until there are sufficient cases to allow meaningful analyses.

Background

The possibilities of injury, and the mutagenic effects of abnormal stimuli on the foetus during early uterine life have been generally recognized in recent years, but have not been fully explored. This Survey demonstrated the effect of low-level obstetric radiation[1,2] and the possible effect of virus infections during pregnancy.[3]

The Bureau of Radiological Health, U.S.A., requested that the investigations on the foetus of ultrasonic tests be made, and this information has been recorded since 1972. So far numbers are insufficient for meaningful analyses. We planned to carry out the other investigations from the slightly earlier date of 1970.

Plan of Project

Questions are included in the interview forms as follows:

These questions are asked of both case and control parents, and are followed through to the antenatal doctor or clinic where the mother's claims are either confirmed or denied. At the present time, we are achieving an 80% interview success rate, and a further 85% success rate in the return of records in response to our queries to those in charge of antenatal care. When sufficient numbers are accumulated, the number of positive and negative replies can be totalled for both case and control children.

3. National Morbidity and Survival Figures

Purpose and Background

The Survey has been in existence since 1955 and covers, as far as possible, all cancer deaths from 1953 to 1960 of children under 10 years in England, Wales, and Scotland, and of children under 16 years from then till 1976. Surviving children have been notified to us since 1962, and there are now 10 completed years of children diagnosed in the years from 1962 to 1971. This timing allows for a 5-year follow-up. There are thus well over 20,000 cases now in the Survey, and practically all but the most recent are already on magnetic tape. I consider it very desirable that much of this accumulated data should be analyzed and published.

Plan of Project, Methods, and Techniques

So far, the data from the Survey have been computed by the Childhood Cancer Research Group in Oxford, and survival analyses are now available for the following main diagnostic categories: lymphoid leukaemia, myeloid leukaemia, monocytic leukaemia, other and unspecified leukaemia, Hodgkin's disease, non-Hodgkin's lymphomas, Wilms' Tumour, C.N.S. malignant tumours, neuroblastoma, bone tumours, other solid tumours, and benign and unspecified tumours. In addition to this, and using the same data, it would be possible, and important, to produce figures showing the incidence of various categories of the disease, and to demonstrate changes occurring over the period of 20 years. Using these figures in conjunction with survival

figures, it might be possible to correlate them with the effects of different methods of therapy known to have been introduced during this period.

4. The Hazards of Chemotherapy

Purpose

Since it is at present becoming obvious that radiotherapy for malignancy carries a risk of loss of growth, infertility, and the possibility of a second neoplasm, it is desirable that the possible hazards of chemotherapy should also be investigated. This might be undertaken by this Survey.

Background and Method

In the Survey there are already over 90 cases in which a second primary neoplasm has been identified. Some of these can be directly related to previous irradiation (6 cases of leukaemia; and 12 cases of osteosarcoma, 10 of which followed after irradiated retinoblastoma, 5 local and 5 distal, 1 after an irradiated medulloblastoma, and 1 after Hodgkin's disease). Other secondary malignancies appear to follow a possible genetic predisposition toward a special category of neoplasia, for example, the cases of varying types of C.N.S. tumour which follow congenital and often familial neurofibromatosis. These figures give rise to concern about intensive courses of chemotherapy.

If it is possible to continue the Survey long enough for cases to accumulate, it might be possible to collect a series of cases treated only with chemotherapy, and to search these for possible hazards: i.e., secondary neoplasms might be demonstrated, and by a long-term follow-up we could assess the risks of failure of growth, intellectual impairment, and the chance of infertility or further genetic damage as shown in the offspring.

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2. Bithell, J. F., and Stewart, A. M. 1975. Pre-natal irradiation and childhood malignancy: A review of British data from the Oxford Survey, British Journal of Cancer, 31, 271.

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APPENDIX A

Key to Margin Codes

If there is no mark this means that the variable has been recorded throughout the survey.

1. Data collected from 1953-55 only.

2. Data collected but not on tape since 1961.

3. Data collected, but not on tape since 1970.

4. Data collected and on tape since 1971.

Variables Collected by Oxford Survey of Childhood Cancers

Sex of Case and Control.
Sib position for Case and Control.
Month and year of birth, onset of disease, diagnosis, death, or last follow-up.
Age difference between Case and Control.
Details of Control choice.
Place of birth of Case and Control (domiciliary or hospital).
Diagnosis on death certificate or cancer registration.
Final diagnosis (after abstraction of medical data).
Pregnancy X-ray of mother, Case and Control.
Age of Case mother and father at birth of index child, also Control.
Dates of parents' births and marriage.
Number of pregnancies, Case and Control.
Twin pregnancies, Case and Control.
Male births, Case and Control.
Female births, Case and Control.
Zygosity and sex of twins, Case and Control.
Congenital defects, Case and Control.
Sibling congenital defects, Case and Control.
Consanguineous marriages, Case and Control.
Pregnancy X-ray details (reason, result, and date), Case and Control.
Pregnancy X-ray, number of films, Case and Control.
3 Pregnancy X-ray, details of hospitals, Case and Control.
Other X-rays of mother, Case and Control.
2 Other X-rays of father, Case and Control.
Child's infectious diseases, Case and Control.
Child's other diseases with intervals, Case and Control.
1 Child's consumption of coloured sweets and soft drinks, Case and Control.
1 Child's exposure to television, Case and Control.
1 Child's contact with pets, Case and Control.
3 Paternal consumption of tea and coffee, Case and Control.
3 Blood groups, Case and Control mother, and Case child.
3 Grandparents' cause of death, age of death, other serious illnesses, Case and Control.

3 Cancers in other relatives (aunts, uncles, and first cousins of index child), Case and Control.
Mother's pregnancy illnesses with dates, Case and Control.
Mother's other previous illnesses, Case and Control.
2 Father's illnesses, Case and Control.
Radiotherapy, mother and father and index child, Case and Control.
Medical data source.
Social class, Case and Control.
Local Authority region at death, Case.
Coding cancer cases according to ICD and MOTNAC numbers.
Child's previous diagnostic X-ray (site, reason), Case and Control.
Dates and age in months of case child at onset, diagnosis, death or latest follow-up.
4 Details of birth (mode, position, and complications), Case and Control.
4 Breast feeding, Case and Control.
4 Parental smoking habits, Case and Control.
4 Mother's smoking habits during relevant pregnancy, Case and Control.
4 Mother's drug administration during relevant pregnancy, Case and Control.
4 Summary of child's immunization, Case and Control.
4 Details of mother's use of contraceptive pills, Case and Control.
4 Ultrasonic tests during relevant pregnancy, Case and Control. (since 1972)
4 Local Authority region at birth (Case and Control) and death (cases) through whole period of survey.
4 Parents' previous drugs for severe illnesses, Case and Control.
4 Parents' occupational exposure to radiation (to petroleum, industrial dusts, and chemicals, since 1976), Case and Control.

Coverage of Parents' Occupations

Year	Mother	Father
1953-60	Occupations before and after marriage.	Occupations as stated on child's death certificate.
1965-70	Occupations with dates.	Ditto.
1971	Current occupation and previous occupational exposure to radiation, etc.	Ditto.

RECOMMENDATIONS FOR FUTURE RESEARCH

Until recently, little attention has been focused on occupationally related reproductive hazards. As a result, awareness of the occupational setting as a factor in the etiology of these problems is limited. Of concern is significant alteration in the physiological process of reproduction including adverse effects such as: heritable changes in genetic material, adverse effects on sperm, infertility, pregnancy loss, prenatal and postnatal growth retardation, physiologic and behavioral diseases in offspring, structural malformations, malignancy induced during gestation and other transplacental effects. The significance of effects at each stage of reproduction needs to be evaluated separately. The utilization of available techniques and the development of better methodology are essential in order to adequately assess potential etiologic factors related to these adverse effects. As a result of the Workshop, four areas that require attention have been identified. Summaries of recommendations in each of these areas follow:

A. Epidemiologic Study for the Assessment of Reproductive Hazards

Human reproductive wastage is a significant problem. An estimated 15% of couples trying to have children are infertile,[1] and it is estimated that 10-15% of recognized pregnancies end in spontaneous abortion.[2] Five to six percent of live born children have congenital anomalies.[3] Congenital heart disease occurs in human populations at the rate of about 5 per 1,000 live births, however, only 5% of the cases have a known genetic or environmental etiology.[4] Prematurity and low birth weight are two of the most significant problems in obstetrics today since these factors are associated with more than 50% of neonatal deaths,[5] are associated with low I.Q.'s, and neurological abnormalities.[6]

There is substantial evidence that certain agents commonly found in the occupational setting can affect normal sexual functions and the ability to produce healthy offspring. It is essential to consider the possibility that additional environmental and occupational agents exert a toxic effect on human reproduction and are responsible in part for reproductive wastage. Even a small percentage increase in affected pregnancies due to occupational exposures would result in a large absolute number of such pregnancies. However, considering the vast number of substances in the workplace, there have been few studies undertaken to evaluate their potential effect in the role of human reproduction. This is true despite considerable evidence indicating that occupational/environmental agents can adversely affect reproduction in laboratory animals.

At present, there is no organized and systematic effort to collect and collate information on occupational factors in relation to reproductive hazards. Immediate action is required commensurate with that directed toward other environmentally related diseases such as cancer.

Recommendations:

1. A short occupational history form should be developed and standardized to facilitate collection of data in a standardized manner. This

form should be used by the medical community and by existing data gathering organizations, including registries, on both the national and international level.

2. The comprehensive National Institute for Occupational Safety and Health Reproductive History Questionnaire is recommended for use in collecting data for studying possible reproductive effects of specific hazardous substances, or work environments. This questionnaire should be used with its accompanying interviewer instructions to standardize interview methods and optimize comparability of data.

3. All stillbirths (20 or more weeks gestation or 500 or more grams) should be certified.

4. Occupational histories of both parents should be recorded on all:

   a. birth certificates;

   b. stillbirth certificates;

   c. death certificates for children to the age of 16 years.

5. Epidemiologic assessment of a larger spectrum of endpoints should be made to measure transplacental effects.

6. Data sources such as the Oxford Survey of Childhood Cancer and the Collaborative Perinatal Project should be more fully utilized and supported.

7. Other existing data sources should be surveyed and assessed for their potential in determining reproductive hazards in relation to occupational exposure. The need for new data banks should be assessed.

8. Existing birth defects registries in the Scandinavian countries should be encouraged to coordinate their activities and supported so that larger data bases may be generated to permit greater statistical power.

B. Whole Animal Testing For the Assessment of Reproductive-Developmental Hazards

The underlying motivation in considering nonhuman test systems is that the human population should be spared serving as the primary test species for toxic agents in the environment. Regardless of imperfections of animal test systems, the response of laboratory animal species to environmental agents should be determined prior to human exposure. The use of animal systems also is essential for chemicals currently being produced. The shortcomings of traditional epidemiological studies in assessing reproductive hazards would tend to increase the need for establishing presumptive hazards based on animal experiments. Additionally, since humans should not be deliberately exposed to known reproductive toxicants, dose-response relationships and the determination of mechanism of action must be elucidated from laboratory animal research.

Recommendations:

1. An adequate test of reproductive toxicity in laboratory animals should include exposure to test materials throughout the sensitive stages of life. Exposure of male and female animals should begin prior to mating in order to cover the periods of spermatogenesis and ovulation, and continue throughout gestation to early neonatal life. In classical teratological testing, exposure to test materials has been limited to maternal animals only during the organogenesis period, an approach that does not address toxic effects exerted on other sensitive processes such as gametogenesis and blastogenesis.

2. The evaluation of transplacental effects has been limited primarily to the assessment of gross abnormalities in structural development. Endpoints also should include adult fertility as well as embryolethality, stillbirths, pre- and postnatal growth retardation, minor structural abnormalities, and functional deficits in the offspring such as behavioral and physiological abnormalities.

3. Standard carcinogenesis testing usually ignores the potentially greater susceptibility of the fetus and neonate which have immature immune systems relative to adults. While no agents are known at present that are carcinogenic exclusively to the fetus or neonate, the immature organism has been shown to be more sensitive to some carcinogenic agents than adults. Carcinogenesis testing protocols should be extended to include prenatal and neonatal exposure periods.

4. The selection of exposure dose and route in animal testing should model the human exposure condition. Comparability in routes of administration and dose interval are essential considerations. In teratological testing, the highest dose level should not exceed the maternal maximum tolerated dose (MTD)* and at least two lower dose levels at some standard fraction of the MTD should be employed. The relationship between doses causing adult toxicity versus embryotoxicity should be determined. Exposures at dose levels below those causing excessive maternal toxicity would reduce the occurrence of false positive results. In all cases, the litter should be considered the experimental unit of analysis for statistical purposes.

5. At present, there is no animal test system for reproductive risk assessment that has been validated against known human teratogens. There are numerous examples of seeming inconsistencies between results of teratogenesis tests in animals and epidemiological data. This apparent problem may be partially resolved if the recommended principles for animal testing are adopted, and if adequately conducted epidemiological studies addressing multiple endpoints are undertaken. It should be recognized that the current state of knowledge in teratology is insufficient to permit reliable extrapolation of toxicity data from laboratory animals to the human condition. However, a positive response in experimental animals should be considered as presumptive of human risk on a qualitative basis.

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*MTD is defined as that dose level causing no more than a 10% weight reduction in exposed animals, and no deaths.

6. Although any chemical with mutagenic activity may pose a teratogenic risk, teratogenic effects can also result from nonmutational mechanisms. Thus, even if a chemical is demonstrated to have negligible mutagenic activity, reproductive and teratologic studies should still be performed.

7. There is a pressing need for more definitive and economic test systems for assessing teratogenic risks. Results with organ and whole embryo culture are encouraging, but there has not been sufficient validation of these systems to recommend them as standard procedures. Research efforts in development of *in vitro* teratogenesis test systems utilizing organ and whole embryo culture, rather than single cells, are strongly encouraged.

C. Non-human Test Systems for Detecting and Characterizing Mutagens

There are three types of mutational events: gene (point) mutations, and structural and numerical chromosomal aberrations. Test methods in mutagenicity usually measure one of the events to the exclusion of the others. Consequently, one must employ a battery of methods to detect agents in the environment capable of producing mutations.

The following systems are listed with a view toward their utility in the area of occupational exposure, with emphasis on their ability to assess potential reproductive hazards in humans. To categorize a chemical as a mutagen there should be multitest evidence of its activity. This, however, does not imply investigation by each method listed. A detailed explanation of these systems can be found in reference 7.

Test Systems:

1. For Gene Mutations

    a. Salmonella-microsome test
    b. Chinese hamster ovary cells
    c. Mouse lymphoma L5178Y
    d. Tradescantia
    e. *Drosophila* sex-linked recessive lethal test
    f. Specific locus test in the mouse
    g. Host-mediated assay and body fluid analysis

2. For Structural Chromosomal Alterations

    a. Mammalian cells
    b. Micronucleus test
    c. *In vitro* cytogenetics
    d. Dominant lethal test
    e. Heritable translocation test

3. For Numerical Chromosomal Alterations

    a. *In vitro* anaphase analysis
    b. Metaphase II analysis in gametes

Recommendations:

1. All *in vitro* methods should be coupled with metabolic activation procedures. It should be recognized, however, that such *in vitro* metabolic activation systems may only approximate the metabolism that occurs in the mammal.

2. Under no circumstances should a chemical be presumed non-mutagenic solely on the basis of *in vitro* tests, and conversely, testing of a mutagen identified by *in vitro* procedures should be examined by *in vivo* methods.

3. Nondisjunctional assays in *Drosophila* should be further evaluated with chemical agents as has been accomplished with x-irradiation.

4. The only method to analyze nondisjunction in females is the analysis of meiotic stages in oogonia. Due to the relatively time-consuming preparation, application of this technique for general screening does not appear advisable at this time. This method should be employed for special problems.

5. The heritable translocation and specific locus tests should be reserved for special studies rather than employed as general screening techniques. The remaining procedures should be sufficient to identify a chemical as a mutagen. In certain instances in which a chemical is not active in the latter procedures, but where population exposure is widespread, e.g., food additives, the heritable translocation test or specific locus test should be employed to further insure safety.

D. Advanced Warning Systems for Detecting Mutagens in Human Populations

An estimated 6% of all newborns have some type of congenital malformation[3] and 2% of all newborns have a demonstrable genetic defect.[8] In order to minimize this genetic burden, it is imperative that we identify and control any agent capable of inducing mutational events in man. Ongoing research programs in the development of human test systems for identifying mutagens have resulted in a number of tests that have demonstrated their ability to detect many environmental agents that induce genetic lesions. These tests include *in vivo* cytogenetics and body fluid analysis.

The fact that the data are being derived from humans is the major advantage of these procedures. Additionally, since these procedures can be used in animal models they tend to bridge the gap between animal and human studies, thus offering the possibility of verification and correct interpretation of results from nonhuman test systems. These toxicological procedures play a unique intermediate role between the presumptive relevance of animal studies and confirmatory results obtained by classical epidemiological procedures. For example, these procedures have identified adverse effects from occupational exposure to radiation, benzene, epichlorohydrin, lead, styrene, dibromochloropropane, and vinyl chloride. However, there is a major obstacle to the meaningful, large-scale application of these available tests, in working populations, i.e., at present it is extremely difficult to identify and gain access to populations exposed in the occupational setting.

Recommendations:

1. Since there are several practical procedures available for detecting mutations in man, it is essential that management, labor, and government determine the most feasible approach and initiate a surveillance program to identify and eliminate exposures to genetic hazards among industrial workers.

2. The highest priority for a surveillance program should be given to industrial populations taking into consideration both the number of individuals exposed in the workplace and in the general environment. In addition, persistence of these chemicals in the environment should be considered.

3. Any detectable adverse effects from these human surveillance systems should be considered of highest relevance and should lead to immediate action by management, labor, and government.

4. Despite negative results by appropriate animal testing, new products should be evaluated for mutagenic activity prior to widespread public usage by chromosomal studies and by analysis of body fluids of exposed workers.

5. Because study results indicate a relatively high incidence of chromosomal anomalies among abortuses, as contrasted with the incidence among live births, cytogenetic examination of abortus material is encouraged, as the power of the test for detecting mutagenetic damage is much greater with a relatively smaller sample of the units being studied.

6. Available methods for monitoring human populations have already detected several chemicals as genetic hazards. Further research is needed to expand the spectrum of genetic lesions that can be detected. Promising techniques where preliminary data are available, but where further developmental research is needed and encouraged include alkylation of macromolecules, fluorescent study of nondisjunction in sperm, sister chromatid exchange procedures, and *in vivo* anaphase analyses. Better methods for measuring gene mutations and improved procedures for detecting meiotic chromosomal effects in man must be developed.

E. Recommendations for Education

Further development in the curricula of nursing, osteopathic, allopathic medical, and other related health institutions and health programs toward an awareness and understanding of occupational factors in the etiology of disease, and specifically of those factors associated with reproductive hazards, is essential. One strategy to insure improvements in medical training programs may be to establish working contacts with accreditation associations. Additional efforts should be directed toward the ascertainment of a better occupational history for the purpose of developing data bases to further assess associations of such exposures and reproductive hazards. The availability of more specific information should increase the sensitivity of surveillance. Improved medical history protocols which include adequate information for assessing reproductive hazards must be more widely used by health professionals.

National Institute for Occupational Safety and Health and Occupational Safety and Health Administration funded programs should include worker education with regard to reproduction hazards. Educational programs for the dissemination of information already available would result in a better understanding of this problem by workers, employers, members of the medical community, and governmental agencies. More research training grants should be established to encourage students to enter the field of reproductive toxicology.

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