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Detection of Low Levels of Urinary Mutagen Excretion by Chemotherapy Workers Which Was Not Related to Occupational Drug Exposures

Richard B. Everson,[1] Jennifer M. Ratcliffe, Peter M. Flack, Dennis M. Hoffman, and Arthur S. Watanabe

Epidemiology Branch, Biometry and Risk Assessment Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [R. B. E., P. M. F.]; Industry Studies Branch, Division of Surveillance, Hazard Evaluations and Field Studies, National Institute of Occupational Safety and Health, Cincinnati, Ohio 45226 [J. M. R., A. S. W.]; and University Hospital, Health Sciences Center, State University at Stony Brook, Stony Brook, New York 11794 [D. M. H.]

ABSTRACT

Urine specimens from a total of 26 subjects who were either nurses, pharmacists, or pharmacy technicians engaged in the preparation, handling, or administration of cancer chemotherapeutic agents were analyzed for the presence of mutagenic substances. Assays were performed using bacterial strains TA98 and TA100 in the *Salmonella*/mammalian microsomal mutagenicity assay developed by Ames *et al.* (Mutat. Res., *31:* 347–364, 1975). Findings were compared with results from similar assays of urine specimens for 38 hospital personnel not exposed to cancer chemotherapeutic agents. There was no evidence of an association between occupational exposure to chemotherapy drugs and the presence of mutagenic substances that could be detected by this assay procedure in either specimens of filter-sterilized urine or extracts of urine concentrated with XAD-2 resins. An association was observed, however, between smoking and increased urinary excretion of mutagens. None of the observed associations was changed substantially by statistical adjustment for the occupational category of the subject (nurse or pharmacist), hospital of employment, or values of concurrent solvent controls for the mutagenesis assays. Associations with occupational exposures were not changed by controlling for smoking history. In addition to the large increases for smokers, testing of extracts of urine from nonsmokers with bacterial strain TA98 yielded mutagenicity values that averaged about 50% higher than values for solvent controls. Similar small increases were observed in previous published reports of human urine mutagenicity assays using tester strains TA1538 and TA98. We found little evidence to suggest that the small increases observed for nonsmokers were associated with technical factors such as the presence in the extracts of histidine or other substances promoting bacterial growth or contamination of the specimens during collection or extraction procedures. Since it appears that technical factors can be excluded, we believe that the increases were associated with urinary excretion of low levels of mutagen by a high proportion of subjects tested. The lack of an association of mutagenicity with occupational exposure to chemotherapeutic drugs may have been due to protective measures at the hospitals surveyed and suggests that, with appropriate procedures, these agents can be administered in a manner such that human exposure cannot be detected using this approach. In view of the known genotoxicity of some chemotherapeutic agents and the finding of positive urine mutagenicity in other studies where employees may have had less adequate protection, strict adherence to the use of containment devices, personal protection, and appropriate operating and disposal procedures for all personnel who have contact with these agents are strongly recommended.

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[1] To whom requests for reprints should be addressed.

INTRODUCTION

Many of the agents used for cancer chemotherapy are known to be teratogenic, mutagenic, and carcinogenic (6, 30, 41, 45, 50, 58). Because of the wide use of cancer chemotherapy in recent years, the extent of exposure of individuals engaged in the manufacture, preparation, and administration of chemotherapeutic drugs is of considerable concern (26, 61). Assays of human urine samples with the *Salmonella*/mammalian microsome mutagenicity test developed by Ames *et al.* (3, 59) or similar assays (35) have revealed evidence for increased urinary excretion of mutagenic substances by smokers (14, 32, 42, 54, 59), passive smokers (8), individuals receiving chemotherapy for cancer (41), or other drugs including metronidazole (11) and nitrofurantoin (57) and, most recently, by individuals ingesting cured meat products (4). In occupational studies, increased excretion of mutagens in urine was associated with exposures to epichlorohydrin (36), as well as carbon electrode (46), rubber (19), and other chemical manufacturing (13, 34). In addition, two published studies suggest increased mutagen excretion among pharmacists and nurses preparing or administering cancer chemotherapy (18, 43); several studies did not find evidence of this association (23, 24, 51, 55); and in one report mutagen excretion associated with administration of chemotherapy was observed only among nurses who smoked (7). Mutagenesis assays of body fluids have been the subject of two reviews (15, 35).

Because of limitations and conflicting data in these reports, the small numbers of individuals sampled, and the likelihood of significant differences in procedures for handling these agents at different sites, we studied the excretion of mutagenic substances by chemotherapy workers at an additional two hospitals. In the course of this study, we investigated and modified procedures for conducting and interpreting mutagenesis assays of human body fluids that could facilitate their use in epidemiological studies.

MATERIALS AND METHODS

Subjects. Table 1 presents characteristics of subject groups included in this study. At Hospital A, four groups of subjects were included. The two exposed groups consisted of ten nurses and nine pharmacists who routinely prepare chemotherapeutic drugs and had handled chemotherapeutic drugs within a 48-h period prior to urine sampling. The two control groups consisted of (*a*) 15 unexposed nurses, research assistants, or administrative personnel and (*b*) 10 pharmacists or pharmacy technicians who did not routinely prepare chemotherapy drugs and had not handled drugs for 72 h prior to urine samplings. For each subject, all urine voided during a single 8-h workshift on either a Wednesday, Thursday, or Friday was collected and frozen on dry ice. Sample volumes varied from 50 to 1200 ml for different individuals. Characteristics of the subject groups were determined from self-administered questionnaires.

Table 1

Characteristics of subjects included in the study

Exposed subjects handled, prepared, or administered cancer chemotherapy agents on the day of and/or the day prior to urine collection.

Group	No. of subjects	Mean age	% male	% smokers	% exposed on the: Day of collection	% exposed on the: Day before collection
Hospital A						
Exposed nurses	10	33	0	30	100	100
Exposed pharmacists	9	33	78	22	66	66
Unexposed nurses	15	33	7	33	0	0
Unexposed pharmacists	10	29	70	50	0	0
Hospital B						
Exposed nurses	4	28	0	0	100	[a]
Exposed pharmacists	3	27	33	0	33	100
Unexposed pharmacists	13	27	54	46	0	0

[a] Zero % for Monday collection and 100% for Wednesday collection (see text describing subjects at Hospital B).

Procedures used for handling antineoplastic drugs at this institution have been previously published (29). All but four subjects who handled chemotherapeutic drugs used both a vertical laminar airflow biological safety cabinet and disposable gloves for protection. In a vertical hood, the airflow is directed away from the operator, out of exhaust ports at the top of the unit. In a horizontal hood, air is exhausted out of the opening through which the operator works, increasing the potential for exposure. Some 23 drugs were handled by the exposed subjects. When these drugs were tested directly using the assay system and the bacterial tester strains utilized in this study, 9 of these 23 drugs were mutagenic (Adriamycin, 1,3-bis(2-chloroethyl)-1-nitrosourea, cyclophosphamide, dacarbazine, daunomycin, hydroxyurea, melphalan, *cis*-platinum, streptozotocin), and 9 were not mutagenic (actinomycin D, *l*-asparaginase, bleomycin, 1-β-D-arabinofuranosylcytosine, methotrexate, mitomycin-C, vinblastine, vincristine, vindesine) (6, 27, 39, 41, 43, 45, 48, 60). One study reported that 5-fluorouracil was not mutagenic when directly tested (45), but in a second study 5-fluorouracil was mutagenic when tested either directly or when urine from patients exposed to the drug was tested (41). We were unable to find reports concerning the mutagenicity of four other drugs [AMSA,[2] 4′-epi-Adriamycin, etoposide (VP-16), galactitol] for the tester strains used in this study. AMSA was mutagenic for bacterial strain TA1537 (21) and mitomycin-C with strain UTH 8414 (43). All exposed subjects handled at least one of the known mutagens during the days urine specimens were collected. Additional information collected from subjects included chronic medical conditions, use of medications and vitamins, and exposure to common beverages, saccharin, and current smoking.

At Hospital B, three groups of subjects were included: 4 nurses who routinely prepared and administered chemotherapeutic drugs; 3 pharmacy technicians who prepared chemotherapeutic drugs on the day of and/or the day prior to urine sampling; and 13 pharmacists or pharmacy technicians who did not prepare drugs for 72 h prior to sampling (although 10 had worked with chemotherapeutic drugs at some time in the past). Samples were obtained at four times from the four nurses at this hospital: (*a*) on Monday just prior to a work shift after a weekend off duty; (*b*) during and after that shift; (*c*) 2 days later prior to a work shift; and (*d*) during and after that shift. Sample volumes varied from 23 to 500 ml. The spectrum of chemotherapeutic drugs used was somewhat narrower than cited for Hospital A, with most subjects reporting use of Adriamycin, bleomycin, cyclophosphamide, vincristine, and/or 5-fluorouracil. All subjects used at least one drug that was a mutagen when tested directly in the assay system used for this study (6, 27, 39, 41, 43, 45, 48). Disposable gloves were generally worn while these compounds were being handled; in contrast to Hospital A, however, chemotherapeutic drugs were mixed in a horizontal hood. Characteristics of the subjects are included in Table 1.

Chemicals. Aroclor 1254 was purchased from Analabs, Inc., North Haven, CT; 2-acetylaminofluorene, 2-aminoanthracene, glucose-6-phosphate, and β-glucuronidases [types VIII (bacterial in 50% glycerol solution) and H-1 (Helix pomatia, partially purified powder)] from Sigma Chemical Co., St. Louis, MO; and NADP and NADPH from Boehringer Mannheim, Indianapolis, IN. Nutrient broth was obtained from Oxoid Corp., London, England; and agar from Difco Laboratories, Detroit, MI. Other chemicals were the purest available commercial reagents.

Bacterial Strains. *Salmonella typhimurium* tester strains TA98 and TA100 were generous gifts of Dr. Bruce Ames, University of California at Berkeley. Periodic testing revealed appropriate sensitivity to crystal violet and resistance to ampicillin. These bacteria are histidine dependent unless reverted to histidine independence by a back mutation.

Urine Specimen Processing. Urine specimens were collected directly in 500-ml polyethylene containers (Nalgene No. 2114-0016; Rochester, NY), frozen immediately after collection, coded, shipped to the laboratory on dry ice, and stored at −80°C until use. The specimens were coded so that the laboratory personnel running the assays had no knowledge of the exposure status of the subjects being tested. After thawing, 3 ml of each specimen were filter sterilized using 0.45-μm filters (Millex; Millipore Corp., Bedford, MA) and used in the mutagenesis tests of unextracted urine and for creatinine determinations. The remainder of the unsterilized urine was passed through glass Econo columns (0.7 x 10 cm; Bio-Rad, Richmond, CA) containing 700 mg of a nonpolar resin (XAD-2; Applied Science Labs., State College, PA). Previously autoclaved columns were prepared on the day of use by addition of resin and three 10-ml washes with acetone (allowing the acetone to remain on the column at least 10 min between washes) and three 10-ml methanol washes followed by a rinse of about 300 ml of distilled water. The urine was passed through the column (up to 500 ml for samples from Hospital A and 250 ml for samples from Hospital B) at a rate of 1.5 to 2.0 ml/min. Flow was regulated with a three-way metering stopcock (Bio-Rad, Richmond, CA). The column was purged with nitrogen for 30 s, rinsed with 10 ml of distilled water to attempt to remove residual histidine, and again purged with nitrogen for 30 s. The columns were eluted with 10 ml of methanol into a glass vial at a rate of approximately 1.0 ml/min. The methanol was evaporated under a slow stream of nitrogen at a temperature of 37°C to complete dryness and the sample frozen at −80°C until use. Immediately before its first use, the frozen extract was resuspended in 0.4 ml of DMSO per 100 ml of urine. Sterility was specifically tested and confirmed for about 30 of the specimens. Creatinine concentration of urine samples was determined using materials and methods included in a reagent kit provided by Sigma Chemical Co. (Kit No. 555-A).

Mutagenesis Assays. Liver homogenate from male Sprague-Dawley rats induced with Aroclor 1254 was prepared, and mutagenesis assays

[2] The abbreviations used are: AMSA, 4′-(9-acridinylamino)methanesulfonanilide; DMSO, dimethyl sulfoxide; LQA, lawn quantitation assays; PED, plating efficiency determination.

were conducted as described by Ames *et al.* (3) with modifications and use of β-glucuronidases as described by Everson *et al.* (17), except that each plate received 2.5 ml of top agar. Positive controls were run in each experiment, including 2-acetylaminofluorene and 2-aminoanthracene. In addition, several sets of duplicate solvent control plates were run per experiment.

To help in the interpretation of these mutagenicity studies where there was sufficient sample (generally when more than 200 ml of urine were available), toxicity of the extracts was estimated by PED. This was measured by plating extract or the control solvent DMSO, about 500 bacteria obtained by diluting a fresh overnight culture of strain TA98 or TA100, S-9 activation mix, and top agar containing an excess of histidine (4.5 mM); incubating 44 h at 37°C; counting colonies; and calculating the proportion of colonies formed by bacteria on treated *versus* control plates. [This procedure may overestimate toxicity in comparison with the standard assay conditions where larger quantities of bacteria (about 1×10^8) are inoculated on plates.]

In addition, the possibility that an increase in revertant colonies might have been observed because of increased numbers of bacteria in the background lawn was investigated by a LQA (17). Briefly, uniform cylinders of agar were isolated from mutagenesis plates, the bacteria on their upper surface were suspended with a Polytron, the suspension was diluted and plated with an excess of histidine, and the plates were incubated 44 h at 37°C. The ratio of colonies on test plates and control plates was used to compare numbers of viable bacteria on the lawn of test and solvent control mutagenesis plates. Because the bacterial dilutions for PED assays and the LQA counts for solvent controls varied for different experiments, results for these assays are presented as ratios of test and control counts calculated within individual experiments.

Data Presentation and Statistical Analyses. Most data from these mutagenesis assays are presented as the average number of revertant colonies per plate from all plates tested at a particular condition (typically duplicate plates from each of two separate experiments). The urine extracts were suspended in 0.4 ml of DMSO per 100 ml of urine processed, so that each 0.1 ml of extract assayed resulted from processing 25 ml of urine. For some data presentations, net revertants corrected for creatinine concentration in the sample were calculated by subtracting the mean number of revertants on control plates from numbers of revertants on plates testing a particular extract in an individual experiment, and dividing the remainder by the milligrams of creatinine in urine processed to form the extract. This presentation has the advantage that it controls both for differences in solvent control values of different experiments and for differences in the concentration of urine. Both these refinements have been suggested by some authors (19, 54); however, others (12) caution that such derived data provide the reader with only indirect information about the actual number of revertants observed, limiting the interpretability of data. Such transformations also produce a statistical dependence in the data by subtracting the same control value from counts for more than one test sample and add to uncertainty about the appropriateness of applying statistical procedures. Therefore, in most instances actual counts of mutant colonies were presented and statistically analyzed. Distributional properties of revertant counts and LQA results as well as logarithmic, log-log, and square root transformations of these were investigated using the Shapiro-Wilk test prior to statistical analyses, and data were then transformed in the manner best approximating normality. In most cases the logarithmic transformation was used. Statistical testing was by *t* tests, analysis of variance, and Kendall's τ rank correlation procedure. Most analyses were performed using programs from the Statistical Analysis System (SAS Institute, Cary, NC).

RESULTS

Control Assays. For assays where urine extracts were tested, plate counts for solvent control assays using 0, 0.05, and 0.1 ml of DMSO averaged (mean ± SD) 27 ± 4, 29 ± 6, and 27 ± 9 for TA98 and 151 ± 24, 151 ± 22, and 144 ± 22 for TA100. Plate counts for solvent control assays from experiments testing filter-sterilized urines were similar, and means for saline controls are presented in Table 2. Control values for both strains were normally distributed after logarithmic transformation and analysis of variance showed no difference associated with dose of DMSO, so results of mutagenesis assays for amounts of DMSO up to 0.1 ml were pooled for presentation in Table 2 and Charts 1 and 2. In other reports, amounts of DMSO in this range have generally not affected revertant counts (38), although a small difference (a 6 to 7% decrease) was observed in a very large data set in which 0.1 ml of DMSO was used (37). An analysis of variance for log-transformed values using all sets of solvent control plates suggested significant differences among control values for different experiments ($P < 0.01$ for either bacterial strain). These differences were not sizeable, however; means for different experiments ranged from 26 to 36 colonies (28 ± 3.5) for TA98 and 122 to 163 colonies (147 ± 14) for TA100. Also, the potential impact of variation in control counts for different experiments was lessened by inclusion of test samples in more than one experiment and by use of averaged values for presentation and analyses, and it was controlled for in the statistical analysis by analysis of variance techniques.

Positive controls included plates tested with 25 and 50 μg of 2-acetylaminofluorene which yielded 1590 ± 336 and 5702 ± 1409 revertants with TA98 and 566 ± 46 and 1764 ± 417 revertants with TA100, and other plates tested with 2, 5, and 10 μg of 2-aminoanthracene which yielded 591 ± 171, 2988 ± 1283, and 5450 ± 1069 revertants with TA98 and 846 ± 376, 2858 ± 1490, and 3133 ± 926 revertants with TA100.

Mutagenesis Assays of Specimens Collected from Nonsmokers at Hospital A. Table 2 presents means and standard deviations of revertants for assays of 0.05 ml of urine extracts and solvent control assays. Among samples from nonsmokers at Hospital A, little difference was observed between exposed and unexposed subjects. However, for most exposure groups, the numbers of revertants in assays of urine extracts were higher than the numbers of revertants in assays of solvent controls. The mean differences between assays of extracts and solvent controls by subject group averaged over 50% for TA98 but 20% or less for TA100 (at most, 181 *versus* 150 revertant colonies). While univariate plots for the data (Charts 1, *A* and *B*) suggest these means could be heavily influenced by a few samples, plots of cumulative percentage (cumulative relative frequency distributions; Chart 2, *A* and *B*) indicate that the similarity of results for different exposure groups and the difference between results for extracts and solvent controls extend over a large portion of the distribution. Samples from two unexposed nurses had 96 and 182 revertants per plate for TA98, numbers that appeared to differ from the rest of the distribution. A reason for these increases was not apparent in data collected on the questionnaire. In addition, 20 samples from subjects at Hospital A (when sufficient specimen was available) were tested at multiple doses. These dose-response curves generally support a dose-dependent increase in the number of revertants (Chart 3).

Investigation of toxicity of the extracts did not substantially change interpretations. At a dose of 0.05 ml, PEDs showed toxicity exceeding 50% for only 1 of 23 urine specimens tested. (In addition, no bacterial revertants were present on mutagenicity plates testing 0.05 ml of urine extract from one exposed nurse.

Table 2

Mutagenesis assay results

Mutagenicity of urine extracts for TA98 and TA100 is shown. Plates contain β-glucuronidases, S-9 activation mix, bacteria, and 0.05 ml of solvent (DMSO) or urine extract from the subject group indicated. Results for all assays conducted on an individual sample were averaged, and those averages are used to calculate means and standard deviations indicated. Results for solvent controls (DMSO controls for assays of extracts and saline controls for assays of filter-sterilized urine) were calculated by determining the average number of histidine revertants per plate for each set of control plates used in experiments testing specimens from the hospital indicated. To allow comparison with results from other studies, results for extracts were presented both as revertants per plate and as revertants on test plates minus revertants on control plates from the same experiment, divided by mg of creatinine in the sample tested. Results are also included for tests of filter-sterilized urine specimens and saline control assays.

Group	Revertants from testing 0.05 ml of urine extract expressed as					Revertants from testing 0.3 ml of filter-sterilized urine		
	No. assayed	No. of revertants		Test-control/mg creatinine		No. assayed	No. of revertants	
		TA98	TA100	TA98	TA100		TA98	TA100
Hospital A								
Nonsmokers								
Exposed nurses	7	47 ± 7[a]	178 ± 24	1.77 ± 0.64	1.77 ± 2.04	7	45 ± 8	264 ± 64
Exposed pharmacists	7	42 ± 14	166 ± 23	0.94 ± 0.72	1.26 ± 2.52	7	48 ± 19	300 ± 131
Unexposed nurses	10	64 ± 45	181 ± 46	2.80 ± 1.97	2.49 ± 3.10	10	45 ± 11	237 ± 64
Unexposed pharmacists	5	44 ± 6	157 ± 17	1.18 ± 0.65	0.22 ± 1.10	5	48 ± 16	284 ± 81
Smokers								
Exposed nurses	2	63 ± 16	163 ± 25	6.24 ± 4.57	1.55 ± 4.22	2	39 ± 6	223 ± 59
Exposed pharmacists	2	88 ± 63	192 ± 7	7.73 ± 2.35	11.91 ± 14.41	2	41 ± 9	225 ± 50
Unexposed nurses	5	158 ± 235	215 ± 83	8.13 ± 9.99	4.84 ± 3.98	5	54 ± 26	271 ± 155
Unexposed pharmacists	5	113 ± 131	196 ± 56	3.77 ± 5.10	5.11 ± 10.28	5	50 ± 9	254 ± 52
Solvent controls	14	27 ± 6	148 ± 23			5	28 ± 5	134 ± 9
Hospital B								
Nonsmokers								
Exposed nurses								
Prior to Monday shift	3	50 ± 9	212 ± 37	0.74 ± 0.30	1.66 ± 3.39		53 ± 9	260 ± 54
During Monday shift	4	32 ± 3	187 ± 26	−0.19 ± 0.27	3.93 ± 3.99	4	55 ± 11	243 ± 54
Prior to Wednesday shift	3	37 ± 14	186 ± 35	−0.23 ± 0.87	−0.01 ± 2.64	4	59 ± 12	304 ± 89
During Wednesday shift	4	32 ± 6	181 ± 30	−0.20 ± 0.52	1.36 ± 1.88	4	67 ± 21	366 ± 115
Exposed pharmacists	3	36 ± 3	189 ± 30	0.96 ± 0.55	2.54 ± 1.94	3	54 ± 10	282 ± 66
Unexposed pharmacists	7	38 ± 8	180 ± 24	0.60 ± 0.40	2.02 ± 1.83	7	72 ± 16	374 ± 150
Smokers								
Unexposed pharmacists	4	64 ± 52	188 ± 32	1.76 ± 1.54	2.67 ± 1.75	4	71 ± 25	380 ± 199
Solvent controls	40	28 ± 5	150 ± 22			9	36 ± 5	177 ± 27

[a] Mean ± SD.

Because only 0.12 ml of the extract was available, PEDs were not obtained for this sample.) Mutagenicity results for the two samples showing toxicity were deleted from results shown in Table 1 and the charts. While findings suggest that toxicity did not frequently limit assessment of mutagenicity at the 0.05-ml dose, at a dose of 0.15 ml of urine extract per plate, some 27% of the samples (8 of 30) had PEDs of less than 50%. The high frequency of toxicity at the 0.15-ml dose suggested that higher doses of extract could not be tested without encountering toxicity. Because of difficulties in interpreting results in the presence of toxicity, we limited doses of extract to 0.1 ml for subsequent assays of specimens collected at Hospital A and all assays of specimens collected at Hospital B.

Table 2 presents results of urine mutagenicity assays expressed as net revertants/mg of creatinine. Differences between these creatinine-adjusted values for different subgroups are parallel to those for extracts alone; most are positive, suggesting increases in numbers of revertants in comparison with controls. Results from testing a 0.3 ml of filter-sterilized urine samples are also presented in Table 2. Although there were substantial increases in numbers of revertant colonies compared with solvent controls, these did not differ among groups of subjects with different occupational drug exposures. We have previously shown for the mutagenicity assay used in this study that increases in numbers of revertant colonies of up to about 3 times control values can be attributed to increased growth of bacterial lawn probably associated with histidine present in urine (17). For TA98, only one sample had a value exceeding 3 times the control; that sample, from a nurse in the unexposed group who was a smoker, produced 93 revertants for the filter-sterilized urine. It also yielded the highest value for 0.05 ml of extract, some 579 revertants, suggesting the presence of a mutagenic substance in the filter-sterilized sample that was concentrated in the extract. Two other filter-sterilized samples, one from an exposed pharmacist and one from an unexposed nurse, had values exceeding 3 times the controls for TA100 (559 and 541, respectively). These two samples also had high LQAs (6.0 and 4.4, respectively), suggesting even this increase in revertants could be accounted for by bacterial lawn growth. Neither specimen had high values when urine extracts were tested.

Mutagenesis Assays of Specimens Collected at Hospital B. Mean values for samples from four nurses obtained during work shifts when they administered chemotherapeutic drugs showed no evidence of increased mutagen excretion compared with values for samples collected prior to these work shifts. Likewise, assay results for samples from three exposed pharmacists were similar to those for samples from seven unexposed pharmacists (Table 2; Chart 1, *C* and *D*). Combining values for samples from exposed work shifts of nurses and exposed pharmacists and comparing them with values for unexposed nurse

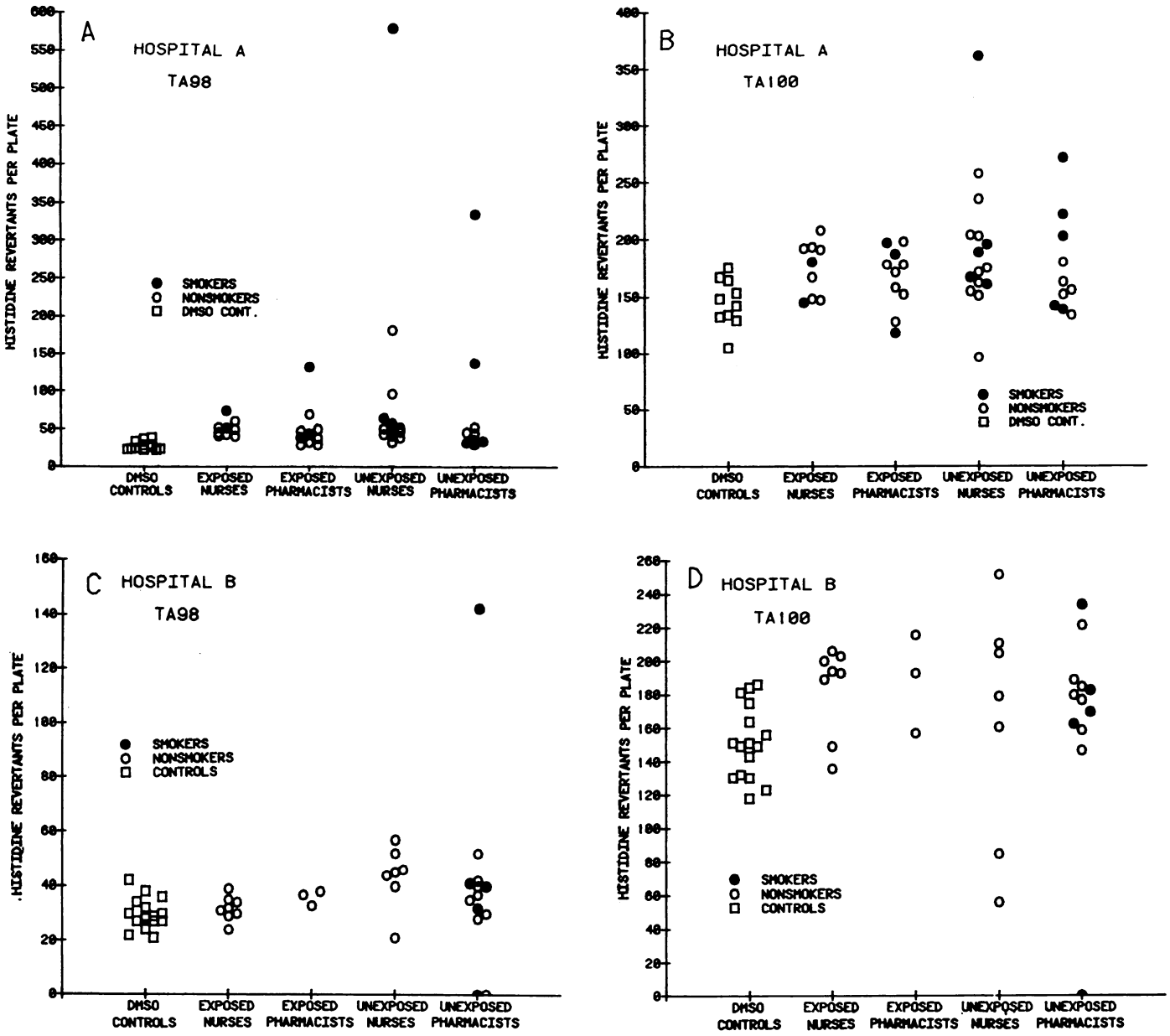

Chart 1. Mutagenicity assay results for solvent (DMSO) controls and extracts of urine from subjects exposed or unexposed to handling chemotherapeutic drugs, by hospital studied. Plates contain bacterial strain TA98 or TA100, β-glucuronidases, S-9 activation mix, and 0.05 ml of DMSO or 0.05 ml of urine extract from the subject group indicated. Results are expressed as the average number of histidine revertants per plate for each set of control plates or for all assays conducted on each individual urine specimen extract, typically duplicate plates in each of two experiments.

work shifts and unexposed pharmacists showed no differences in urine mutagenicity that were evident on cumulative frequency charts. Assay results for extracts of urine from each of the exposure groups, however, tended to be higher than those for DMSO controls (Table 2; Chart 2, *C* and *D*). Extracts from two unexposed pharmacists were toxic to test bacteria; no revertant colonies were seen when 0.05, 0.01, and 0.005 ml of extract or 0.3 ml of filter-sterilized urine samples were tested. Results for these samples were excluded from Table 2 and the charts. Dose-response curves, which were obtained for 0.05- and 0.1-ml samples of nearly all these extracts, were similar or showed smaller increases than were observed for Hospital A (Chart 3, *C* and *D*). Tests of a 0.1-ml specimen of extract from one nonsmoking unexposed pharmacist revealed revertant counts that were 3 times control counts; no explanation for this increase was apparent from the information on the questionnaire. Data from the subjects at Hospital B, expressed as the net of revertants on plates testing extracts minus revertants on solvent control plates corrected for creatinine, also showed relatively little increase. In some cases, values for test plates were lower than control values, probably because of modest toxicity of some extracts or random variation in assay results. Testing by PED was not done for specimens from Hospital B.

Analyses of the Combined Data for Subjects at Both Hospitals. Chart 4, *A* and *B*, presents cumulative frequency distributions for the combined data for subjects in all occupational exposure groups at both hospitals, comparing assay results for 18 current smokers with assays results for 53 nonsmokers. Use

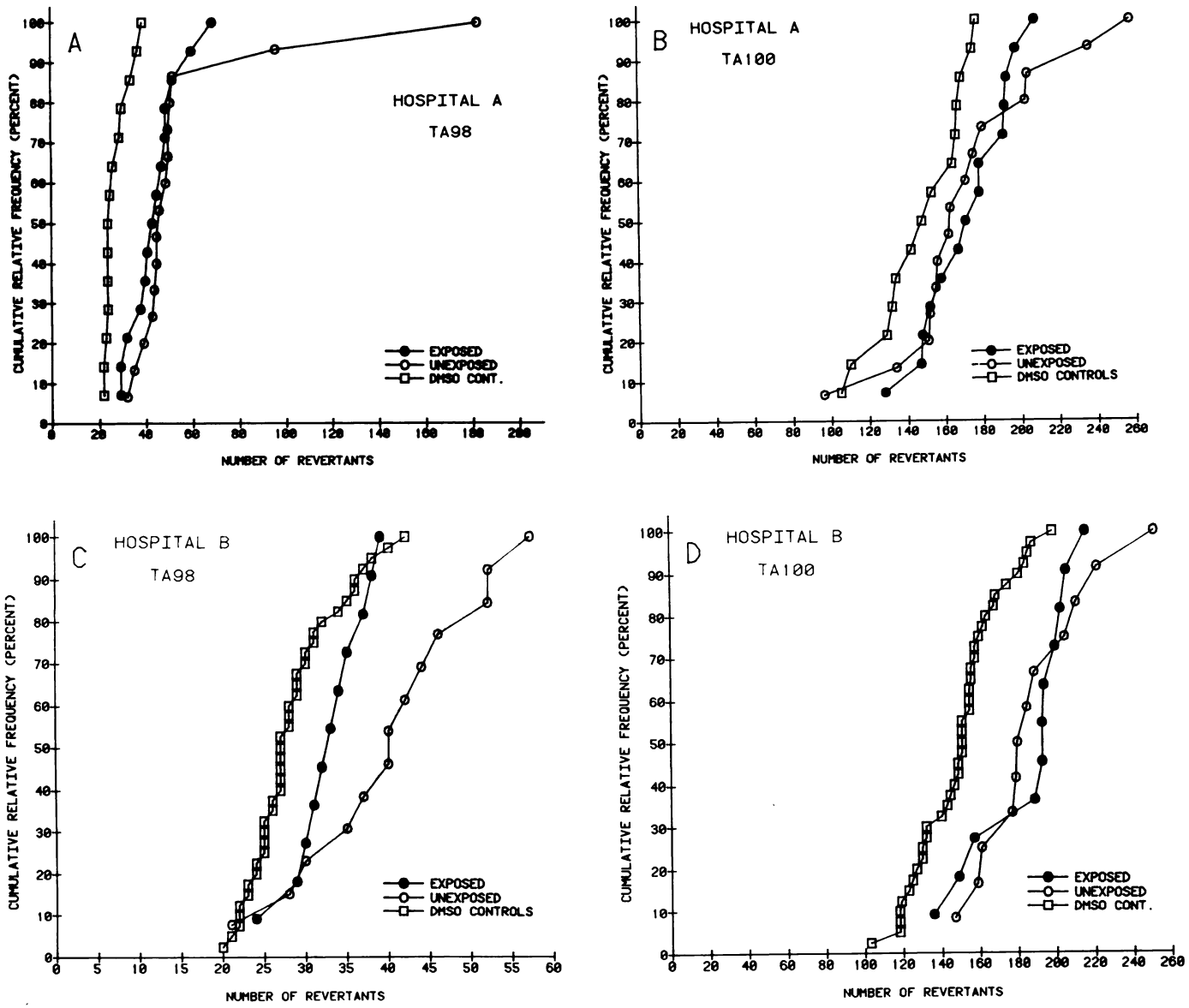

Chart 2. Cumulative relative frequency distributions for results of mutagenesis assays of specimens obtained from nonsmokers at Hospital A (*A* and *B*) and Hospital B (*C* and *D*). Cumulative relative frequency distributions portray the percentage of values less than a given number of revertants, facilitating comparison of entire distributions of data. *A* and *C* show revertant counts for bacterial strain TA98, and *B* and *D*, counts for TA100. Results are illustrated for 0.05-ml samples of DMSO or individual extracts from exposed or unexposed subjects. Note the change in scale between *A* and *C*.

of tobacco was moderate; 3 subjects reported smoking one cigarette per day or less, and only 5 subjects reported smoking 20 cigarettes per day or more. Curves for smokers and nonsmokers appear to diverge at about the 50th percentile for TA98, suggesting an effect for almost half of the smokers studied. An effect can be seen in less than 20% of subjects for strain TA100. Among smokers, there was a relatively weak but highly significant correlation between the average number of cigarettes used per day and revertant counts in assays using tester strain TA98 (τ, 0.31; $P < 0.001$) but not using TA100 (τ, 0.09; $P < 0.34$). Although increased revertant counts associated with smoking were observed for a minority of subjects, they tended to strongly affect means shown in Table 2. In addition, data in Table 2 also suggest that unexposed smokers tended to have higher values than exposed smokers.

To examine the aggregate of the data for trends associated with exposure to handling chemotherapeutic drugs and to control for variables including hospital, occupational category (nurse or pharmacist), the solvent control values for experiments in which the specimen was tested, age and sex of the subjects, and whether subjects were smokers or nonsmokers at the time of the study, analysis of variance was performed including these variables in a model of the log transformed values for revertants found by testing extracts with TA98 and TA100. These findings should be interpreted with caution, because log transformation did not fully normalize the data, because some terms used in the model may be interdependent, and because of the multiplicity of models that could be considered. For 0.05 ml of extract, both smoking history and hospital were associated with the frequency of revertants on mutagenic plates at a significance level of $P < 0.01$ for TA98: no variables were significantly associated with TA100. Findings were similar for the smaller numbers of extracts

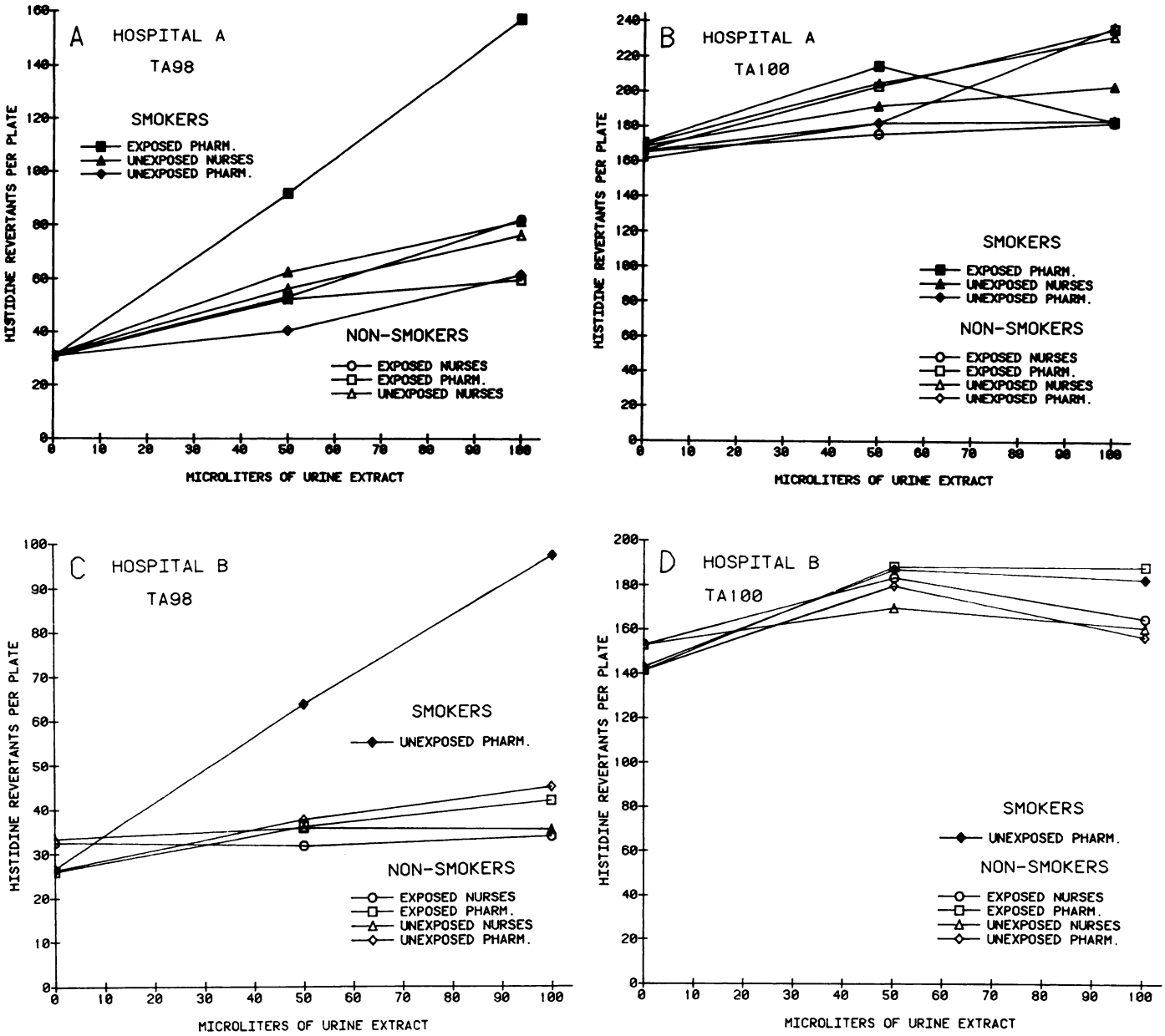

Chart 3. Dose-response curves for mutagenicity assays of DMSO and extracts of urine specimens from nonsmokers with bacterial strains TA98 (*A* and *C*) and TA100 (*B* and *D*). Results for all assays of an individual sample at each dose were averaged and used to calculate averages of subject groups indicated at each hospital. Only individuals for whom assay results were available for 0.05 ml and 0.1 ml of extract were included; most groups contained three to five subjects.

tested at a 0.1-ml dose. Combining data for all doses and strains, only the smoking history was significantly associated with the log transformed revertant count ($P < 0.002$). In none of these models was there a significant association between revertant counts and exposure to chemotherapy.

DISCUSSION

Associations between Mutagenesis Assay Results and Occupational Exposure to Cytotoxic Drugs. Similar results were observed for urine mutagenesis assays of samples from nonsmoking nurses and pharmacists who were exposed or not exposed to chemotherapeutic agents, thus providing no evidence for an association between their occupational exposure and urinary excretion of mutagenic substances. As in previous studies (14, 54, 59), there was evidence for the presence of mutagenic substances in urine samples from a large proportion of smokers. Strongly positive assays were also observed for a few nonsmokers in both exposure groups; information from questionnaires did not suggest an exposure responsible for these increases. A recent study finding mutagenicity in urine samples following ingestion of fried pork or bacon (4) suggests one possible source of these increases; dietary information relevant to fried meat exposure was not obtained because our study was designed prior to this report.

The absence of an increase in urinary mutagen excretion among chemotherapy workers in this study is consistent with results of several recent studies but differs from two other reports. Falck *et al.* (18, 20) used urine extracts produced by a

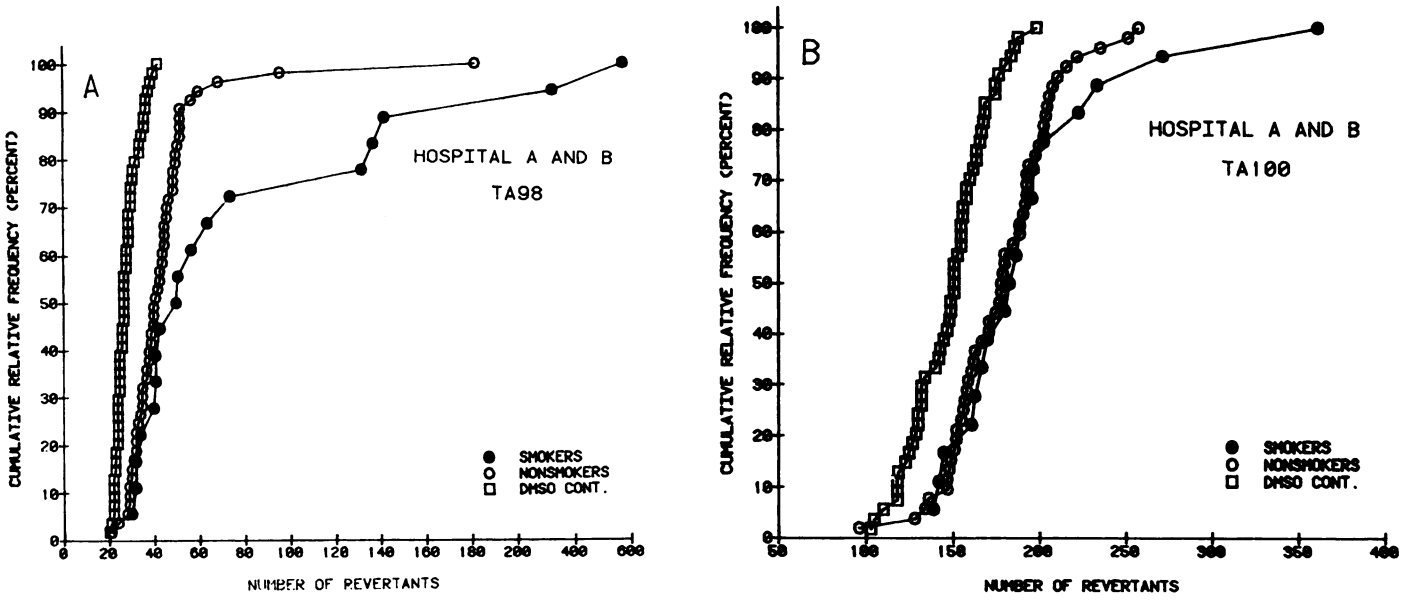

Chart 4. Cumulative relative frequency distributions for mutagenesis assays comparing DMSO controls and urine extracts from smokers and nonsmokers at both hospitals for bacterial strains TA98 (*A*) and TA100 (*B*).

modification of the same extraction procedure used for our study but tested for mutagenicity with a bacterial fluctuation assay instead of the plate assay. Seven exposed nurses and 25 control subjects were included in their study. The exposed nurses showed a 2 to 3-fold increase in revertants compared to the controls, significant by *t* tests. Using the assay procedure reported by Yamasaki and Ames (59), Staiano *et al*. (51) found no evidence of mutagenic activity in urine from eight hospital pharmacists who routinely mix chemotherapeutic drugs in vertical laminar flow hoods. Although frequently cited, interpretation of these reports is limited by their brief presentation as letters to the editor, providing no information regarding issues such as the presence of substances promoting bacterial growth in extracts, toxicity of extracts, replicability of assays, and the appropriateness of statistical analyses. Such issues can strongly affect results: for example, Harrington *et al*. (25) suggest that fluctuation assays can be altered by the presence of growth-promoting substances in test samples and recommend that survival determination be routinely used to avoid artifactual positive results.

A more recent study by Nguyen *et al*. (43) also reported evidence of mutagen excretion in the urine of six pharmacy personnel when they mixed chemotherapeutic drugs in open-faced, horizontal laminar flow hoods but not when vertical flow biological safety cabinets were used. Their study, like that of Staiano *et al*. (51) and our study, used a modification of the assay procedure of Yamasaki *et al*. (59). Several modifications of the assay procedure differed from procedures used in our study, including incubating urine samples at 37°C for 16 h in the presence of β-glucuronidase and testing extract from large and variable amounts of urine. Both the possibility of mutagen formation during incubation with β-glucuronidases [similar to that observed by Miller and Stoltz for isoniazid (40)] and the observations that mutagen excretion occurred only for specimens obtained after several days of work with chemotherapeutic drugs [which seems improbable given the relatively rapid clearance of most of these agents (9, 30)] raise questions concerning the interpretation of this study. Nonetheless, its findings suggest that excretion of mutagenic material was associated with handling of chemotherapeutic agents by at least four exposed pharmacy personnel when horizontal but not vertical laminar hoods were used.

Another recent study (7), using methods similar to our study, reported a modest increase (22% in median values) in revertant counts from urine mutagenicity assays of smokers who were exposed to or handled chemotherapeutic drugs. The increase only occurred for strain TA100 and was not observed for nonsmokers. However, for TA98, unexposed smokers had higher numbers of revertants than exposed smokers. The author contended that smoking was not associated with increased revertants using TA100. Results seem difficult to interpret, however, because of the small increase in revertant counts for assays of specimens from subjects exposed to drugs and difficulty in excluding with assurance a confounding effect of smoking.

Three additional recent studies of small numbers of chemotherapy workers found no association between exposures to mutagenic drugs and the induction of mutation by substances in urine (23, 24, 55). Two of these studies reported nearly the same number of positive fluctuation assays for extracts of urine from both exposed and unexposed subjects. The authors suggested that these positive assays were associated with the presence of enhancers of bacterial growth in the extracts from both subject groups (23, 55) but had no direct test to control for this possibility. A biochemical assay for the presence of histidine, however, revealed that some histidine was present in the urine extracts (23). Biochemical analyses, however, may not quantitate the amount of histidine that is biologically available to the tester bacteria. Aeschbacher *et al*. (1) have shown that some forms of histidine with methyl substitutions or histidine that is bound to large molecules (at least some of which would probably be measured in the biochemical assays) does not support growth of test bacteria.

Several other studies of individuals who handle chemotherapeutic drugs suggest effects from exposure to these agents. Norppa *et al*. (44) found slight increases in sister chromatid

exchange frequencies for oncology nurses compared with office workers, but not compared with other nurses. Waksvik *et al*. also found slight increases in sister chromatid exchange frequency and larger increases in chromosome gaps among nurses who administer these drugs (56); a similar study by Stiller *et al*. (52), however, found no effect for nine exposed subjects. Jagun *et al*. observed increased levels of thioether excretion among 15 nurses who handled cytotoxic drugs (31); excretion of thioethers has been proposed as a measure of exposure to alkylating agents (49). Reynolds *et al*. (47) noted that nine pharmacy and nursing personnel had symptoms after mixing AMSA, a relatively new cancer chemotherapeutic agent, in horizontal hoods. These nine individuals included one who had an urticarial rash on three separate occasions after mixing the drug, one with nausea and vomiting, and seven reporting lightheadedness, nausea, headache, and malaise. No reactions to AMSA were noted when vertical hoods were used. Hirst (28) reported that, on several occasions after administering cyclophosphamide, the urine of two nurses contained evidence of the drug by gas chromatographic analysis. Taken together these observations strongly suggest that individuals mixing or administering chemotherapeutic agents can receive at least low doses of exposure to these agents.

The lack of association between occupational exposure to chemotherapeutic agents and urinary excretion of mutagenic substances in our study may reflect a lack of sensitivity of the mutagenesis test system used. Reports in the literature indicate, however, that with the bacterial tester strains used in this study at least 9 of the 23 agents used were mutagenic when tested directly (6, 27, 39, 41, 43, 45, 48, 60), and several of these were positive when urine of humans (41) or experimental animals given the drug was tested (45). Direct tests of as little as 1 μg of Adriamycin (less than 0.01% of a typical clinical dose) were positive (45). Of all the cytotoxic drugs used, those positive in mutagenicity assays tended to also be positive in carcinogenicity studies (6, 30, 41, 45, 50, 58), suggesting that the assay procedure used would have identified compounds of greatest concern. Several other agents used were not mutagenic when tested directly (6, 39, 41, 45, 48). Some may be metabolized to mutagenic derivatives. Alternatively, the lack of association between handling chemotherapeutic drugs and mutagen excretion may indicate a true lack of ambient exposure or adsorption of ambient levels of the drugs; may be falsely negative because of a lack of excretion of mutagenic chemicals, loss of mutagenic activity during collection, processing, or storage; or a qualitative, quantitative, or procedural failure of the assay technique to detect mutagenic materials actually present in urine. These considerations and those discussed above in reviewing other studies emphasize the range of possible technical approaches to mutagenesis assays of body fluids. The exact method used in any given study will inevitably represent a compromise, including a number of arbitrary decisions (15, 35).

Evidence of Low Levels of Mutagen Excretion among Nonsmokers. Although occupational exposure had no effect on mutagenesis assay findings in our study, small but consistent and statistically significant differences were observed between assays of extracts and solvent controls run simultaneously under identical conditions. There are several possible explanations for these differences. (*a*) As discussed above, small amounts of histidine or other substances enhancing the growth of test bacteria may be present in the extracts. This would allow growth of the histidine-dependent bacteria in the background lawn of the top agar of mutagenicity plates used for these assays; the increased numbers of bacteria would then result in increased numbers of spontaneous revertants (17, 59). This possibility was investigated by running lawn quantitation assays for all available extracts from Hospital A. The mean LQA was not increased for extracts (Chart 5). While the mean LQA could be reduced by toxicity of the extracts for tester bacteria, none of the extracts used was toxic by PED, and the cumulative frequency distribution does not suggest that the LQA means were strongly influenced by toxicity. Thus, no increase in bacteria counts for background lawns for these assays was suggested, making it unlikely that the presence of histidine was responsible for increases in the observed frequencies of revertants. [This was consistent with our finding in previous studies of a strong increase in bacterial lawns when sterilized urine samples are tested directly (17), but little increase when urine extracts are tested (16). In addition, the quantities of histidine that Gibson (23) found in XAD-2 extracts of urine appear to be large enough to affect fluctuation assays but not the plate assays used in our study. They found an average of 0.027 μmol of histidine per extract from 12.5 ml of urine, whereas we have reported that 0.11 μmol would be required to increase the spontaneous revertant rate for TA98 by 50% in the plate test used (17).] (*b*) Mutagenic substances may have contaminated glassware, columns, resin, water samples, or solvents used to produce the extracts. This was investigated by processing several 250- and 500-ml samples of distilled water in the same manner as urine samples (including frozen storage in similar specimen containers). Based on results for between 15 and 20 assays for each group, extracts of water samples yielded 27 $\pm$ 4 and 150 $\pm$ 23 revertants, and DMSO controls for these same experiments yielded 29 $\pm$ 5 and 158 $\pm$ 30 revertants, respectively, for strains TA98 and TA100. Thus, no evidence of contamination was observed. (*c*) The differences between extracts and solvent controls may be due to urinary excretion of a small amount of mutagen by a high proportion of the subjects tested. This may reflect either endogenous formation of mutagenic substances or environmental exposures to mutagens. The data appear to support mutagen excretion as the source of increased revertant counts for nonsmokers in this study, but limitations in the precision of these mutagenesis assays and the assays used to exclude possibilities a and b above suggest that this conclusion should be considered tentative and in need of confirmation by other studies. The likelihood that mutagen excretion was the source of the increased revertant counts among nonsmokers is supported, however, by results for several studies of mutagenicity of human urine which found increased numbers of revertants for urine extracts compared with solvent controls (4, 7, 8, 13, 22, 34, 46, 55, 59). Unlike our study, however, these studies did not use assays to determine increased growth of bacterial lawns by substances contained in the urine extracts, making it impossible to exclude the effect of growth-promoting substances in the extract as a cause for the positive assays.

The presence of small amounts of genotoxic substances in human urine specimens is also suggested by a report of an increase in the frequency of sister chromatid exchanges induced by extracts of urine specimens from a high proportion of subjects (5). Recent studies finding urinary mutagen excretion following

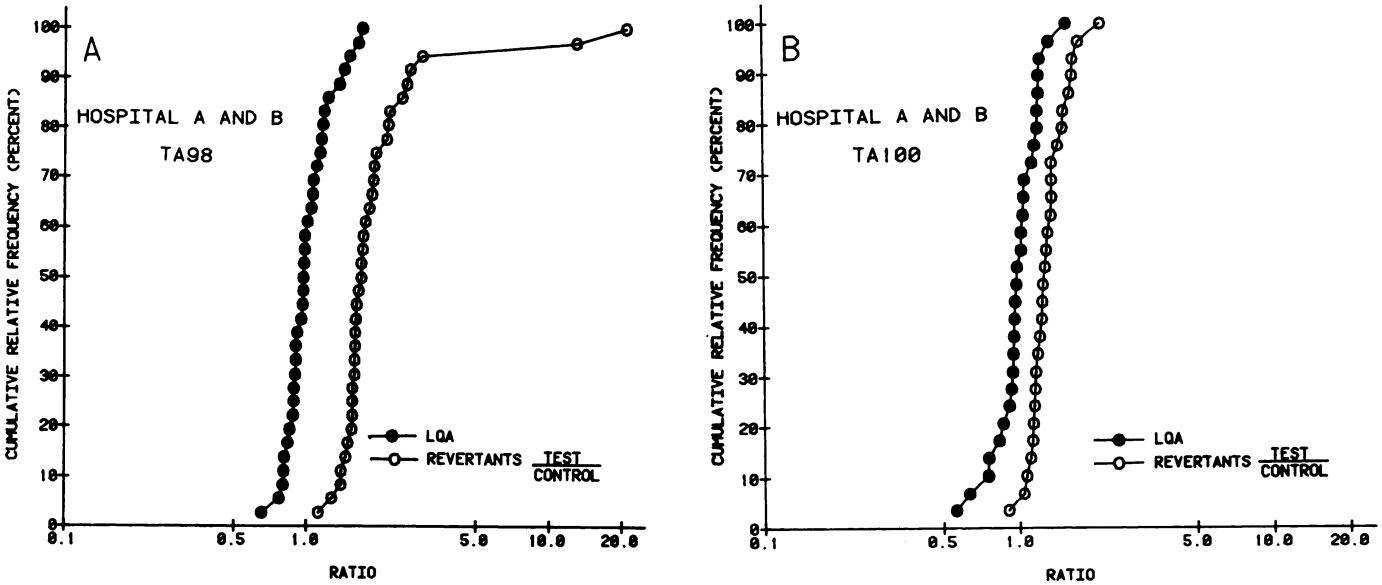

Chart 5. Cumulative relative frequency distributions for the ratio of assays for growth of the bacterial lawn on test and control plates (called lawn quantitation assays or LQA in the text) and the ratio of revertants on test and control plates for assays of specimens from nonsmoking subjects at both hospitals. A ratio consistently exceeding 1.0 for the revertant counts demonstrated an increased number of revertants on the test plates. This could not be accounted for by growth-promoting substances in the extracts because the ratio of LQAs centered near 1.0, indicating that growth of the bacterial lawn was the same on test and control plates.

fried pork or bacon meals (4) and passive smoking (8) suggest sources of exposure to mutagens not covered by our questionnaire that could be responsible for these increases. Other possibilities would include exposure to other mutagens in diet (2) or in the hospital environment.

Handling of Chemotherapeutic Drugs. In addition to variation that might be ascribed to the laboratory assays, inconsistent findings in studies of mutagen excretion among individuals handling cytotoxic drugs may have resulted from variations in procedures used by hospital staff to dispense and administer these agents. Preparation may have been done on open benches, sterile hoods with a horizontal air flow that would exhaust aerosols and powders produced in the hood toward the operator, or sterile hoods with a vertical air path that would eliminate most potential exposure, especially if the hood is vented to the outside. Administration may have been performed by staff using no protective devices or using gloves and gowns; and protection afforded by nursing procedures for injection and for handling excreta from treated patients is likely to vary in different settings. Even where procedures are standardized, unusual occurrences ranging from cracked vials to clogged i.v. infusions or unexpected vomiting may cause episodic exposures. Assays would have to be conducted daily for several weeks to exclude these sorts of episodic exposures.

Several of the literature reports cited above (43, 47, 51) suggest that the potential for exposure to chemotherapy agents is greater where horizontal laminar flow hoods are used for drug preparation than where vertical flow biological safety cabinets are used and that nurses who administer the drugs may also be at increased risk of exposure. The use of special precautions, including biological safety cabinets, personal protective equipment, and safe housekeeping practices such as those recommended by the American Society of Hospital Pharmacists and others (26, 29, 53, 61), minimizes potential exposures to these agents from either typical or exceptional events. Other approaches may help investigate the extent that medical personnel are exposed during handling and administration of cytotoxic drugs. Examining experiences of technicians using radionucleotides for diagnostic imaging or therapy may be instructive, since highly sensitive monitoring for contamination from radioactivity can be readily accomplished. Detecting exposure during routine procedures for handling cytotoxic drugs also might be done by applying highly sensitive chemical assays (10, 28, 33, 55). This approach only detects exposure to individual chemicals studied, however, rather than the multiple agents that may affect single bacterial mutagenicity assays.

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