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Concentration of mutagens from urine by adsorption with the nonpolar resin XAD-2: Cigarette smokers have mutagenic urine

(detecting carcinogens in urine or aqueous liquids/*Salmonella* test)

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ABSTRACT **A method is described for concentrating mutagens/carcinogens from human urine about 200-fold for subsequent assay in the *Salmonella*/mammalian microsome mutagenicity test. The method is also applicable for other aqueous liquids and for other *in vitro* tests for mutagens/carcinogens. The urine (up to 500 ml) is put through a column with a 1.5-cm^3 bed volume of XAD-2 (styrene-divinylbenzene polymer) and the adsorbed material is then eluted with a few milliliters of acetone. The acetone is taken to dryness and the residue is dissolved in dimethyl sulfoxide. This is the *urine concentrate* that is assayed for mutagenicity. Various mutagens/carcinogens have been added to human urine and the recoveries have been measured after adsorption on XAD-2, XAD-4, and Tenax GC (diphenyl-*p*-phenylene oxide polymer). We propose that this method be used in monitoring the urine of human populations and of experimental animals in toxicological studies.**

It is shown with this procedure that cigarette smokers have mutagenic urine while nonsmokers do not.

Humans are being exposed to a wide variety of environmental chemicals that are mutagens/carcinogens (1–4). A number of rapid *in vitro* systems for detecting these chemicals have been developed (1), such as the *Salmonella*/mammalian microsome mutagenicity test (5). The *Salmonella* test is about 90% accurate in detecting a wide variety of carcinogens as mutagens (6–9). Chemical mutagens enter people through the diet, or in other ways, and are often excreted unchanged, as conjugates, or as other metabolites in the urine. In order to demonstrate exposure to mutagens/carcinogens it is of particular interest to have a rapid way of examining urine of large numbers of people.

Testing urine directly in the *Salmonella* system has been done (10–14), but only a small amount of urine can be added to the test system because of volume limitations and interfering histidine (10), and therefore only mutagens present in high concentration or those of exceptional potency can be detected. We (10) and others (11) have used solvent extraction techniques for concentrating mutagens from urine, but because of the inconvenience of solvent extraction and methods such as lyophilization and subsequent selective extraction, we have explored resin adsorption techniques. Various nonpolar resins such as XAD-2, XAD-4, and Tenax GC have been used for adsorbing drugs and their metabolites from urine (15–18), and organic compounds from water (19–24) and from sea water (25–27).

MATERIALS AND METHODS

Materials. XAD-2 and XAD-4 resins were washed by swirling and decanting several times with 10 volumes of acetone followed by absolute methanol and distilled water, and then stored in water at 4°. Glass Econo columns, 0.7 cm (inside diameter) × 10 cm (Bio-Rad, Richmond, CA), were filled with distilled water before addition of sufficient washed resin (0.7 g dry weight) to give a bed height of 4 cm (1.5-cm^3 bed volume). Flow was regulated with a 3-way nylon stopcock (American Hospital Supply). Distilled water (about 50 ml) was passed through the resin before use. Tenax GC resin, 0.25 g added per column, was washed with 20 ml of acetone followed by 40 ml of distilled water prior to use.

Resins were from Applied Science Laboratories (State College, PA); phenolphthalein and 8-hydroxyquinoline and their glucuronide conjugates, *p*-nitrophenol, *p*-nitrophenyl sulfate, and β-glucuronidase (*Escherichia coli*) from Sigma; Glusulase (a mixture of sulfatase and β-glucuronidase activities obtained from *Helix pomatia*) from Endo Laboratories (Garden City, NY); [6,7-^{3}H(N)]estradiol 17β-glucuronide and [6,7-^{3}H(N)] estrone sulfate from New England Nuclear.

Urine Samples. Urine samples were collected from smokers and nonsmokers who were on normal diets and who were not taking any medication. The pH values of the urine samples ranged from 6.5 to 7.0. Urine was filtered through Whatman no. 1 filter paper and frozen at −20°, unless otherwise indicated. Samples were thawed and warmed to room temperature shortly before assay. An occasional sample with precipitated matter was refiltered before passing through the column.

Urine samples were collected in polyethylene or polypropylene containers after the evening meal or just before retiring for the night, and in the morning on arising. In some instances urine samples were obtained from smokers over a 24-hr period during which each sample was collected separately.

Reconstruction Assays. A combined urine sample from about six nonsmokers was thawed and known quantities of mutagens were added to various urine volumes. Fresh dimethyl sulfoxide solutions of chemicals were prepared on the day of the assay and 0.1 ml or less was added to the urine. The amount of mutagen added was determined by the sensitivity of the tester strain to reversion by that particular mutagen. Urine samples without added mutagen were included in each assay.

XAD Resins. The urine sample was loaded on the resin (a glass powder funnel on the column facilitated loading) and an effluent flow rate of 2–3 ml/min was regulated by means of the stopcock. All operations were at room temperature. After loading, nitrogen was introduced into the top of the column for a few seconds to remove the residual aqueous phase; however, drying of the resin was avoided. The adsorbed components were eluted into 18 × 150-mm test tubes (glass) with 10 ml of acetone. Each of the eluted fractions was then thoroughly mixed before placement of the tubes in a constant temperature heating block at 60–65° to evaporate the solvent under an atmosphere of nitrogen. After complete drying (usually taking less than 1 hr), dimethyl sulfoxide (spectrophotometric grade) was added to each tube to dissolve the acetone residue, resulting in the *urine concentrate;* 0.4 ml of dimethyl sulfoxide was used per 100 ml of urine unless otherwise specified.

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Table 1. Adsorption and recovery on resins of model compounds added to urine

Compound added to urine	% recoveries on resins		
	XAD-2	XAD-4	Tenax GC
[^{14}C]Histidine (13 ng)	8	—	8
p-Nitrophenol (20 μg)	100	84	91
Phenolphthalein (20 μg)	67	54	79
p-Nitrophenyl sulfate (385 μg)	54	—	0
Phenolphthalein glucuronide (620 μg)	48	—	94
4-Nitro-*o*-phenylenediamine (50 μg)	100	—	100
[^{3}H]Estradiol glucuronide (1 ng)	75	—	63
[^{3}H]Estrone sulfate (0.3 ng)	84	68	84

Urine samples (10 ml) were loaded on the resins as described in *Materials and Methods*. *p*-Nitrophenol and phenolphthalein were assayed by measuring absorbance; their derivatives were hydrolyzed with Glusulase before analysis. The recovery of 4-nitro-*o*-phenylenediamine was based on the mutagenic activity of the extract with TA98 as the tester strain.

Assay of Mutagenicity. In the reconstruction experiments 20- to 100-μl amounts of the *urine concentrate* were assayed with the requisite *Salmonella typhimurium* tester strains. When required for the metabolic activation of specific compounds, S-9 Mix containing 50 μl of liver S-9 from Aroclor 1254-induced rats (5), unless otherwise indicated, was incorporated with the sample in the petri plate. The level of spontaneous reversion due to the control urine extract alone was subtracted from all experimental values. Control mutagenicity plates to obtain a dose–response for each mutagen were included in each assay to quantitate the recovery of mutagenic activity of the resin-adsorbed compound from urine. 8-Hydroxyquinoline glucuronide was assayed in the presence of β-glucuronidase (10). Assay of mutagenicity of *urine concentrates* from smokers' urine was with tester strain TA 1538 in the presence or absence of S-9.

RESULTS

Suitability of Resins: XAD-2, XAD-4, Tenax GC. Three resins appeared suitable as prospects for adsorbing mutagens from urine while not adsorbing histidine. Nonpolar molecules are known to be adsorbed by these resins quite effectively (24–26). The adsorption capabilities of XAD-2 and XAD-4 (styrene-divinylbenzene polymers) and Tenax GC (diphenyl-*p*-phenylene oxide polymer) were tested by adding each of seven model compounds or [^{14}C]histidine urine and determining the percent recovery after adsorption and elution (Table 1). Among the model compounds were several glucuronide and sulfate esters which were used as examples of conjugates known to be excreted in human urine, and we wished to be able to adsorb molecules with some polar characteristics. We decided to use XAD-2 for further studies, rather than Tenax, because it is cheaper and adsorbs even the relatively polar *p*-nitrophenyl sulfate. XAD-2 was also superior to the fairly similar XAD-4 in terms of general recovery. We determined that none of the three resins adsorbed sufficient histidine from urine to interfere with the plate assay for mutagenicity even when large volumes (up to 500 ml) of urine were used.

We compared (data not shown) acetone and methanol (10 ml) as eluting agents for XAD-2 with six of the chemicals in Tables 1 and 2. Recoveries were fairly similar and we have used acetone because of its low toxicity and high volatility.

Reconstruction Assays with XAD-2 and Normal Urine. We have added a series of representative mutagens to human urine and measured the recoveries (Table 2). The recoveries were quite reasonable, with the possible exception of 8-hydroxyquinoline glucuronide. 8-Hydroxyquinoline and its glucuronide were difficult to assay [because of weak mutagenicity and problems of inhibition of the bacteria at higher concentrations (28)] and the recovery values for these compounds are only approximate. We did not detect any mutagenic activity in the pooled normal human urine (from nonsmokers) that we used in these experiments.

Table 2. Recoveries of mutagens added to urine and adsorbed on XAD-2

Compound	Urine volume, ml	Mutagen in sample, μg	Tester strain	S-9/plate, μl	% recovery
Tris-(2,3-dibromopropyl)-phosphate	100	320	TA100	20 A	85, 79
2-Acetylaminofluorene	100	80	TA98	20 A	89
2-Aminofluorene	100	40	TA98	50 P	48
2-Aminoanthracene	100	40	TA98	50 P	21
Furylfuramide	100	0.16	TA100	—	40
Benzo[*a*]pyrene	100	80	TA100	20 A	19
4-Nitro-*o*-phenylene-diamine	100	160	TA98	—	91
Daunorubicin·HCl	100	16	TA98	—	26, 26
ICR191	100	8	TA1537	—	91
8-Hydroxyquinoline	100	320	TA100	50 A	12
	50	320			22
	10	320			47
8-Hydroxyquinoline glucuronide	100	640	TA100	50 A	<15
	50	705			18
	10	640			20

Recoveries were based on the assay of the mutagenic activity of 50- and 100-μl samples of the dimethyl sulfoxide solution of the *urine concentrate* (see *Materials and Methods*). β-Glucuronidase (1000 units) was incorporated in the petri plate for the assay of the glucuronide. As indicated, rat liver S-9 preparations from phenobarbital- (P) or Aroclor- (A) induced rats were incorporated in the assay as needed.

Mutagens in a Smoker's Urine. In order to test the XAD-2 method for concentrating urinary mutagens, we examined the pooled urine of a smoker. Fig. 1 shows that there is mutagenic activity and a dose–response, when the *urine concentrate* equivalent to 12.5 ml or 25 ml of urine is plated with tester strain TA1538 (or the related strain TA98) or with strain TA100. There was no activity with the other tester strains (TA1535, TA1537) tested. The mutagenic activity with strain TA1538 requires the rat liver homogenate fraction, S-9. We have used strain TA1538 for further tests, because this strain has a low background and shows good activity. No additional activity was observed when β-glucuronidase was added to the *urine concentrate* tested with either strain TA1538 or strain TA100 in the presence of the S-9.

Investigation of the Parameters of the XAD Method with a Smoker's Urine. Combined urine samples from a cigarette smoker (who had smoked up to 44 cigarettes per day) were assayed on XAD-2. (*i*) We determined that our standard procedure for evaporating the acetone eluate (10 ml of acetone dried in an 18 × 150-mm test tube at 60–65° under a stream of nitrogen) was comparable to evaporation of the acetone in a rotary evaporator under reduced pressure (250 colonies versus 248 colonies). (*ii*) A column that had been eluted with 10 ml of acetone was eluted subsequently with 10 ml of toluene followed by 10 ml of methanol. The methanol fraction, but not the tol-

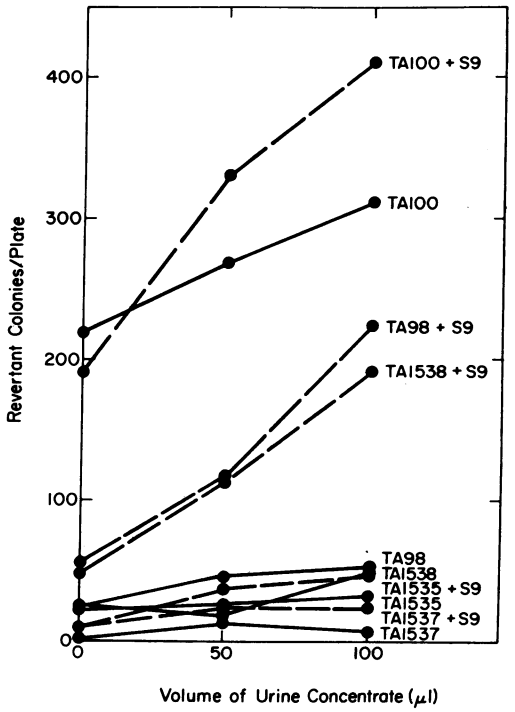

FIG. 1. Mutagenicity of compounds in a smoker's urine adsorbed to XAD-2. Urine samples collected during 1 day from a smoker (GC) were pooled. Aliquots (100 ml) were passed through each of five XAD-2 columns, and the pooled *urine concentrate* was obtained as described in *Materials and Methods*.

uene fraction, had some activity when assayed with S-9 and β-glucuronidase (with both strains TA1538 and TA100). We did not investigate this activity further, because it was small (15%) compared to the mutagenic activity eluted with acetone. (*iii*) We have determined the minimum amount of acetone required to elute the mutagenic activity from the column. Almost all the activity was eluted with 1 ml of acetone (172 colonies) and 3 ml of acetone removed about the same amount (226 colonies) as did 5 ml of acetone (224 colonies). Because we had previously done a number of experiments with various urine samples eluted with 10 ml of acetone we have continued to use this amount for the remaining studies in this paper, though it appears that 3 ml would be satisfactory for desorption of the mutagenic components of smokers' urine. (*iv*) We have put aliquots of pooled smokers' urine ranging from 10 ml to 500 ml through the standard XAD-2 column and found that the calculated activity per 100 ml of urine decreased as we increased the volume (3120 colonies per 100 ml for a 10 ml sample; 2036 for 50 ml; 1498 for 100 ml; 1200 for 100 ml; 1141 for 150 ml; 406 for 500 ml). Thus there appears to be a competitive adsorption effect of urinary components when larger aliquots of urine are used. The total mutagenic activity recovered in the *urine concentrate* increased, however, indicating the possible value of using as large a volume of urine as possible when screening urine for mutagens.

The Influence of Histidine from Urine on Background Mutagenesis. The urine we have examined from nonsmokers showed no, or minimal, mutagenicity. In those cases where there appeared to be a very slight activity, however, it became important to see if this could be due to an effect of extraneous histidine added with the sample. A small amount of histidine from urine is entrapped in the XAD resin bed because the column is not washed with water, before eluting with acetone in our standard protocol, to avoid the loss of any weakly adsorbed material. We estimate that about 0.8 ml of urine might be trapped in the standard XAD column and proportionately more in a larger column, as calculated from the experiment in Table 1 and similar experiments. The amount of histidine in human urine is in the range of 0.7–1.3 μmol/ml (29). We thus estimate that about 0.1 μmol of histidine is added to the mutagenicity test plate per 100-μl aliquot of the *urine concentrate*. This additional 0.1 μmol of histidine (there is some histidine in the plates and in the S-9 fraction) will raise slightly the number of spontaneous revertants, e.g., for 1538 from 46 to 62 colonies and for TA100 from 200 to 225 colonies. Strains TA98 and TA100 (but not TA1538) show an increase in spontaneous mutation rate with small amounts of additional histidine that cause an appreciable growth of the background lawn, which was not observed in the experiment with urine. This increase in the number of spontaneous revertants can be 3- to 4-fold greater than that observed under standard conditions and one should be attentive to the density of the lawn and be aware of this possible artifact.

In recent experiments, completed after the results reported in this paper, we have used a 1.5-ml water wash after passing the urine through the column (using a stream of nitrogen to remove the aqueous phase before and after the water wash) and before the elution by acetone. This water wash removes about 75% of the histidine that is trapped by the column (determined with [^{14}C]histidine) and does not remove any appreciable amount of the mutagenicity from smokers' urine. We recommend this procedure for general screening because it minimizes the possibility of any artifacts due to traces of histidine when strains TA98 and TA100 are used.

Assay of the Mutagenicity of the Urine from Nonsmokers. We have examined 37 urine samples from 21 nonsmokers for mutagenicity (Fig. 2). None of these urine samples had any appreciable mutagenic activity and no significant difference was seen between morning and evening samples. For TA1538 we normally expect a doubling of the control value and a dose–response relationship before we call a result positive. In general there was no dose–response relationship with the two aliquots of nonsmokers' *urine concentrate* assayed relative to the control without the *urine concentrate*. The sample with the highest mutagenic response may be of borderline significance. We have not corrected for the approximately 20 colonies on the sample plates that we think are additional spontaneous revertants generated during extra bacterial growth caused by the histidine added from the urine. Thus the true baseline in Fig. 2 is likely to be about 20 revertant colonies.

Assay of the Mutagenicity of the Urine from Cigarette Smokers. We have examined the urine of 10 cigarette smokers (who smoked from 15 to 44 cigarettes per day) for mutagenicity. Of seven smokers who inhaled and smoked ordinary cigarettes, all had mutagenic urine. We have taken 24 urine samples from three of these seven smokers (CF, GC, and SP) at various times (excluding only morning samples voided before the start of smoking for the day). All of these samples, except for two of borderline significance, were mutagenic and showed a good dose–response relationship. Two smokers smoked low-tar cigarettes; one (WS) who normally smoked these cigarettes had mutagenic urine and one (CF) who switched to low-tar cigarettes for a day did not. Two smokers who did not inhale did not have significant urinary mutagenic activity.

Kinetics of Excretion of Mutagens. We have compared the mutagenicity of urine voided before retiring in the evening with that voided on arising the following morning (Fig. 2*B*). There was no appreciable mutagenic activity in eight paired (evening/next morning) urine samples from nonsmokers. This was also the case for the two smokers who were noninhalers. In the case of the eight pairs from smokers, the evening sample was always mutagenic while the following morning sample was

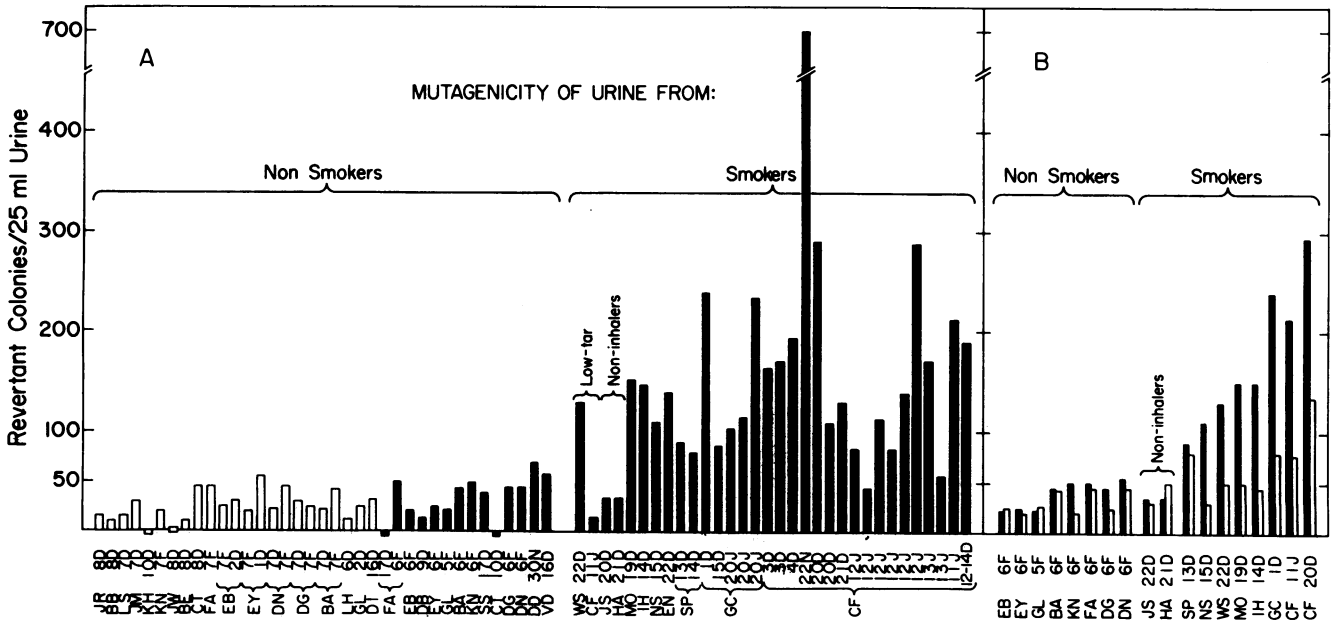

FIG. 2. Urine samples (usually 200 ml) from 10 smokers and 21 nonsmokers were treated as described in *Materials and Methods* and the *text* and assayed for mutagenicity. In a few samples where less urine was used, the dimethyl sulfoxide was reduced proportionately. The least square line for the control, 50-, and 100-μl points was determined and the mutagenicity (after subtracting the control value) for a 100-μl aliquot (25 ml of urine) is plotted. The results from smokers whose urine samples were assayed on more than one occasion are grouped together. Each bar is identified by the individual's initials, and the date of the sample collection (indicated by the day and the first letter of the month: Nov., Dec., Jan., Feb.). A black bar represents the mutagenic activity of a urine sample taken on retiring in the evening (or in *A* for multiple samples from smokers taken on the same day) and a white bar represents one taken on arising in the morning. (*A*) Urine voided at various times; (*B*) evening/next morning samples.

always less or not mutagenic. The occasional significant mutagenic activity in the morning following a 6- to 10-hr period of nonsmoking indicates that there may be an appreciable time period for clearance of some mutagens.

DISCUSSION

The development of rapid, sensitive *in vitro* assays for mutagens such as the *Salmonella*/mammalian microsome test (5), and the demonstration that about 90% of chemical carcinogens can be detected by this system, makes it possible to detect the variety of mutagens/carcinogens in the environment that are contributing to cancer, genetic birth defects, and other diseases caused by DNA damage.

One approach is to test the reasonably pure chemicals to which humans are exposed, both those on the market and those under development. Another approach is to test the complex mixtures that people ingest, or inhale, or absorb through their skin.

A completely different approach is to analyze feces and urine for mutagenic products. The analysis of mutagens in human feces appears quite promising (30). We have been interested in analysis of mutagens in urine (10, 13), as have other groups (11, 14). The analysis of the mutagenicity of urine is complicated by several technical problems. Urinary metabolites are usually present in low concentrations and relatively little urine can be added directly to the *Salmonella* test system because urinary histidine interferes in the test. In addition, urine contains a variety of conjugates such as glucuronides and sulfates that are not split by the enzymes in *Salmonella* or in the mammalian liver added to the system. This latter problem can be overcome by adding β-glucuronidase (10, 11) or sulfatase to the petri plate. In the present study we have explored the various types of adsorbant resins for concentrating mutagens from urine. Because most carcinogens and mutagens likely to be effective must penetrate the cell membrane, they tend to be nonpolar unless they are taken up by specific transport systems. Thus the use of a resin such as XAD-2 seems particularly convenient because it is quite effective at adsorbing relatively nonpolar compounds in water (19–27) or urine (15–18), while letting histidine pass through. Even some of the conjugates of nonpolar compounds, such as glucuronides and sulfates, stick to the resin (Tables 1 and 2).

Because one can concentrate the urinary material several 100-fold, it is quite feasible to put the equivalent of 25 ml of human urine on a petri plate. At this concentration, in all but two borderline cases, the 37 urine samples from nonsmokers examined on strain TA1538 showed no significant mutagenicity. We have examined the urine from some of the nonsmokers on strain TA100 as well. A few samples showed some weak activity on this strain.

Several lines of evidence suggested that we should be able to detect some mutagenic activity in the urine of cigarette smokers. We had previously reported that cigarette smoke condensate is quite mutagenic on strain TA1538 in the presence of rat liver (or human autopsy liver) homogenate (31) and this has been confirmed in several additional studies (32–34). We had found an activity of about 1240 revertant colonies per mg of tar or about 29,000 colonies per cigarette containing 23 mg of tar. About 800 mg of tar would be inhaled per day by a smoker smoking 40 cigarettes containing 20 mg of tar each. It seemed likely that a good fraction of this tar would be absorbed and appear in the urine because an appreciable amount of the nicotine in the tar is absorbed (possibly 10%) and appears in the urine (35, 36). It is also known that there is a higher risk of bladder cancer among smokers (37).

Our analysis of smokers' urine shows that all of the seven smokers examined who inhaled and smoked ordinary cigarettes had significant mutagenic activity in their urine. Of the 28 samples taken from these smokers at different times (excluding the urine first voided on arising), all but two showed consider-

able mutagenic activity (Fig. 2A). We have also examined the mutagenic activity of urine after the overnight period of nonsmoking (Fig. 2B). In all eight pairs the night urine showed more activity than the morning urine; in four cases the activity of the morning urine was still significant and in four cases the activity had dropped to below the limits we consider significant. Thus, it appears that there is much less mutagenic activity in urine after the interval of 6–8 hr of sleep. We have also found that smokers who did not inhale showed no significant mutagenic activity (Fig. 2). A detailed kinetic study would be complicated by individual variation in amount of urine voided, dilution of urine, diet, etc.

It is of considerable interest to know whether the wide-spread use of low-tar cigarettes will be less hazardous (38). If mutagenic activity is a reflection of carcinogenic activity, low-tar cigarettes may, in fact, be less hazardous. The important factor appears to be the total amount of tar one is exposed to, because all tar appears to have approximately the same mutagenic activity per mg (34). We have analyzed urine of two smokers who were smoking low-tar cigarettes. One smoker who normally smoked low-tar cigarettes had mutagenic urine and one who switched to low-tar cigarettes for the day did not. Clearly, an analysis of the quantitative aspects of mutagenicity of urine as related to metabolic and genetic differences in smokers, and type and number of cigarettes smoked, would be interesting.

We propose that this method be used to examine the urine of a large population of nonsmokers for mutagenicity in an attempt to detect unsuspected mutagens/carcinogens. (For general screening we would recommend using the maximum amount of urine that can be obtained from an individual and washing the column with 1.5 ml of water before elution with acetone.) It would also be of interest to examine particular populations that are likely to be absorbing significant doses of mutagens, such as women dyeing their hair (4) or children in sleepwear treated with add-on flame retardants (3). When new drugs are tried on humans this method should be used to examine the urine of the patients for mutagenicity as an adjunct to the standard toxicological tests in animals.

The method is of sufficient practicality to make it useful for routine screening of animals used in toxicology testing as an adjunct to the standard *Salmonella* plate test. There are several reports in the literature of the detection of mutagens in animal urine that could not be detected in the standard plate test (as cited in ref 13).

The XAD-2 methodology presented here should also be useful in examining the mutagenicity of pollutants in water. Several studies have shown the utility of XAD-2 for adsorbing nonpolar chemicals from water (19–27). It also should be useful in examining a variety of other aqueous fluids that are consumed by humans.

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