4

URINE MUTAGENICITY ASSAYS

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INTRODUCTION

Chemical mutagens can be defined as agents capable of causing heritable changes in genetic material (Sorsa et al., 1982). Various bacterial mutagenesis techniques can be used to analyze urine, feces, and breast fluid for such mutagenic chemicals. For over 15 years the presence of mutagens in human body fluids has been used as an indicator of systemic exposure to a wide range of mutagenic chemicals. Because mutagenesis testing reveals the presence of many types of mutagens in the analyzed sample, the procedure is especially useful in detecting multiple or unspecified mutagenic agents. Currently, the urine mutagenesis assay is one of the most widely used techniques to monitor populations for mutagenic exposures.

As a sample body fluid, urine has several advantages. It is a major route of elimination for xenobiotics. Urinary material consists of substances already concentrated by the kidneys (Eisenstadt, 1983). Urine is an easy body fluid to collect, and its long-term storage is somewhat simpler than that of blood, feces, or breast milk or fluid. Accordingly, the vast majority of human studies in mutagenesis have been conducted on urine specimens. The outstanding disadvantage in using urine as a specimen for mutagenic testing is the relatively short cycle of urine production and excretion. Because urine is manufactured continually and expelled every few hours, it is unsuitable as an indicator of long-term or cumulative exposures. An additional problem is the possibility that certain mutagens may be underrepresented in urine.

BIOLOGICAL BASIS OF URINE MUTAGENESIS TESTING

The principal methods of testing urine for mutagenicity are the Ames test and a derivative method, the fluctuation test. The biological basis of both tests is the ability

of a test solution to induce specific mutations in selected bacteria. The mutations may be either *base-pair* or *frameshift* mutations (McCann, 1983). In base-pair mutations, one nucleotide base in a matched pair within a DNA strand is replaced by another base. If this substitution occurs at a critical location in the gene, the resulting protein product is dysfunctional (Fig. 4-1). In a frameshift mutation, a nucleotide is either added or deleted in a sequence of base pairs. As a result, the reading frame shifts for every point after the alteration (Fig. 4-2). Again, the result can be the inability to function in producing a critical product (McCann, 1983).

In the early 1970s, Ames and his colleagues developed numerous strains of *Salmonella typhimurium*, each designed to exhibit specific mutations that can be mutated back to a normal state ("reverted") by a wide variety of mutagens. Several strains, each with its own unique mutations and suitable for detecting particular mutagens, were designated as the *tester strains* of the mutagenesis assay.

The enabling mechanism of the Ames test for mutagenicity is the dependence of *Salmonella* on the amino acid histidine. Tester strains are genetically engineered mutants unable to produce this essential substance. When incubated on agar plates enriched with histidine, the bacteria will grow until the histidine in the media is exhausted. If a mutagen is mixed with the bacteria before incubation, those bacteria reverted by the mutagen will be able to produce histidine for themselves, and thus continue growing after the added histidine is gone. The presence of the mutagen can then be detected by the *Salmonella* colonies that develop (Ames et al., 1975). The assay's ability to quantitate mutagenicity varies widely, depending on the compound tested; some investigators report only qualitative results obtained with the Ames method (Tennant et al., 1987). The fluctuation test for mutagenicity is based on the same principle, but uses an additional organism, *Escherichia coli*, which has been altered to be deficient in its ability to produce tryptophan (Sorsa et al., 1981). The fluctuation test also employs test tubes or wells rather than plates as containers. Table 4-1 summarizes methods of performing both tests, which are more fully discussed later.

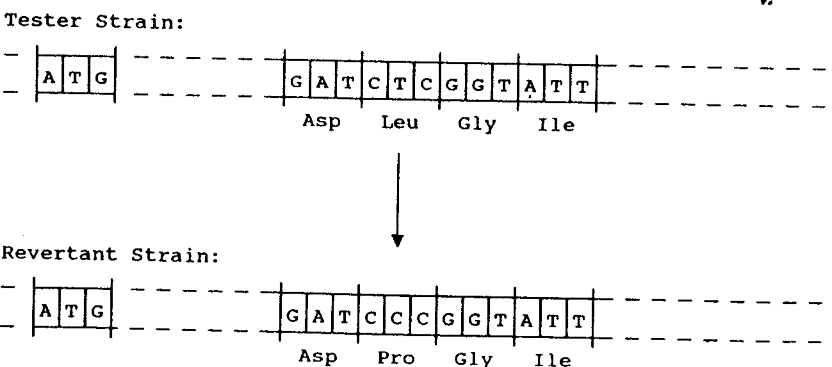

Figure 4-1. Base-pair mutation. (after McCann, 1983)

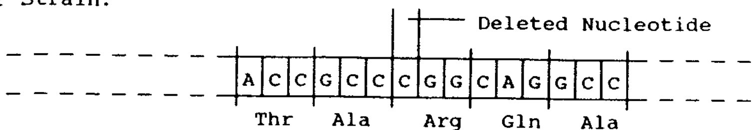

Figure 4-2. Frameshift mutation. (after McCann, 1983)

ISSUES IN SPECIMEN COLLECTION AND PREPARATION

Methods of sample collection and preparation vary slightly among investigators. Understanding of some key points in specimen preparation, however, is crucial to the valid interpretation of study results. The major issues are sample concentration, choice of elution fluid, and enzymatic sample activation.

Sample Concentration

Sample concentration is a technique employed to reduce total volume while retaining all mutagens present in the specimen. Concentration is desirable because the small volumes of liquid mixed with the test organisms may not contain sufficient amounts of mutagen if they are unconcentrated. Up to several hundred milliliters of urine are slowly poured through a column containing XAD-2 resin, which selectively adsorbs nonpolar molecules from the sample (Eisenstadt et al., 1983). The column is rinsed with distilled water and refilled in a wash step (eluted) with a solvent that is then evaporated. The resulting dried specimen concentrate is resuspended, usually with dimethylsulfoxide (DMSO). This elution product, containing the mutagen "collected" by the XAD-2 column, is then used to perform the mutagenesis assay. Although most studies employ this technique, some use unconcentrated urine.

Table 4-1 Specimen Preparation and Testing Protocols

A. Collect random or timed urine specimen. Preserve chemically or by refrigeration
B. Activate specimen with S-9 liver extract and/or β-glucuronidase. Incubate overnight at 37°C
C. Concentrate specimen
  1. Pass through XAD-2 resin column
  2. Wash column
    a. with distilled water
    b. with solvent (e.g., acetone)
  3. Evaporate eluate
  4. Resuspend dried residue with solvent [e.g., dimethylsulfoxide (DMSO)]

Ames Method

A. Mix sample concentrate with *Salmonella* tester strains and histidine
B. Plate onto solid agar
C. Incubate 48 hrs at 37°C
D. Score by subtracting number of colonies on control plates from number of colonies on test plates

Fluctuation Method

A. Dispense fluid medium into about 50 test tubes/sample wells
B. Inoculate with tester organisms
C. Add sample concentrate to half of tubes
D. Incubate 72–96 hours at 37°C
E. Read turbidity by comparison with standard
F. Compare difference between controls and samples with Chi-square or *t*-test

Elution Techniques

An important question in sample concentration is the choice of solvent used to extract bound molecules from the concentration column. An inappropriate extraction procedure could underrepresent the amount of mutagen present in a specimen, or even result in no mutagenic material being recovered from the column. Ames recommended that the solvent used be suitable for the suspected mutagens (McCann and Ames, 1975). For general screening, like that done in most studies using human urine, he suggested an extraction procedure applicable to a wide variety of metabolites. The most common choice is a solvent, such as acetone or methylene chloride, with resuspension of the dried extract in DMSO (Eisenstadt et al., 1983; Maron et al., 1981).

Activation/Deconjugation

Activation of the urine sample with enzymes and microsomal liver preparations is a recommended specimen preparation technique (McCann, 1983). This step is based on the fact that many chemicals are not themselves mutagenic, but are transformed into mutagenic forms through hepatic activity (McCann, 1983). Activating the specimen by adding a microsomal extract of rat or human liver (known as S-9 extract) allows the transformation of promutagens into mutagenic forms in vitro. It should be noted that S-9 activation is not the exact equivalent of metabolization by the target organ, but rather is an in vitro surrogate.

Similarly, some conjugated metabolites—molecules that have been bound with other substances by the liver to aid in eliminating them from the system—are nonmutagenic when excreted in urine, but were mutagens in vivo. Adding enzymes, such as β-glucuronidase, to the urine sample deconjugates these substances and permits detection of their mutagenic nature in the body (Falck, 1982). Everson (1986) notes, however, that a potential problem in activating specimens is that mutagenicity may be wrongly attributed to biologically inactive substances that have been activated only in vitro.

Additional Considerations

Exposures of interest in studies using urine mutagenesis testing include cigarette smoking, dietary items, therapeutic drugs, and occupational exposures. There is some evidence that the time of day may be a factor in the mutagenicity of casual urine samples collected from tobacco smokers: in two studies, the mutagenicity of samples

collected in the evening was greater than that of samples taken the following morning (Yamasaki and Ames, 1977; van Doorn et al., 1979). Because mutagenicity seems to be a short-lived phenomenon, and because presumed peak values may be of as much interest as average values over time, single urine samples are employed at least as often as timed 24-hour specimens. In occupational exposure monitoring, a major use of urine mutagenicity testing, 24-hour urine collection is extremely impractical (Aitio and Järvisalo, 1985) and is seldom used. Some studies attempt to correct for the low mutagenic potential of extremely dilute urines by adjusting sample volumes so that they are equivalent to a stated concentration of creatinine, an excreted substance commonly used as a measure of renal function. Using differing volumes of urine sample and reporting results per millimole of creatinine minimizes the effect of differences in the renal function of each individual.

The final factor to be considered in specimen collection and preparation is endogenous urine histidine. Certain people, such as those on high protein diets, may excrete histidine, which increases the overall bacterial growth on the test plates and possibly increases the rate of spontaneous reversion. When the sample is concentrated by column extraction, there is evidence that the distilled water wash step greatly reduces retention of urine histidine (Yamasaki and Ames, 1977; Recio et al., 1982). Alternatively, techniques are being developed to quantitate the amount of background growth, which identifies urines containing endogenous histidine (Everson, 1986).

AMES TEST PROCEDURE

The urine sample is centrifuged or filtered to remove particulates. When indicated, enzymatic deconjugation of the sample is performed by 37°C incubation overnight with β-glucuronidase. The specimen is then extracted by the XAD-2 elution column and solvent-extraction procedure described previously. This step also sterilizes the specimen, preventing growth of unwanted organisms that might be present in the urine.

Tester strains of *Salmonella typhimurium* are mixed with the specimen and with media containing enough histidine to permit a few generations of growth. Most of the literature is based on tests of five *Salmonella* strains: TA100 and TA1535 (base-pair mutations), and TA1538, TA98, and TA1537 (frameshift mutations). Currently, the recommended strains for general screening are TA97 (replacing TA1537), TA98 (replacing TA1538), TA100, and TA102 (Maron and Ames, 1983). Dr. Bruce Ames will supply these organisms on request.* It is important to understand that the different tester strains are specific for either base-pair or frameshift mutation; test organisms should be carefully selected to insure detection of the specific DNA damage that is suspected or should be employed in test panels designed to detect both mutation types.

The test plate is then incubated for 48 hours at 37°C. During incubation, those bacteria that revert to histidine production by specimen mutagens will establish colonies. Spontaneously reverted bacteria will also colonize the test plates. The test is scored by subtracting the number of spontaneously mutated colonies occurring on

*As of this writing, Dr. Ames' address is Department of Biochemistry, University of California, Berkeley CA 94720.

control plates from the total number of colonies on test plates (Ames et al., 1975; Maron and Ames, 1983; McCann, 1983; Everson, 1986).

Sensitivity and Specificity

In a survey of selected agents, Ames and colleagues estimated the sensitivity of the Ames test at 90 percent, specificity at 87 percent, and positive predictive value at 92 percent (McCann, 1983). These figures were based on tests with known carcinogens directly added to the tester strains. In another report of direct testing of carcinogens, Tennant et al. (1987) estimated sensitivity at 45 percent (95% C.I. 30-61), specificity at 86 percent (95% C.I. 68-96), positive predictive value at 83 percent (95% C.I. 63-95), and negative predictive value at 62 percent (95% C.I. 50-73). Estimates of sensitivity and specificity vary considerably, depending on the compounds being tested. In addition, sensitivity is probably increased and specificity decreased by the use of S-9 liver extract in the Ames test. Because compounds are unlikely to be metabolized to the same extent in human tissues, S-9 activation could result in a larger number of false-positive results when interpreted for humans.

Thus, substances identified as mutagens by the Ames method are likely to cause mutations in vivo, at least to the extent that S-9 activation reflects target tissue metabolization. Because the test is designed to detect only specific point mutations, however, a negative result may not indicate the absence of all mutagenic activity within a specimen. McCann (1983) lists several important carcinogens that have tested negative in the Ames mutagenesis assay: carbon tetrachloride, DDT, dieldrin, and other chlorinated hydrocarbons. Some of these agents have been detected as mutagens in different short-term assays, whereas others are believed to produce mutagenic metabolites with half-lives too short to be identified by any current short-term methods. It should also be recalled that nonmutagenic agents may be involved in carcinogenesis, as is the case with asbestos. This mineral fiber is thought to cause cancer through its physical properties as an irritant. Another example is diethylstilbestrol (DES), which most likely promotes carcinogenesis in already initiated cells. Failure to detect entire classes of potentially mutagenic or carcinogenic compounds is a significant disadvantage of the Ames procedure.

Interindividual and Intraindividual Variability

One factor that greatly influences variability in the Ames test is the metabolic capability of study subjects. One can expect to find both interindividual and intraindividual variability unrelated to exposures of interest, based on short-term or long-term fluctuations in hepatic and renal function. For a general discussion of variability, see "Human variability" and "Determinants of variability" in Chapter 3.

FLUCTUATION TEST PROCEDURE

The fluctuation test is based on the same biological principle as the Ames test: reversion of mutated bacteria. The most commonly used organism in this procedure is *E.*

coli, and tryptophan is the product of the gene that reverts back to its normal state. Some investigators also employ Ames' *Salmonella* strains as test organisms in this procedure.

Sample collection and preparation is a modified version of the Ames protocol. A fluid medium containing tryptophan is dispensed into about 50 test tubes or wells, which are then inoculated with the tester strains of *E. coli*. Current recommended strains are WP2 uvrA, and CM561 exrA (Green et al., 1976). The concentrated urine specimen is then added to half the tubes. After 37°C incubation for 72–96 hours, reverted bacteria will have grown to a sufficient volume to produce a qualitatively assessed level of turbidity (positive versus negative) in the tubes compared to a standard. The difference in the number of positive control tubes and test tubes is evaluated by a Chi-square or Student's *t*-test (Green et al., 1976; Collings et al., 1981; Falck, 1983).

An automated method for this assay has recently been developed (Falck et al., 1985). A major advantage, apart from labor savings, is the ability to monitor the kinetic activity of sample wells. By detecting the shape of the growth curve, one can gain more information about the mutagens present in the sample; one can also detect toxicities that prevent the tester strains from surviving.

OTHER URINE MUTAGENESIS TESTS

Two other procedures occasionally are used to analyze the mutagenicity of urine: sister chromatid exchange (SCE), presented in Chapter 6, and a forward-mutation assay that measures mutations in the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) genetic locus of mammalian tester cells. In the sister chromatid exchange method, urine is used as the test substance in an attempt to induce mutations within human lymphocyte cell lines. With the HGPRT assay, mammalian cells are mixed with urine and scanned for mutations that permit cell survival under test conditions. Details of the method are beyond the scope of this chapter.

STUDIES OF URINE MUTAGENICITY IN HUMANS

The investigation of urine mutagenicity in humans has been conducted in three major categories: life-style exposures, such as tobacco smoking and dietary factors; exposures resulting from disease or medical therapy; and occupational exposures. Few of these studies were designed as epidemiologic research. Many early investigations were planned to test modifications of the method, and others reflect the genetic and toxicologic interests of their authors. In general, sample sizes are very small, often because the study involved some experimental exposure. Possible systematic biases are rarely if ever addressed, and potential confounders (apart from smoking) are only occasionally taken into account in design or data analysis. Tables 4-2 through 4-5 summarize the urine mutagenesis studies.

Tobacco Smoking and Urine Mutagens

Cigarette smoking is the most widely reported life-style exposure that produces mutagenic urine. Because of smoking's known carcinogenicity, smokers have often been chosen as subjects in tests of methodologic refinements. Table 4-2 summarizes results of the tobacco smoke studies.

In a test of specimen extraction procedures, Yamasaki and Ames (1977) assayed urine samples from 10 cigarette smokers (including three who did not inhale) whose tobacco consumption ranged from 15–44 cigarettes per day, along with urine samples from 21 nonsmokers. All participants denied taking medications and consumed "normal diets." Morning and evening specimens were collected and frozen until analysis, when the samples were concentrated using XAD-2 resin columns eluted with acetone and resuspended with DMSO. Samples were activated with S-9 liver homogenate extract and β-glucuronidase.

Smokers' urines showed significant mutagenicity to *Salmonella* strain TA1538 (up to 700 revertant colonies/25 ml urine), whereas nonsmokers produced no mutagenic specimens. A background of up to 50 revertant colonies/25 ml urine was considered negative. Some of the lowest mutagenic responses were shown by the noninhaling smokers and two low-tar brand smokers. Pooled specimens from one smoker were concentrated twice and showed a higher level of mutagenicity as more urine was added to the test plates. For nonsmokers and noninhalers, morning and evening mutagenicity were essentially the same, whereas the samples of inhaling smokers were always mutagenic in the evening and less or nonmutagenic the following morning.

In an 8-week long study, Jaffe et al. (1983) collected urine samples each week from 13 smokers and five nonsmoking controls. They did not monitor diet and medications or give specimen collection times. Specimens from the five nonsmokers showed no mutagenic activity. Specimens from smokers, however, demonstrated a 10-fold range in values, from 0.14–1.4 revertants/μmole creatinine. Although there was a positive association overall between tobacco consumption and mutagen excretion, no dose-response could be observed on the basis of either the tar content of the cigarettes or the number of cigarettes smoked.

Table 4-2 Mutagenicity Studies of Exposure to Tobacco

Reference	Sample preparation	Sample activated with	Tester strains	*N*	Reported results
Yamasaki & Ames	XAD-2/acetone/DMSO	S-9/β-glucuronidase	TA1538	31	+
Jaffe et al.	XAD-2/acetone/DMSO	S-9	TA98	18	+
Van Doorn et al.	XAD-2/acetone/DMSO	S-9	TA1538	20	+
Mohtashamipur et al.	XAD-2/methanol/DMSO	S-9	TA98	12	+
Recio et al.	XAD-2/acetone/DMSO	S-9	TA98	35	+
Falck et al.[a]	XAD-2		TA98/WP UVRA	39	+
Bos et al.	XAD-2/acetone/DMSO	S-9	TA1538	18	+
Aeschbacher & Chappuis	XAD-2/acetone/DMSO	S-9/β-glucuronidase	TA98/TA100	27	+

[a]Fluctuation method.

Van Doorn et al. (1979) studied mutagenicity in five nonsmokers and 15 smokers, all of whom were men taking no medications and on normal diets. Smokers were evenly divided into three groups: those who smoked 1–10, 11–20, or more than 20 cigarettes/day. Smokers of less than 10 cigarettes/day showed no significant difference from the five nonsmokers in urine mutagenicity, approximately $0.3–0.4 \times 10^6$ reverted bacteria/mmol creatinine. Urine mutagenicity in those who smoked 11–20 cigarettes was significantly different from that of nonsmokers ($p < .05$), roughly 2.5×10^6 bacteria/mmol creatinine. Samples from those who smoked over 20 cigarettes/day were significantly more mutagenic than samples from those who smoked 11–20 cigarettes ($p < .01$), about 3.5×10^6 bacteria/mmol creatinine. Similar gradients were reported for concentrations of urinary thioethers, such as mercapturic acids, which are nontoxic and nonmutagenic by-products of potentially carcinogenic alkylating substances present in cigarettes.

A threshold effect was also reported by Mohtashamipur et al. (1985). In this study, designed to test the effectiveness of three sample concentration methods, 12 subjects of both sexes were divided into "nonsmoking" and smoking groups (but nonsmoking is defined here as 1–5 cigarettes/day). Using a XAD-2/methanol/DMSO concentration protocol with S-9 activation and *Salmonella* TA98, the investigators found no mutagenicity in the urine samples of the "nonsmoking" group. Smokers produced mutagenic urine (up to 60 mean revertants/plate), but the investigators could make no correlation between the number of cigarettes smoked and amount of mutagenicity.

Recio et al. (1982) collected evening urines from 16 nonsmokers and 19 smokers who smoked an average of one pack per day. Samples were plated at three concentrations per subject: 40, 60, and 80 μL of concentrate (corresponding to 10, 15, and 20 ml of urine). Again, no significant mutagenicity was found among nonsmokers. A large variation in mutagenicity was reported for smokers, ranging from 31–162 revertants/plate (mean 80 revertants/plate). Although two low-tar brand smokers and a pipe smoker scored some of the lowest mutagenic values, Recio could find no relationship overall between number of cigarettes (self-reported by subjects) or amount of tar and urine mutagenicity. Recio also presents results of a series of assays on a single smoker who increased his cigarette consumption over 8 successive days. The result is a fairly linear dose-response between number of cigarettes and level of urine mutagenicity.

In a study of the fluctuation test, Falck (1982) examined three groups of Finnish military recruits. Group A consisted of 13 men who smoked medium-tar cigarettes; group B had 14 low-tar smokers, and group C comprised 12 nonsmokers. Because all subjects were recent service inductees and were living on-post, they were comparable in age, general health, and extraneous exposures encountered during the study period. Cigarettes were supplied daily to each subject, and consumption was comparable between the two smoking groups. Evening urine specimens were collected at the end of the first, fourth, and seventh weeks, and were then concentrated in XAD-2 resin columns. Each specimen was split into parts, half of which were S-9 activated and half which were not. The samples were then dispensed into test tubes containing suspensions of either *Salmonella* TA98 or *E. coli* WP2 uvrA. Only activated samples of smokers' urine showed mutagenic activity in the *Salmonella* mixture (creatinine-adjusted means of 17.7 ± 6.0 for group A, 17.3 ± 5.9 for group B, and 6.5 ± 4.1 for group C, $p < .001$ for both groups A and B when compared with group C). No correlation could be found between urinary mutagens and the amount of tar per cigarette.

In a study of passive smoking, Bos et al. (1983) enclosed eight nonsmokers in a poorly ventilated room for 6 hours with 10 heavy smokers. The subjects voided just before the start of the experiment and collected all urine for the succeeding 12 hours. Twelve-hour samples were also collected the day before and the day after the exposure. In addition, Bos and colleagues sampled the room air the day before, the day after, and during the test by bubbling about 500 L of air through cylinders of chilled hexane. The air samples were then evaporated to dryness, and resuspended in DMSO. Both air and urine samples were plated with *Salmonella* TA1538. The study showed statistically significant differences between values for urine from nonexposed and exposed nonsmokers (mean colony number of 2.8 and 3.7, $p < .02$). The increase in nonsmokers' urine mutagenicity was judged to be about 4 percent of the urine mutagenicity of the active smokers in the experiment. Mutation assays of room air produced values up to 10 times higher for smoky samples than for samples taken when the room was empty.

Aeschbacher and Chappuis (1981) compared the urine mutagenicity of cigarette smokers and coffee drinkers in two experiments. In the first, they divided 15 subjects into three groups: two nonsmoking groups (six persons each), one of which drank instant coffee, and a group of three who both smoked and drank coffee. The experiment lasted 4 days, during which all subjects followed a standard diet, drank coffee as assigned (12 g instant coffee per day), and smoked as usual (20–30 cigarettes per day). All urine was collected during this period, with acetic acid preservation and refrigeration of each subject's pooled specimens. Half of each pooled urine was deconjugated with β-glucuronidase, then both conjugated and deconjugated aliquots were concentrated and activated. The investigators found a dose-response relationship between the amount of smokers' urine concentrate plated and the number of revertants, ranging from 137 colonies/plate for a DMSO control to 315 colonies/plate with 100 μL of sample concentrate. There was no mutagenicity found in the urine of nonsmokers, regardless of coffee consumption.

In the second experiment, 12 nonsmokers and 6 smokers were given 1 L of water to drink, and their urines were collected and preserved. A week later the subjects ate a standard breakfast and drank 1 L of coffee, then fasted for 7 hours. Smokers consumed cigarettes as desired (ranging from 7 to 18). All urine was collected with preservative. As in the first experiment, all nonsmokers' urines were negative. There was only a slight increase in the urine mutagenicity of the smokers after they drank coffee rather than water; 68 ± 11 colonies/plate compared with 54 ± 7 colonies/plate at 200 μL of sample concentrate. In both experiments, the deconjugation step reduced the number of revertant colonies detected, perhaps because the high sample volumes resulted in concentrates with bacteriocidal potency.

Diet and Mutagenicity

Several studies, summarized in Table 4-3, have examined the urine mutagenicity of foods and food preparation methods. Baker et al. (1982) conducted two series of experiments with five subjects. In the first, the participants fasted for 24 hours and were allowed only nonalcoholic liquids, including coffee, milk, soft drinks, and juices.

Table 4-3 Mutagenicity Studies of Dietary Exposures

Reference	Exposure	Sample preparation	Sample activated with	Tester strains	N	Reported results
Baker et al.	Fried pork	XAD-2/acetone/ DMSO	S-9/β-glucuronidase	TA98/TA100/TA1535/ TA1537/TA1538	5	+
Dolara et al.	Fried pork	XAD-2/acetone/ DMSO	S-9	TA1538	8	+
Sousa et al.	Fried ground beef	XAD-2/acetone/ DMSO	S-9/β-glucuronidase	TA98/TA100	4	+
Sousa et al.	Red wine/ grape juice	XAD-2/acetone/ DMSO	S-9/β-glucuronidase	TA98/TA100	5	–

They then consumed a morning meal of either fried or microwaved pork or bacon, followed by another 24-hour liquid diet. All urine for the entire 48-hour period was collected as individual samples and frozen. In the second test, subjects ate a light lacto-ovo-vegetarian meal the preceding evening, then breakfasted on bacon. Urines were collected before breakfast and for 4 hours after.

Neither study detected any increase in urine mutagenicity in samples taken after microwave-prepared meals. The first test, a 48-hour experiment, showed that most mutagenicity was detected in the first 2–4 hours after ingestion of the fried foods, although activity continued for up to 24 hours. For both experiments, the mutagenicity of the subjects' urine was about 30 percent that of the mutagenicity detected in assays of the fried foods themselves.

Dolara et al. (1984) modified the Baker fasting protocol by adding two experimental groups: one group followed a lacto-ovo-vegetarian diet for 1 day before eating the test meal; the other group was permitted food during the urine collection period after the test meal. Dolara found a slight to moderate increase in mutagenic activity after the test meal in all of the test groups. The recovered urine mutagens accounted for only about 0.6 percent of the ingested dose, according to assays of food samples. Dolara notes that differences in the degree of char on the meat surfaces could help explain the variation between his results and Baker's.

Results similar to Baker's were reported for the ingestion of fried beef by Sousa et al. (1985,B). In addition, Sousa tested the mutagenicity of red wine and grape juice consumed by five nonsmoking subjects (Sousa et al., 1985,A). He detected no urine mutagenesis in *Salmonella* TA98 or TA100, with or without S-9 activation. Deconjugation with β-glucuronidase also produced no activity. In contrast, extracts of the beverages themselves showed mutagenicity. Concentrated urines spiked with beverage concentrates were also mutagenic.

Liver Disease and Urine Mutagens

In mammals, metabolic transformation of xenobiotics is a major protective role of the liver. Substances absorbed into the bloodstream are enzymatically converted to forms that improve biological availability or facilitate elimination. During this process, potentially mutagenic agents may be activated or frank mutagens may be altered to inactive products. Thus, liver diseases, such as cirrhosis, may have important implications for the metabolism and excretion of mutagens.

There are two possible mechanisms by which liver dysfunction could influence mutagen metabolism. First, organ damage disrupts the production of enzymes needed to degrade mutagenic agents in the system. In addition, severe liver damage may lead to portal-vein shunting, a condition in which some of the portal vein blood is put into the systemic circulation without being processed by the hepatic system. Both situations thus result in potentially prolonged systemic contact with mutagenic agents. Table 4-4 summarizes studies of mutagenicity in liver disease.

Conflicting reports of urine mutagenicity in cirrhosis have been published by Gelbart and Sontag (1980) and Everson et al. (1983). Gelbart and Sontag concentrated 12-hour urines from five nonsmoking cirrhotic patients and compared them with urine concentrates from 12 healthy smokers and 15 healthy nonsmokers. They found 60-390 revertants/25 ml urine in the urine of the cirrhotics, 70-200 revertants/25 ml urine among smokers, and essentially negative results among nonsmokers. The authors note that three of the five patients had alcoholic cirrhosis with slight elevations in some liver enzymes. None of the patients was drinking at the time of the study, but all were in poor general health and poorly nourished.

In contrast, Everson et al. (1983) reported no increase in mutagens in the urines of 12 cirrhotics (including one smoker) or 9 patients with other liver disease (including three smokers). Five smokers (four apparently healthy and one with rheumatoid arthritis) were also analyzed. Each of the liver disease patients was taking numerous medications and exhibited some abnormal laboratory values. Twelve-hour urines were collected, concentrated, and tested with and without S-9 extract activation. Only the smokers showed an increase of mutagenicity, and no dose-response could be established based on amount of concentrate plated.

Exposures From Medical Therapy

Urine mutagenicity has been investigated in a number of medical therapeutics including: cancer chemotherapeutics, coal tar, metronidazole, niridazole, and praziquantel (Table 4-4). Minnich et al. (1976) assayed random urines from patients receiving cancer chemotherapeutic agents. Mutagenic activity was observed for 19 patients receiving cyclophosphamide and four on 5-fluorouracil, whereas no increase was reported for patients receiving melphalan or one person taking mitomycin C.

To evaluate the effect of coal tar and ultraviolet (UV) light, Wheeler et al. (1981) collected urines from 2 healthy nonsmoking volunteers and 12 nonsmoking and 2 smoking psoriasis patients being treated with UV light and coal tar. Of the 14 psoriatic patients, 12 displayed urine mutagenicity, ranging from 42-496 revertants/20 ml urine among nonsmokers to 213–1,100 revertants/20 ml urine for the two smokers. The volunteers, who received UV and coal tar treatments 1 week apart, also displayed urine mutagenicity levels of 16–493 revertants/20 ml urine.

Metronidazole and niridazole were found to be mutagenic by Legator et al. (1975). Six patients receiving metronidazole provided urine samples 1 hour after taking the drug each day for 10 days. Up to a 12-fold increase in mutation frequency over control plates resulted, with the most activity starting at about day 8. Speck et al. (1976) reported that paper chromatography of mutagenic urine from patients who received metronidazole yielded the unmodified drug and at least four of its known urinary metabolites. In the Legator study just mentioned, a urine sample of the single

niridazole patient showed a 50-fold increase in mutagenicity on day 2 after treatment. Roxe et al. (1980) attribute the mutagenic effect of niridazole to the metabolic activation of the pigments excreted by patients receiving this drug.

The antihelminth praziquantel was investigated by Obermeier and Frohberg (1977). Three patients submitted five urines each, which were activated with S-9 extract and deconjugated with β-glucuronidase and arylsulfatase. No increase in urine mutagenicity could be detected in *Salmonella* TA98 and TA100.

Occupational Exposures

Many occupations have been examined for possible mutagenic exposures. Exposure to anticancer drugs among nurses and pharmacists is the focus of the largest number of studies. Other subjects of investigations include worker exposures in chemical manufacturing, the rubber industry, foundries, and exposure to iron oxide particles (Table 4-5).

Exposures Among Health-care Workers

Falck et al. (1979) used the fluctuation assay to examine urine mutagenicity among nurses handling cytostatic drugs. Urine samples from 7 nonsmoking nurses, 10 nonsmokers receiving chemotherapy, and 32 unexposed nonsmoking controls, were tested. Mutagenicity of patients was significantly higher than that of nurses, but the nurses' urines were significantly more mutagenic than those of controls ($p < .001$).

In a similar study design, Bos et al. (1982) collected 24-hour urines from 32 nurses or patient care personnel exposed to cytotoxic drugs and from 29 controls not in contact with patients. The study included both smokers and nonsmokers. In tests with *Salmonella* TA100, mutagenicity among exposed nonsmokers was not increased compared with nonsmoking controls. Among smokers, exposed subjects were significantly more mutagenic than controls, who smoked a similar amount.

In three more investigations of cytostatic drug exposure, Staiano et al. (1981), Nguyen et al. (1982), and Everson et al. (1985) reported opposite, although not necessarily conflicting, results. Neither Staiano nor Everson found an increase in urine mutagenicity in hospital pharmacists working with vertical laminar flow hoods that draw aerosols up, preventing worker contact. Nguyen et al., however, reported a doubling of mutagenicity in the urines of four of six pharmacists working with a horizontal laminar flow hood, which protects materials inside from bacterial contamination but directs aerosols toward the worker.

Manufacturing and Industrial Exposures

Among Italian factory workers exposed to petroleum coke and pitch, Pasquini et al. (1982) found increased urine mutagenesis during and after work hours, but not before work or on Sunday, a day off. Ten exposed male workers and 16 unexposed controls from the same plant provided urines on Monday and Friday mornings on arising, and on Monday and Friday at the end of work. Both exposed and unexposed groups contained smokers. With tester strain *Salmonella* TA98, exposed workers showed significantly more mutagenicity than controls in urine specimens collected during or after work. Before work, and for a 12-hour specimen collected on a Sunday, the two groups showed essentially no difference.

Table 4-4 Mutagenicity Studies of Disease States/Therapeutic Exposures

Reference	Exposure	Sample preparation	Sample activated with	Tester strains	*N*	Reported results
Gelbart & Sontag	Cirrhosis	XAD-2/acetone/DMSO	S-9/β-glucuronidase	TA100	32	+
Everson et al.	Cirrhosis/other liver disease	XAD-2/acetone/DMSO	S-9	TA100/TA1538	26	−
Minnich et al.	Cytotoxic drugs	None	S-9/β-glucuronidase	TA1537	24	+/−
Wheeler et al.	Coal tar/UV light	XAD-2/acetone/DMSO	S-9	TA98	16	+
Legator et al.	Metronidazole/niridazole	Freeze-drying	S-9	TA1535/TA1538	7	+
Obermeier & Frohberg	Praziquantel	XAD-2/acetone/DMSO	S-9/β-glucuronidase	TA98/TA100	3	−

Table 4-5 Mutagenicity Studies of Occupational Exposures

Reference	Exposure	Sample preparation	Sample activated with	Tester strains	N	Reported results
Falck et al.[a]	Cytotoxic drugs	XAD-2/acetone/DMSO	S-9	TA98 WP2 UVRA	49	+
Bos et al.	Cytotoxic drugs	XAD-2/acetone/DMSO	S-9	TA100 TA1538	61	+/−
Staiano et al.	Cytotoxic drugs	XAD-2/acetone/DMSO	—	Not given	8	−
Nguyen et al.	Cytotoxic drugs	XAD-2/acetone/DMSO	—	TA98 TA100 TA1535 TA1538/TA1975	9	+
Everson	Cytotoxic drugs	XAD-2/acetone/DMSO	S-9	TA98 TA1538	26	−
Pasquini et al.	Petroleum coke/pitch	XAD-2/acetone/DMSO	S-9/β-glucuronidase	TA98	26	+
Kreibel et al.	Chemical plant/coke oven	XAD-2/methylene chloride-acetone/DMSO	S-9	TA100 TA1538	198	+
Dolara et al.	Chemical manufacturing	XAD-2/methylene chloride/DMSO	S-9	TA100 TA1538	70	+
Laires et al.	Mineral oils/iron oxide	XAD-2/acetone/DMSO	S-9/β-glucuronidase	TA98 TA100	33	+
Falck et al.[a]	Rubber industry	XAD-2/acetone/DMSO	S-9	TA98 WP2 UVRA	36	+
Ahlborg et al.[a]	Chemical	XAD-2	S-9	TA98 WP2 UVRA	109	+
Scarlett-Kranz et al.	Sewage treatment	XAD-2/DMSO	S-9	TA100	236	+

[a] Fluctuation method.

In chemical plant and coke oven workers, Kreibel et al. (1983) found increased revertants for smokers regardless of occupational exposure, as well as an increase in mutagenicity among exposed nonsmokers in one of four test configurations. They collected urine samples from 198 persons, including 101 chemical workers, 43 coke plant workers, and 54 controls. Fifty-one percent of the chemical workers, 65 percent of coke plant workers, and 20 percent of controls smoked. The authors characterize their results as showing "modestly but significantly higher" mutagenicity levels in the urines of both chemical workers and coke oven workers. They caution, however, that smoking was the main determinant of urine mutagenicity in the study.

Dolara et al. (1981) obtained 24-hour urines from 35 workers at two small chemical plants and selected a control group of 35 nonexposed subjects from the same age group. All participants were men, and some in each group smoked. Concentrated specimens were plated with *Salmonella* TA1538 and TA100 with and without S-9 activation. In TA1538 comparisons among nonsmokers, exposed workers were significantly different from controls only when the specimen concentrate was activated. Smokers of both exposed and nonexposed groups were not significantly different in either configuration of TA1538. With TA100, smokers and nonsmokers in the control group showed significant urine mutagenicity, but both classes of workers far exceeded controls in mutagenesis ($p < .01$).

Laires ét al. (1982) conducted a study of exposure to mineral oils and iron oxide particles. They collected urine samples at the end of the working day from two groups of people: 17 workers exposed to both oils and iron oxide and 16 workers exposed to mineral oils only. Both study groups contained smokers. Mutagenicity was significantly higher in the workers exposed to both mineral oils and iron oxide ($p < .002$). Among nonsmokers in both study groups, the urinary mutagenicity of those exposed to iron oxide was significantly higher than the urinary mutagenicity of those exposed to mineral oils alone ($p < .04$).

Using the fluctuation test, Falck et al. (1980) asked 20 rubber industry workers (nine of whom smoked) and 16 factory office controls (including seven smokers) to provide specimens after working 4 days of a 5-day week. Mutagenesis with the *E. coli* WP2 uvrA tester strain was marked among exposed workers regardless of smoking status. Among exposed workers, the mean values were 727.1 and 501.1 revertants/mmol creatinine for smoking and nonsmoking workers, respectively, versus 13.2 and 34.7 revertants/mmol creatinine for smoking and nonsmoking controls, respectively. Results of *Salmonella* tests, however, implicated smoking over occupational exposure: smokers had mean values of 717.1 and 298.2 revertants/mmol creatinine for exposed and nonexposed workers, respectively, versus 149.8 and 25.1 for nonsmoking workers and controls, respectively. All results were statistically significant.

Ahlborg et al. (1985) examined workers in a plant producing a large variety of chemicals and explosives. Twelve areas of specific exposure were identified, each involving different employees. One hundred and nine workers completed questionnaires on smoking and alcohol use, diet, health, and medications. Each worker provided three urines: one after 4 weeks vacation, one before work, and one at the end of a work shift. Half of each concentrated sample was activated with S-9; *E. coli* WP2 uvrA and *Salmonella* TA98 were the tester strains employed in this fluctuation assay. When all subjects were considered together, mutagenicity was significantly increased for the occupationally exposed group using TA98 without S-9 activation, but no significant

differences were observed for other test configurations. The increase is almost totally explained by a large rise in urine mutagenicity in workers handling trinitrotoluene (TNT). Examining each exposure separately showed that two groups had an increase in mutagenicity measured using activated TA98 (workers handling TNT and those handling hexaminetetranitrate), but the differences failed to reach statistical significance.

In a study of toxic wastes from industrial sewage, Scarlett-Kranz et al. (1986) compared 164 sewage treatment workers from 14 New York State plants with 72 water treatment workers. Each subject completed a questionnaire on smoking and drinking habits, length of employment, and position in the plant. Random urine samples were concentrated and plated with *Salmonella* TA100, both with and without S-9 activation. The frequency of urine mutagenicity was significantly higher among sewage workers than among water treatment workers, both with and without sample activation. Models using presence/absence of mutagens as the dependent variable were constructed (one each for activated and unactivated samples) to assess such possible confounders as smoking, alcohol use, or exposure to wood stove smoke at home. The activated sample model produced an adjusted odds ratio of 12.9 (95% C.I. 4.5–37.4) for employment in a sewage treatment plant. Smoking was not significant in the model; the authors theorize that the approximately equal percentage of smokers in each occupation or the time of specimen collection (end-of-shift, when presumably few opportunities to smoke had occurred during the day) minimized the effect of smoking in these data. In the model for unactivated samples, the adjusted odds ratio is 2.2 (95% C.I. 1.2–4.3) in favor of water treatment employment.

ISSUES IN THE INTERPRETATION OF URINE MUTAGEN STUDIES

Results of mutagenicity studies using the Ames or fluctuation tests can only be regarded as approximate, even apart from a consideration of study design issues. McCann (1983) discusses reasons for this, which are fundamental to the method. First, it must be remembered that the exact amount of mutagenicity shown by the sample cannot be determined, because the rates at which mutagenic constituents are converted into active forms by S-9 extract and the rates at which these metabolites decay are both unknown. Second, calculation of results should be based on the number of bacteria at risk for reversion. This number, however, is unavailable: only the number plated is known. Thus, a urine containing large amounts of a mutagen or other substances highly toxic to the tester strain may be scored incorrectly because the toxic urine killed a high proportion of tester organisms. The automated fluctuation procedure discussed earlier can monitor the kinetic activity of the test. A warning thus provided by abrupt shifts in the growth curves of tester strains may provide at least a partial solution to this problem.

Beyond methodologic problems, the basic relationship between in vivo mutagenesis and the presence of urine mutagens is not understood. Schulte (1987) notes that xenobiotic exposures result in a wide range of measurable biological responses. A consensus has yet to be reached on which of these responses reflect actual pathologic processes and which are adaptive. Until the presence of urinary mutagens can be more directly linked to specific disease, interpretation of positive mutagenicity tests must remain conservative. Presently, most investigators view positive tests only as indicators of exposure to possible mutagens.

Even given the limitations of the procedure, the inconsistency of findings in studies of urine mutagenicity are nonetheless striking. Positive studies vary considerably in their estimates of mutagenicity for similar exposures, and several exposures have been studied repeatedly with conflicting results. Everson (1986) advances five possible reasons for variation in mutagenicity results:

1. Heterogeneity in the nature and extent of exposure of different study populations to the agent or process being studied.
2. Variable exposure of study populations to unstudied confounders.
3. Differences in assay techniques causing false-positive or false-negative results.
4. Confounding by toxic or growth-promoting substances in sample extracts, resulting in toxicity to or growth of tester strains.
5. Inadequate or inappropriate statistical analysis of data.

He points out that factors 1, 2, and 5 are traditional concerns of the epidemiologist and will become even more important in the interpretation of mutagenicity testing as methodologic improvements address factors 3 and 4. Everson also notes that positive results in assays of urine mutagenesis might be interpreted as indicative of a superior ability to eliminate systemic mutagens, thus reducing in vivo exposure. He reasons that if mutagens are abundantly present in urine, they may be evidence of faster or more complete elimination from the body. If so, the mutagen's time in contact with target tissues may well have been less than average.

Another problem is that only mutations at specific genetic loci are detectable. Although one can partly address this by using multiple organisms, one cannot capture the full spectrum of mutagenic potential with these organisms. Finally, mammalian systems may differ in important aspects from genetically altered tester organisms like *Salmonella* and *E. coli*, for instance in the action of DNA repair systems and in vivo metabolic activation of promutagens. Brusick (1982) notes that basing direct estimates of risk on this method is inappropriate, both because of these organismic differences and because of the secondary nature of urine as an indicator of exposure.

SUMMARY

1. Mutagens are substances capable of causing heritable genetic changes. Mutagenicity testing detects the presence of a wide spectrum of mutagens in body fluids such as urine, feces, and breast milk.

2. Mutagenesis testing can be performed on any body fluid. Because of the ease of collection and storage, urine is most commonly used. Collection and handling of samples is noninvasive, relatively simple, and inexpensive.

3. The physical and chemical properties of a specific mutagen govern the rate at which it is metabolized within the body and then excreted. The metabolic characteristics of mutagens may vary somewhat, depending on the body fluid being tested. In general, however, mutagenicity will begin to appear within hours after exposure to the

agent of interest. Persistence of mutagens in urine is evidently brief: many studies report diminished mutagenicity within 24 hours or less after exposure.

4. For some mutagenic agents, reports of specificity and sensitivity of the urine mutagenesis assay are high, although published estimates vary. Not all mutagens are detectable through these tests, however; chlorinated hydrocarbons, for example, are potent mutagens that are not revealed by mutagenesis testing. Nor will the test differentiate between several potentially confounding exposures; it only reveals the cumulative effects of all recent detectable exposures. The fact that not all carcinogens are mutagenic may be an additional consideration in evaluating the appropriateness of mutagenicity testing.

5. Interindividual variability is high in mutagenesis assays, at least partly as a result of differences in subjects' liver and kidney function, which directly affects the metabolization and excretion of xenobiotics.

6. Details of sample preparation and test methodology may affect the validity and comparability of results. Areas of concern include sample extraction and activation and tester strains employed.

7. Simulating mammalian function in bacteria by adding enzymes and microsomes in vitro may not accurately model these processes in vivo. Both false-positive and false-negative results may occur.

8. The relationship between the presence of urine mutagens and in vivo mutagenesis has not been established. Most investigators interpret positive test results narrowly, as evidence of exposure to a potential mutagen.

Urine mutagenesis testing provides a useful tool in the evaluation of broad categories of potentially mutagenic exposures in occupational or community settings. The test is relatively simple, it is inexpensive to perform, and it can be carried out on samples that are easy to obtain and handle. It detects mutagenic activity that results from a broad range of exposures, a distinct advantage for most applications. Although sensitivity and specificity vary with the compounds detected, urine mutagenesis testing compares favorably with other short-term mutagenesis tests (Tennant et al., 1987).

Disadvantages of the assay include: interindividual and intraindividual variability, its inability to detect some classes of mutagenic chemicals, and disparities between microsomal sample activation with S-9 compound and the actual behavior of metabolized compounds in vivo.

Until very recently, human studies of urine mutagenesis testing have been limited to methodologic or pilot studies. Of those reviewed here, only the study by Scarlett-Kranz et al. (1986) could be described as a well-designed and properly analyzed epidemiologic study. The human studies literature, then, is limited in its value for drawing overall conclusions. Currently, a more serious limitation to the use of mutagenesis testing may be the lack of valid interpretations for positive results of the assay. At present, no definite link has been forged between mutagenic urine and disease. The most pressing need for further study in urine mutagenesis is the validation of a clear relationship between mutagenicity in human urine and morbidity.

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