A Simple Method for the Detection of Mutagens in Urine: Studies with the Carcinogen 2-Acetylaminofluorene

(carcinogen activation and detection/2-nitrosofluorene/Salmonella typhimurium/β-glucuronides)

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Contributed by Bruce N. Ames, November 19, 1973

ABSTRACT We described previously a simple test on petri plates for detecting many carcinogens as mutagens using an especially sensitive set of bacterial strains to detect mutagens and a rat, or human, liver homogenate for carcinogen activation. We now extend the utility of the method for the detection of mutagenic metabolites in urine. The addition of commercial β-glucuronidase (EC 3.2.1.31) to the petri plates along with the urine, liver homogenate, and bacteria allows detection of metabolites that are excreted in urine as β-glucuronide conjugates. By this method mutagenic activity is readily demonstrated with urine of rats that were administered as little as 200 μg (1.6 mg/kg) of the carcinogen, 2-acetylaminofluorene. In this case the major urinary metabolite that we detect appears to be a glucuronide conjugate. We propose that the method be used for the screening of human urines in order to detect mutagenic metabolites of drugs and of dietary components. It may also be useful for testing of urinary metabolites of drugs and food additives in experimental animals.

We have previously described a very sensitive and simple bacterial test system for detecting chemical mutagens (1-4). The compounds are tested on petri plates with specially constructed mutants of Salmonella typhimurium as tester strains. Four tester strains have been selected for sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by a variety of mutagens. One strain can be used to detect mutagens causing base-pair substitutions and three to detect various kinds of frameshift mutagens. In addition to the histidine mutation, each tester strain has two additional mutations that greatly increase their sensitivity to mutagens: one causes loss of the excision repair system and the other loss of the lipopolysaccharide barrier that coats the surface of the bacteria. We have shown that by adding a microsomal activation system of rat (or human) liver to the petri plates a wide variety of carcinogens can be activated to mutagens and thus detected easily (3). Thus, an important aspect of mammalian metabolism can be duplicated in an in vitro test. The present study examines the testing of urine in the combined bacterial/liver system. A wide variety of metabolites of drugs and other ingested compounds appear in the urine. Since many metabolites are excreted in urine as glucuronides, we added β-glucuronidase (EC 3.2.1.31) to our test plates. The method is demonstrated by administering the carcinogen, 2-acetylaminofluorene (AcAF), to rats and detecting mutagenic metabolites in the urine.

AcAF, like many other compounds, gives rise to a large number of metabolites (5-11). The oxidation products N-hydroxy-2-acetylaminofluorene (N-OH-AcAF), N-hydroxy-2-aminofluorene (N-OH-AF), and 2-nitrosofluorene (NOF), are thought to be proximate or ultimate carcinogens formed from AcAF and 2-aminofluorene (AF) by the microsomal systems of liver (5). They are more carcinogenic than the parent compounds at the site of application in rodents and react with nucleic acids to various extents (5, 12-15). We have previously shown that NOF, N-OH-AF, and N-OH-AcAF are very active frameshift mutagens and that AcAF, when incubated with liver homogenate on petri plates, can be activated to frameshift mutagens (2-4). All of these fluorene compounds mutate a repetitive C-G sequence in our Salmonella tester strain TA1535 (3, 17). Several conjugates of AcAF metabolites have also been described. The sulfate and acetate esters of N-OH-AcAF are extremely reactive forms and are thought to be possible ultimate carcinogens (5, 12, 13, 15). The glucuronide of N-OH-AcAF has been found in urine of animals given AcAF (8-11, 16), and the glucuronides of AF and N-OH-AcAF have been found after incubation of AcAF with liver extracts (9).

MATERIALS AND METHODS

Materials. AF was purchased from Schuchardt (Munich), AcAF from Aldrich, and the other fluorene compounds were gifts of the Millers and Bartsch (4). Dimethylsulfoxide (Schwarz/Mann spectrophotometric grade) was used as a solvent for N-OH-AF, and the other compounds were dissolved in ethanol.

Bacterial β-glucuronidase (type V-A, 130,000 units/g) and β-glucosidase were purchased from Sigma. Stock solutions (50 mg/ml) were prepared and sterilized by passage through 0.45-μm membrane filters.

Animal Experiments. Male albino rats (Sprague-Dawley/Bio-1, Horton Animal Laboratories, Oakland, Calif.), weighing 125 ± 5 g, were used. Pairs of rats were housed together in polycarbonate metabolism cages designed for the separate collection of urine and feces. Rats were given free access to water and Purina laboratory chow. Rats were injected intraperitoneally with AcAF suspended in 0.9% NaCl-1.75% gum acacia solution (18). Urine was collected in dark, ice-chilled flasks containing a drop of chloroform. 24-hr samples (about...
15 ml per rat pair) were collected, centrifuged to remove debris, and stored at -15° for 1 or 2 days until assayed. Before they were assayed, the samples were filtered through 0.45-μm membrane filters.

**Assay of Urine Samples.** Mutagenesis assays were performed as described (2, 3) with strain TA1538 of *Salmonella typhimurium*, our tester strain which can detect the variety of active metabolites of AcAF. The urine (or urine concentrate) to be tested was added to the top agar along with the bacteria and other additions. For activation of compounds by liver homogenate, 0.25 ml of S-9 Mix (3) was incorporated into the top agar. The 9000 × g liver homogenate fraction (S-9) was from a phenobarbital-treated rat and was prepared as described (3).

Where indicated, β-glucuronidase (0.1 ml of the sterile, 50 mg/ml solution) was added to the top agar. β-Glucuronidase is the most expensive single component of the assay, costing about 25 cents per 5 mg. Although we have used 5 mg for our assay, we have found that adding only 0.5 mg per assay did not cause a significant drop (<15%) in mutagenic activity, and we recommend this lower amount for general screening.

Urine concentrate was prepared after treatment with glucuronidase and extraction with chloroform. To 10 ml of urine was added 10 ml of buffer (1 M sodium acetate, pH 6.0) and 0.2 ml of glucuronidase solution and the mixture was incubated for 24 hr at 37° in a 50-ml extraction flask. Samples were extracted with 10 ml of chloroform with gentle swirling for 1 hr, and then the chloroform layer was drawn off and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 0.25 ml of ethanol. (Dimethylsulfoxide presumably would also be a good solvent for dissolving urine concentrates, as it is nontoxic to our bacteria and liver homogenate. Up to 0.25 ml of either solvent does not interfere with the test system.) Chloroform was found to extract the metabolites of 2-AcAF satisfactorily; diethyl ether proved to be unsatisfactory because of a residue that was toxic to the bacteria.

**RESULTS**

*Addition of Urine and β-Glucuronidase to the Assay System.* A series of experiments was done to determine how much urine and β-glucuronidase could be added to the standard assay system without interference. Up to 0.5 ml of rat urine could be added to a pour plate without interfering with mutagenesis by 2-AcAF. Larger volumes caused the top layer to become watery, and the bacterial colonies were difficult to count. Some human urine samples produced a heavy background lawn when 0.5 ml per plate was added: this effect was probably due to higher levels of histidine in the urine, reflecting the higher protein diets of the human donors. At 0.25 ml of urine per plate satisfactory results were obtained in all of the samples tried. Up to 0.05 ml of urine concentrate in ethanol (see Methods) and 5 mg of β-glucuronidase could be added to the pour plate without interference.

**Standard Curves and Reconstruction Experiments with AcAF Metabolites.** Fig. 1 shows the relative potency of the different AcAF metabolites in reverting the histidine mutation in strain TA1538 (2-4). Over the range of concentrations tested, the dose–response curves were linear. NOF was the most active mutagen, producing about 8000 revertants per μg on a plate. N-OH-AF was second most active, producing 2800 revertants per μg. N-OH-AF is somewhat unstable, however, in aqueous solution, and is oxidized spontaneously to NOF (5). Thus, the mutagenic activity of N-OH-AF could be due in part to its conversion to NOF on the pour plate. N-OH-AcAF and AF were moderate and weak mutagens, producing, respectively, 4.3, and 0.75 revertants per μg. AcAF had no activity at levels up to 200 μg per plate.

Treatment by a liver homogenate fraction (S-9) caused a dramatic increase in the mutagenicity of AF, AcAF, and N-OH-AF (Table 1), presumably by metabolizing these compounds to NOF. The mutagenic activity of NOF was progressively reduced by increasing levels of S-9, which could be explained by its being degraded to less active compounds.

Before doing experiments in vivo, we did reconstruction experiments with some of these available metabolites of 2-AcAF by adding N-OH-AcAF, N-OH-AF, and NOF to normal rat urine, allowing the urine to stand for 24 hr at 0° in the dark, and then determining the recovery by mutagenic assay (without the liver homogenate). N-OH-AcAF was completely stable, but the two reactive compounds, NOF and N-OH-AF, rapidly lost activity under these conditions. Therefore, we would not have detected them if they were excreted in urine.

**Mutagenic Testing of the Urine of Rats Given AcAF.** Table 2 summarizes the results of experiments in which rats were injected with 0.2–10 mg of AcAF and their urine tested for mutagens. A major part of the metabolites that can be detected as mutagens are conjugated as glucuronides. These can be detected with great sensitivity when β-glucuronidase is used to split the glucuronides and the liver homogenate (S-9) is used to metabolize the N-OH-AcAF (and possibly other compounds) to the more active metabolites, presumably.
Table 1. Effect of liver homogenate treatment on mutagenicity of fluorene compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>μl S-9 added/plate</th>
<th>His(^+) revertants on plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>25</td>
</tr>
<tr>
<td>2-Aminofluorene (100 μg)</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7400</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>3440</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>4470</td>
</tr>
<tr>
<td>2-Acetylaminofluorene (100 μg)</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8600</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>14,300</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>13,400</td>
</tr>
<tr>
<td>N-Hydroxy-2-acetylaminofluorene (10 μg)</td>
<td>0</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10,500</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>9800</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>9100</td>
</tr>
<tr>
<td>N-Hydroxy-2-aminofluorene (0.5 μg)</td>
<td>0</td>
<td>1480</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1720</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>228</td>
</tr>
<tr>
<td>2-Nitrosofluorene (0.1 μg)</td>
<td>0</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>243</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>65</td>
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</table>

Pour plates were prepared as described in Methods, incorporating into the top agar TA1538, 5-10 μl of test solution, and 0.5 ml of S-9 Mix containing various amounts of S-9 as indicated.

NOF and N-OH-AF. When tested directly (without S-9 or glucuronidase treatment), urine from rats given the highest dose of AcAF had low but significant activity, but urine from rats given lower doses had only questionable activity. Treatment of the urine by either S-9 Mix or glucuronidase alone increased its mutagenicity slightly, and treatment by both S-9 Mix and glucuronidase together increased activity dramatically, even with rats given the lowest dose tried (200 μg/rat; 1.6 mg/kg). The 40X concentrate of glucuronidase-treated urine was somewhat active without S-9 activation and most active with S-9 activation. Rats given higher doses of AcAF had greater activity in their urine, although the relationship of dose to activity was not linear. This effect is probably the result of thresholds in the rat for metabolism of AcAF and a balance in the bacteria between reversion and killing by the fluorene mutagens. The urine, or urine concentrate, from rats injected with the vehicle only showed no mutagenic activity.

An experiment was done in which the rats were fed 2-3 mg AcAF per day in their chow and urine was collected over a 24-h period. Results were similar to those with the rats injected with 10 mg of AcAF. Feeding experiments were not pursued due to the problem of controlling the scattering of the AcAF-containing chow.

**DISCUSSION**

Our in vitro test for detecting carcinogens as mutagens makes use of rat (or human) liver homogenate coupled with an especially sensitive set of bacterial tester strains (2, 3). Thus an important aspect of mammalian metabolism can be supplied in vitro. We present in this paper a method for examining that important class of metabolites that appear in urine. These metabolites may be different from those formed by liver homogenates. Since many mammalian metabolites are known to be excreted as β-glucuronides (8-11, 19, 20), we have added β-glucuronidase to our assay so that this class of metabolites can be detected. Moreover, the presence of liver homogenates during urine testing broadens the scope and sensitivity of the assay, as is seen from the results with the model compound we chose, the carcinogen AcAF.

We have investigated the liver carcinogen AcAF as a model compound for our urine studies because its urine and liver metabolites have been described, and tested for carcinogenicity, by the Millsers and others (5), and we have previously examined a number of fluorene metabolites for mutagenicity. AcAF is not believed to be the primary carcinogen (5). It is not carcinogenic at the subcutaneous site of entry, while three of its liver metabolites, N-OH-AcAF, N-OH-AF, and NOF, are (5). These three active compounds are frameshift mutagens in Salmonella (2, 4) while the parent compound, AcAF, causes frameshift mutations only after oxidation by liver homogenate (3). Our experiments show that with rats given AcAF, almost all of the material excreted in urine that can be activated to mutagens is present as a conjugate releasable by β-glucuronidase. This is presumably the glucuronide of N-OH-AcAF (9-16), which is then further converted by our liver homogenate to more powerful mutagens, presumably NOF and N-OH-AF. The glucuronide of N-OH-AcAF has been reported to react directly, though very slowly,
with DNA (16), but for mutagenesis in our system the glucuronide is much less active than NOF and N-OH-AF. Since nanogram amounts of these powerful mutagens can be detected, mutagenic activity was easily detected in the urine of rats given very low doses (200 μg per rat = 1.6 mg/kg) of AcAF. The sensitivity of the system can be increased still further by the method for concentrating urine metabolites that is described. We would like to caution, however, that AcAF may be particularly favorable for urine testing, and that other classes of compounds, such as polycyclic hydrocarbons, may not be as successful.

One general use for this urine test is in toxicological testing of a compound in rats or other experimental animals. It is not much additional work to test for mutagens in the urine of animals that are being dosed with compounds as part of other toxicology tests. The method also could provide a quick bioassay of the active compounds in a large mixture of metabolites. Legator (21) originally pioneered the idea of combining bacterial indicator strains with rodents in his host-mediated assay. He injected our bacterial tester strains in the (sterile) peritoneum of a mouse, and after giving the mouse a compound that required metabolism to be activated, showed that the bacteria, when reisolated, were mutated. In his experiments with the alkylating agent streptozotocin he also obtained a positive result by testing the rat urine directly (22). The host-mediated assay has some drawbacks due to lack of sensitivity. We think that testing in vitro with liver homogenates (3) is much superior in both simplicity and sensitivity. The use of urine strengthens the in vitro tests in toxicology studies by determining the activity of additional products of mammalian metabolism.

An additional general use for urine testing is in a direct screening of the human population. Humans might be ingesting a variety of mutagens and carcinogens in their diet. Some of these compounds may be identified by these sensitive and inexpensive tests. In addition, patients in hospitals exposed to a variety of drugs can also be tested. Siebert and Simon (23, 24) have done an interesting study in this regard and have examined human urine in cancer patients treated with the mutagen and carcinogen, cyclophosphamide, which needs activation for activity. They have been able to detect the presence of active metabolites in the urine using a mutagenic assay with yeast and have called attention to the usefulness of this sort of test. We think that the methodology described here using bacterial tester strains combined with the liver homogenates (2, 3) is sufficiently sensitive and inexpensive, that it is feasible to start general screening of urine at the present time. We recommend the addition of β-glucuronidase to the system for urine testing. It may be useful to add additional enzyme preparations such as sulfatase and β-glucosidase.* These are available commercially. Many compounds are excreted as sulfate conjugates and β-glucosides can be present in plants in the diet (e.g., cycasin (25) is carcinogenic and mutagenic if the glucosyl group is removed).

The potential hazard for humans of mutagens and carcinogens in the environment is very high, and it is important to screen large numbers of compounds and mixtures of compounds to which humans are exposed. However, it is impractical for both technical and monetary reasons to do this screening by using mammals. Carcinogenicity testing is also extremely expensive and takes years for adequate tests. Microorganisms have been used extensively in mutagenesis testing because they offer great technical advantages and because of the fact that DNA is the genetic material in both bacteria and man. Clearly many different types of test systems will have to be developed and each will have particular advantages and disadvantages. The Salmonella/liver system previously described is simple, inexpensive, and extremely sensitive, and, with the addition of the urine test presented here, we believe it will detect a high percentage of the environmental chemicals that cause cancer and mutations in man. The Salmonella test is demonstrably effective in detecting as mutagens a wide variety of carcinogens, and because of this we think that most all known chemical carcinogens are, in fact, mutagens that cause cancer because of somatic mutation (3) Of course, not all compounds that are dangerous for man will be detected, and there may certainly be a number of compounds that are effective mutagens in bacteria but are not mutagens or carcinogens in man for various reasons. Obviously, the extrapolation of these test results to man is not simple. We suggest that compounds that give a positive response in our test should be considered potentially very hazardous for man and should be scrutinized as to benefit, risk, and need for further testing by other systems.

Note Added in Proof. While this paper was in press, we learned of a similar, independent study by Commoner, Vithayathil, & Henry which will appear in Nature.

This work was supported by A.E.C. Grant AT(04-3)/4, P.A. 156. We thank Edith Yamashiki and Frank D. Lee for their help in various aspects of this work. We thank Joyce McCann, J. A. and E. C. Miller, W. Benedict, D. Nebert, and L. Poirier for a critical reading of the manuscript.


* The commercial β-glucosidase we have tried has an acid pH optimum and does not work sufficiently at the pH of the petri plates (pH 7) to detect cycasin. Incubation of the cycasin with the enzyme at lower pH values liberates the mutagen (25).