Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection

(frame-shift mutagens/aflatoxin/benz(a)pyrene/acetylaminofluorene)

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ABSTRACT 18 Carcinogens, including aflatoxin B1, benzo(a)pyrene, acetylaminofluorene, benzidine, and dimethylnitrosamine, are shown to be activated by liver homogenates to form potent frameshift mutagens. We believe that these carcinogens have in common a ring system sufficiently planar for a stacking interaction with DNA base pairs and a part of the molecule capable of being metabolized to a reactive group; these structural features are discussed in terms of the theory of frameshift mutagenesis. We propose that these carcinogens, and many others that are mutagens, cause cancer by a somatic mutation. A simple, inexpensive, and extremely sensitive test for detection of carcinogens as mutagens is described. It consists of the use of a rat or human liver homogenate for carcinogen activation (thus supplying mammalian metabolism) and a set of Salmonella histidine mutants for mutagen detection. The homogenate, bacteria, and a TPNH-generating system are all incubated together on a petri plate. With the most active compounds, as little as a few nanograms can be detected.

We have previously described the use of a set of mutants of Salmonella typhimurium for detecting and classifying chemical mutagens with great simplicity and sensitivity (1, 2). With this test we have also shown that the active forms of a large number of known carcinogens are mutagens (1–5). The active forms of carcinogens such as aflatoxin, polycyclic hydrocarbons, dimethylnitrosamine, and various aromatic amines are formed by mammalian metabolism, in particular by the TPNH-dependent microsomal enzymes of liver (6–11). The principal limitation of any bacterial system for detecting carcinogens as mutagens is that bacteria do not duplicate mammalian metabolism in activating carcinogens. Mammalian-liver homogenates have been used by Garner et al. (6) to activate aflatoxin B1 to a compound lethal to our bacterial tester strain lacking excision repair, by Malling (12) to activate dimethylnitrosamine to a compound that reverts one of our bacterial tester strains, and by Slater et al. (13) to activate dimethylnitrosamine to a compound lethal for bacteria lacking polymerase I. In this study we have extended this work and shown that carcinogens can be detected as mutagens simply and with great sensitivity by incubation of the carcinogen, a rat or human liver homogenate, and our bacterial tester strain together on a petri plate.

MATERIALS AND METHODS

Compounds. Glucose-6-phosphate, TPN, TPNH, and 2-naphthylamine were obtained from Sigma. Benzo(a)pyrene, 2-acetylaminofluorene, and benzidine were from Aldrich. Dimethylsulfoxide (MeSO), spectrophotometric grade, was obtained from Schwarz/Mann, sodium phenobarbital from Mallinkrodt, aflatoxin B1 from Calbiochem, and 3-methylcholanthrene from Eastman; 7,12-dimethylbenz(a)anthracene was a gift of P. L. Grover. Schuchardt (Munich) was the source for the other carcinogens.

Bacterial Strains used are mutants of S. typhimurium LT-2 and have been discussed in detail (2).

Source of Liver. Male rats (Sprague–Dawley/Bio-1 strain, Horton Animal Laboratories) were maintained on Purina laboratory chow. A week before they were killed, their drinking water was made 0.1% in sodium phenobarbital (14). The rats (250–500 g) were killed by a blow to the head and cervical dislocation; the liver was removed and placed in a sterile, ice-cold beaker. A portion of human liver was obtained from an autopsy of a 77-year-old man who had died 7 hr earlier of heart failure.

Preparation of Liver Homogenate Fraction "S-9". We have used the procedure of Garner et al. (6). All steps were performed at 0–4° with cold and sterile solutions and glassware. The liver (rat livers were 10–25 g each) was washed in an equal volume of 0.15 M KCl, minced with sterile scissors in three volumes of 0.15 M KCl (3 ml/g of wet liver), and homogenized with a Potter–Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged (Sorvall RC2-B) for 10 min at 9000 X g, and the supernatant, which we call the S-9 fraction, was decanted and saved. 1 ml of S-9 fraction contained microsomes from 250 mg of wet liver; the protein concentrations were fairly constant from preparation to preparation except for the human S-9 fraction which was about half, perhaps due to difficulties in homogenization because of its fibrous nature. The fresh S-9 fractions (rat and human) were distributed in 2-ml portions in small plastic tubes (2-ml liquid nitrogen storage tubes/4-Shore-USA, La Jolla, Calif.), quickly frozen in dry ice, and stored at −80° in a Revco freezer. As required, sufficient S-9 fraction was thawed (at room temperature) and kept in ice; the unused portion was discarded at the end of the day.

Mutagenesis Test with the S-9 Fraction. The method without the liver activation system has been described in detail (2). The only modification is the addition of S-9 Mix to the top agar. The S-9 Mix contains per ml: 0.3 ml of S-9 fraction, 8 mM MgCl2, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM TPN, and 100 mM sodium phosphate (pH 7.4). To 2 ml of...
TABLE 1. Activation of carcinogens to mutagens

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Histidine revertants per plate</th>
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<tbody>
<tr>
<td>2-aminoanthracene</td>
<td></td>
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<tr>
<td>6-aminochrysene</td>
<td></td>
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<tr>
<td>2-acetylaminofluorene</td>
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<tr>
<td>benidine</td>
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<tr>
<td>4-aminoazobenzene</td>
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<tr>
<td>4-aminobiphenyl</td>
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<tr>
<td>stilbene</td>
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<tr>
<td>4-aminobiphenyl</td>
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<tr>
<td>trans-Stilbene</td>
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<tr>
<td>4-aminofluorene</td>
<td></td>
</tr>
<tr>
<td>N,N-dimethylaniline</td>
<td></td>
</tr>
<tr>
<td>p-(phenylazo)-aniline</td>
<td></td>
</tr>
<tr>
<td>4-(o-tolylazo)-aniline</td>
<td></td>
</tr>
<tr>
<td>N,N-dimethylaniline</td>
<td></td>
</tr>
<tr>
<td>2-naphthylamine</td>
<td></td>
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<tr>
<td>1-anilinonaphthalene</td>
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<tr>
<td>6-aminochrysene</td>
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<tr>
<td>benzo[a]pyrene</td>
<td></td>
</tr>
<tr>
<td>3-ethylcholanthrene</td>
<td></td>
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<tr>
<td>aflatoxin B1</td>
<td></td>
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<tr>
<td>sterigmatocystin</td>
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Revertant colonies (his\(^+\)) on each plate were scored after 2 days. Numbers underlined are judged to be significantly different from the controls. Rat liver homogenate (S-9 Mix) was added to the plates, indicated. Human S-9 fraction gave qualitatively similar results when 2-aminoanthracene, the flavine derivatives, 4-aminoazobenzene, 6-aminobiphenyl, 6-aminochrysene, and aflatoxin B\(_1\) were assayed with TA1538. The amount chosen for each carcinogen was the one that gave maximum mutagenesis of several amounts tried (see Fig. 1). All of the compounds were added from solutions in MeSO\(_4\) usually 1 mg/ml; the control, lacking compound, had an equivalent addition of MeSO\(_4\).

* The S-9 preparation was from a 200-g rat that was injected intraperitoneally, 24 hr before it was killed, with 16 mg of 3-methylcholanthrene dissolved in corn oil.

† One-third the normal amount of S-9 fraction was used in the S-9 Mix.

‡ We are indebted to Dennis Hsieh for a sample and for suggesting we test sterigmatocystin.

molten top agar at 45\(^\circ\) are added 0.1 ml of the bacterial tester strain culture (2 to 3 \(\times\) 10\(^6\)/ml), up to 0.1 ml of a solution (MeSO\(_4\) or water) of the compound to be tested, and 0.5 ml of S-9 Mix; then the tube is rotated quickly and the contents are poured on the agar plate. The additions and pouring should take less than a minute. The colonies on the plates (his\(^+\) revertants) are counted after a 2-day incubation at 37\(^\circ\).

RESULTS

Combined System for Carcinogen Activation and Bacterial Mutagenesis. We use the TPNH-dependent microsomal-enzyme systems in liver homogenate to activate carcinogens. The activated carcinogens are detected as mutagens. The test assays, on a petri plate, the number of revertant colonies induced by mutagens in the set of histidine-requiring mutants. Two technical improvements greatly simplify the use of the combined bacterial and liver system. The TPNH-generating system and liver homogenate fraction (S-9 Mix) can be incubated directly on the petri plate along with the compound to be tested and the bacterial tester strain. Both rat and human liver preparations can be frozen for many months without loss of activity.

Carcinogens Detected as Mutagens. Table 1 shows that rat liver homogenates can activate 18 different aromatic type carcinogens to mutagens. The compounds were chosen for testing because they were carcinogenic in human or in animals (7, 15). Control values are presented both for the number of revertant colonies on plates with compound and no S-9 Mix, and for the number of colonies on plates

Fig. 1. Effect of S-9 fraction and carcinogen concentration on mutagenesis of TA1538. (A) The procedure was as described in Methods except that the amount of S-9 fraction in the Mix was varied. As the human S-9 had half the protein of the rat S-9, the results with the human material are plotted at half the S-9 amount actually used. 4-Aminobiphenyl (100 \(\mu\)g per assay) had about 0.1 the activity of 2-acetylaminofluorene (50 \(\mu\)g per assay), and the results plotted should be multiplied by 100 rather than 1000. Controls with S-9 Mix, but no compound, and with compound, omitting S-9 Mix, were less than 25 colonies. (B) With human S-9, 0.5 ml was used per assay, and with rat S-9 the usual 0.15 ml was used.
with S-9 Mix and no carcinogen. The control values are low in all cases, indicating that the S-9 Mix is sterile and does not significantly enhance the spontaneous mutation rate characteristic for each tester strain, and that none of the compounds in the table is mutagenic before activation. It can be seen from Table 1 that after activation every compound listed reverts one or both of the frameshift mutants TA1537 and TA1538.

It seems likely that the reactive groups produced from the amino, dimethyamino, and acetylaminogroups of amine carcinogens are the nitroso, hydroxynitroso, and hydroxyacetaminogroups, which are known activation products of liver metabolism (7) and which we have shown convert the original compounds into powerful frameshift mutagens for TA1538 (2, 5). The polycyclic hydrocarbons are presumably activated by liver to the epoxides (8), which react with DNA and are likely to be the true carcinogens (16), and which we have shown are powerful frameshift mutagens for TA1537 and TA1538 (4). Aflatoxin B1, which may require liver activation for carcinogenicity (17) and the related fungal carcinogen sterigmatocystin, are here shown to be, after activation, frameshift mutagens at very low concentrations.

Specificity of Mutagenesis. Each of our four different tester strains is reverted by different mutagens (1–5). The three frameshift tester strains, TA1536, TA1537, and TA1538, have different DNA sequences at the site of the histidine mutation (2) and are reverted by different frameshift mutagens, each of which has a preference for a particular repetitive sequence in DNA (2). This preference can be seen in Table 1: some activated aromatic amine carcinogens revert TA1538, which has a CGCGCGCCG sequence* at the site of the mutation, in preference to TA1537, a strain with a repetitive G sequence, while activated dimethylnitrosanthracene is more active on TA1537. None of the compounds is very active on the frameshift mutation in TA1536, a strain that is particularly sensitive to the ICR frameshift mutagens (2, 18, 19). Two of the compounds can also revert TA1535, an indicator of base-pair substitutions. The fact that different mutagens react at different DNA sequences makes the meaning of quantitative comparisons uncertain.

Carcinogens Inactive in the Plate Test. We have tested a total of 20 carcinogens of the aromatic type. Eighteen of the carcinogens gave a clear positive result as frameshift mutagens after activation, and appear in Table 1. Two carcinogens of the 20 tested, uracilamine or 

N,N-dimethyl-p-(phenylazo)-aniline, did not give a positive result: both have a dimethylamino side chain that presumably requires oxidation for activation. The latter compound is very closely related to several of the azobenzene derivatives in Table 1 that are active, and we have shown that 4-nitrosazobenzene acts directly as a frameshift mutagen without activation (5).

Various carcinogens that are simple alkylating agents cause base-pair substitution mutations and revert the histidine mutation hisG46 (present in our tester strain TA1535) (1, 3). A carcinogen that needs activation before becoming a simple alkylating agent is dimethylnitrosamine. Dimethylnitrosamine has been used to revert hisG46 by Legator (20) after activation in his host-mediated assay, and by Malling (12) after activation in liquid culture with mouse-liver homogenate under oxygen. We have repeated Malling's experiment successfully (using our own rat S-9 Mix), but were unsuccessful in preliminary experiments with our standard plate assay with either hisG46 or TA1535.† In view of the hazards of working with it and the fact that its mutagenicity after activation has been amply demonstrated, we decided not to work with it further.

Variation of the Concentration of Rat or Human S-9 Fraction. The relationship between the amount of rat or of human liver S-9 fraction added and the number of revertant colonies induced was investigated with the compounds listed in Table 1. The results with 4-aminobiphenyl and 2-acetylaminofluorene are shown in Fig. 1A. With most of the compounds tested, human S-9 fraction was less active than rat S-9 fraction (on a protein basis), but the rats were induced with phenobarbital, the human liver was not as fresh as the rat liver, and there is quite a variation in activity in human liver from autopsy (6). The plate assay is not a simple enzyme assay, and various complicated factors influence the yield of revertants, e.g., activation and inactivation of the carcinogen and loss of activity of the S-9 fraction on the plates with time. There is a technical limitation to adding more S-9 than shown in Fig. 1: there is a trace of histidine in the S-9 fraction, and an excess of it results in growth of the background lawn of bacteria, which obscures the revertants.

Variation of the Concentration of Carcinogen in the Assay. The relationship between the amount of carcinogen and the number of revertants induced (with a fixed amount of S-9 fraction) is shown in Fig. 1B. For 2-aminoanthracene, which is activated particularly well by human S-9, at higher concentrations the yield of revertants diminishes markedly. We think that the reason for this is that a mutagen kills bacteria by inactivating many essential genes on the chromosome, in addition to reverting the particular frameshift mutation in the tester strain. The optimum balance between maximum reverision and minimum killing varies with the mutagen, which is specific for particular base sequences, and with the tester strain. This killing has also been observed with many other carcinogens and is often visible as a thinning of the background lawn. Because of this and other complexities of the test, we recommend that if it is desired to maximize mutagenesis for a new compound, a number of assays be done, varying both concentration of carcinogen and amount of S-9.

The activated carcinogens are powerful mutagens and minute amounts can be detected (Fig. 1B and Table 1). With 2-aminoanthracene and human S-9 activation, 1 ng doubled the spontaneous reversion yield of about 20 colonies, and 0.5 µg gave 11,000 revertant colonies. The amount of conversion to the activated form is hard to determine in most cases, but with 2-acetaminofluorene it may be about 40%: we obtain about 2000 revertant colonies per µg of 2-acetaminofluorene, and we previously found about 5000 colonies per µg for 2-nitrosourea or 2-hydroxylaminofluorene (5), which are known metabolic products (7), mutagenic without activation by the S-9 fraction.

S-9 Mix. The liver microsomal enzymes responsible for activating aromatic amines, polycyclic hydrocarbons, and aflatoxins all require TPNH, and it is customary in in vitro

† HisG46 is superior to its derivatives lacking excision repair as a tester strain for these and some other methylating agents, while TA1535 (or TA1530 or TA1930) is better for larger alkylating agents.

* Isono and Yoruno (2) have shown that this is the DNA sequence at which 2-nitrosourea causes a two-base deletion.
systems to a TPNH-generating system such as TPN, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, as well as phosphate buffer, MgCl₂ and KCl (6, 8, 10–12). The optimum concentrations of the components of the S-9 Mix have been determined in experiments similar to those shown in Table 1 with 2-aminofluorene, with both rat and human S-9 fractions. TPN was found to be absolutely essential, and the optimum concentration was determined to be about 4 mM for several of the carcinogens: substitution by TPNH did not enhance mutagenesis. Glucose-6-phosphatedehydrogenase did not enhance mutagenesis and was omitted. The phosphate buffer was necessary for stabilizing the S-9 activity in the top agar.

Addition of glucose-6-phosphate resulted in a slight stimulation with our human liver preparation, and we have left it in the S-9 Mix. We have found no advantage in using purified microsomes as a substitute for our S-9 preparation.

Addition of the S-9 Mix directly to the top agar with the bacteria and carcinogen is more convenient and gives as good, or better, activity than the alternative method of spreading the S-9 Mix on the agar plates before pouring the top agar with the bacteria as an overlay. The short (less than 1 min) exposure of the S-9 enzymes to the 45° top agar does not affect their activity. The procedure recommended is also more convenient than the method described previously by other workers (6, 12, 21), in which the bacteria are incubated in liquid culture with the carcinogen and liver homogenate activation system and then aliquots are plated for assay of mutagenesis.

Stability of the S-9 Fraction to Storage. The S-9 preparation does not lose any activity in activating 2-acetylaminofluorene (experiments as in Fig. 1) after storage at −80°C over a 3-month period. We have done less complete experiments with various carcinogens in Table 1 and have found similar enzyme stability. We have made a preparation of S-9 from frozen rat liver (freshly frozen, stored 1 week at −80°C, thawed overnight in the refrigerator) that showed excellent activity when tested with 2-acetylaminofluorene and 4-aminobiphenyl.

Induction of Liver Enzymes. Many compounds are known that induce elevated amounts of some of the enzymes of the mixed function oxygenase system in liver. We have not investigated this in detail, but have routinely used phenobarbital, which is commonly used for induction of these enzymes. In experiments comparing control rats with those given phenobarbital in their drinking water, we found a 2-fold increase in the ability of the S-9 fraction from the phenobarbital-induced rats to activate 2-acetylaminofluorene and 4-aminobiphenyl. When rats were induced instead by 3-methylcholanganthrene, the S-9 fraction was about 3-times more active in the activation of benzo(a)pyrene (see legend to Table 1) and less active with acetylaminofluorene than from either phenobarbital-fed or control rats.

DISCUSSION

We have shown that after activation by mammalian liver homogenate, 18 known chemical carcinogens of the aromatic type are frameshift mutagens. We (1–5) and others (reviewed in ref. 7) have shown that various other carcinogens are mutagens; some causing base-pair substitutions and others frameshift mutations. We propose that those carcinogens that are mutagens cause cancer by somatic mutation. This hypothesis, which of course is not new, seems compelling in view of this correspondence between carcinogens and mutagens and the precedent that cell regulation can be easily altered by mutation, as demonstrated routinely by bacterial and phage molecular biologists. A heritable change in cell regulation is one of the most characteristic properties of cancer cells.

Many of the most powerful carcinogens cause mutations of a special type: frameshift mutations. Knowledge of the mechanism of frameshift mutation makes the structural requirements for this type of carcinogen understandable and, thus, predictable. The model of frameshift mutagenesis as first worked out by Streisinger and Tsugita and their colleagues (22) was that a flat aromatic molecule such as an acridine, which intercalated in the DNA base-pair stack, could stabilize a shifted pairing in a repetitive sequence of bases. This then led, during DNA replication or repair, to an addition or deletion of base pairs in the DNA sequence. Our work led to another generalization: that the potency of an intercalating agent as a frameshift mutagen could be increased by orders of magnitude if the ring system contained a side chain that could covalently react with DNA (4, 5, 18, 19). The carcinogens tested in this study satisfy the requirement of having a nearly planar aromatic ring system (presumably capable of a stacking interaction with DNA) and a potentially reactive part of the molecule. None of the compounds tested here caused detectable frameshift mutagenesis before treatment with liver homogenate, but all of them were mutagenic afterward, a consequence, we believe, of activation by liver enzymes.

Although many of the more potent carcinogens are frameshift mutagens, many carcinogens, such as simple alkylating agents, cause base-pair substitutions. We believe the potency of the frameshift type is due to the two factors of activation in situ and bifunctionality. The parent carcinogens presumably reach specific tissues (and dissolve in membranes) in their unreactive form and are not activated until they are inside the target cell. A simple alkylating agent could be inactivated by reaction with sulphydryl groups or protein at the site of entry. A ring system capable of a stacking interaction with DNA gives the molecule a particular affinity for DNA as compared with a simple alkylating agent.

Miller and Miller (7) have recently reviewed the field of carcinogen activation and discussed the idea that many carcinogens, among which are many of the carcinogens we have examined, need activation to be carcinogenic. They also review the evidence of many investigators that a number of these active forms are mutagenic in one system or another. We showed that the pure active forms of a number of these carcinogens were potent frameshift mutagens without liver activation and discussed the structure of the carcinogens on the basis of frameshift mutagenesis theory (2, 4, 5). The present results add many carcinogens to the list of frameshift mutagens and show that the active forms can be generated by mammalian-liver homogenates and detected as mutagens with our set of Salmonella tester strains.

It is quite reasonable to use bacteria as a test system for carcinogen detection, because so many carcinogens appear to be mutagens acting on DNA and all DNA is basically the same. We have overcome the limitation of the inability of bacteria to duplicate mammalian metabolism by adding human, or rat, liver homogenates and a TPNH-generating system directly to the petri plates with our Salmonella tester.
strains and the carcinogen. In detection of mutagens causing point mutations, a bacterial test system has many practical and theoretical advantages, among which are the small genome (about $4 \times 10^6$ base pairs), large number of organisms exposed (about $10^8$ per plate), and the positive selection for mutated organisms. The set of *Salmonella* tester strains (2) has three additional advantages: lack of excision repair, loss of the lipopolysaccharide barrier, and scoring of mutations in “hot spots” (23) for frameshift mutagenesis (e.g., the GCGCGCG sequence in TA1538). Scoring of reversion in an easily mutated “hot spot” combined with smallness of the genome aids the test by maximizing reversion relative to killing. Our set of tester strains for detecting frameshift mutagens is not yet complete and, thus, a negative result does not necessarily mean that a compound is not a mutagen.

The *Salmonella* test system can detect carcinogens with great sensitivity. In any system for detecting mutagens one scores mutations in only a small part of the genome. Thus, the revertant colonies we see represent only a tiny fraction ($10^{-2}-10^{-4}$) of the bacteria mutated. Nevertheless, because of the sensitivity of the tester strains and the potency of the activated carcinogens as mutagens, one can detect nanograms of carcinogen: e.g., 0.5 µg of 2-aminoanthracene gives 11,000 revertant colonies, compared to controls of about 30 colonies.

We believe that the coupled system, mammalian liver/Salmonella tester strain, should be used in screening of suspected carcinogens, in isolation of carcinogens from natural materials, and in identification of the active forms of carcinogens. The system provides a rapid, simple, sensitive, and economical method for detecting those carcinogens that cause point mutations. This class, though it includes an impressive array of chemical carcinogens and radiations, probably will not include all carcinogens: e.g., those that cause mutations indirectly by inhibiting mammalian repair. Human autopsy and rat-liver homogenates are both effective in activating carcinogens, and other mammalian tissues could be tested as well. The liver homogenates can be stored at $-80^\circ$ for at least 3 months without loss of activity. The results of testing a compound can be scored in 2 days, and only very small amounts of test compound are needed. Another use of the system could be as an assay for the ability of mammalian tissues to activate carcinogens. In a standard assay, we use the equivalent of 37 mg of wet liver. For a positive response with the most active compound, less than 1 mg of liver could be used.

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