Detection of Mutagenic Impurities in Carcinogens and Noncarcinogens by High-Pressure Liquid Chromatography and the Salmonella/Microsome Test

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ABSTRACT

We have used high-pressure liquid chromatography and the Salmonella/microsome mutagenicity test to look for mutagenic impurities in 11 carcinogens and noncarcinogens. Because of the million-fold range in mutagenic potency observed in the Salmonella test, even trace amounts of potent mutagenic impurities in a nonmutagenic compound could be detected. The mutagenic potency of 7-hydroxy-2-acetylamino fluorene, a noncarcinogen in the standard animal carcinogenicity tests, is shown to be due to a small amount of impurity, which is probably the potent carcinogen 2-acetylamino fluorene. This is discussed in relation to the statistical limitations of animal carcinogenicity tests. We also discuss the role of mutagenic impurities in assessing the mutagenicity of environmental (and industrial) chemicals with high-sensitivity mutagenicity assays, such as the Salmonella/microsome test.

INTRODUCTION

We have previously described a rapid and sensitive bacterial test for the detection of environmental mutagens (reviewed in Refs. 4 and 26). The test measures reversion to prototrophy of a special set of histidine mutants of Salmonella typhimurium and incorporates an aspect of mammalian metabolism by utilizing homogenates of rat (or human) liver for activation of chemicals to their mutagenic forms. Recently, we have validated the test for the detection of carcinogens as mutagens (25, 27). Of 175 carcinogens tested, spanning a wide variety of chemical classes, 90% were mutagenic in the test. Despite the difficulties inherent in defining noncarcinogenicity (25, 26), few of the 107 noncarcinogens tested showed any degree of mutagenicity. Despite the fact that chemicals of the highest purity available were used, in a few cases we found that the classification of a chemical as to mutagenicity or nonmutagenicity was complicated by the presence of mutagenic impurities.

In the past few years, the technique of HPLC3 has been developed to a high level of sophistication (13). The technique is similar to normal liquid column chromatography except that the eluting solvent is pushed through the column by a high-pressure pump. This method can be quite useful as a tool when looking for mutagenic impurities: (a) the relative speed of the method makes it particularly adaptable for use with a rapid in vitro screening method like the Salmonella test; (b) the sensitivity and degree of resolution possible are sufficient to permit separation of chemicals from very tiny amounts of mutagenic impurities that might be present; (c) one can readily obtain amounts of chemicals from the columns sufficient for mutagenesis testing; (d) there are a number of different types of columns and solvents that may be used in HPLC, permitting its application to the separation of a wide range of different types of chemicals; and (e) the breakdown of relatively unstable chemicals can be minimized because of short retention times and because the column operates at ambient temperatures.

We describe here the use of HPLC, in combination with the Salmonella/microsome test, to detect small amounts of mutagenic impurities. We also discuss the problems that mutagenic impurities can create in mutagenesis testing.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained as follows. When possible, chemicals of the highest purity commercially available were used, and purity, when known, is indicated in parentheses. 1-AA (90%), 2-AB (>97%), 2-AAF (>97%), and benzo(a)pyrene (>99%) were from Aldrich Chemical Co., Inc., Milwaukee, Wis. 1-NA and 2-naphthylamine (>99%) were from Sigma Chemical Co., St. Louis, Mo. bis-2,7-AAF, 2-aminoanthracene, and 4-aminoethylbenzene (99%) were from Schuchardt, Munich, West Germany. 3-OH-B(a)P and B(e)P (highly purified) were from the National Cancer Institute, Bethesda, Md. Samples of 7-MB(a)A were gifts of P. Grover and J. Flesher. Samples of 7-OH-2-AAF were gifts of J. and E. Miller and E. Weisburger, and 5-OH-2-AAF was a gift of E. Weisburger. 4-AAF (>98%) and 3-methoxy-4-aminoazobenzene were gifts of V. Simmon.

Solvents. Solvents were from either Mallinckrodt Chemicals, St. Louis, Mo., or Matheson, Coleman and Bell, Cincinnati, Ohio, and were spectroquality grade. All solvents were degassed on the house vacuum prior to use.

Columns. Columns (purchased prepacked) were, for the benzopyrenes and 7-MB(a)A, a reverse-phase Micro C18 column (10-μm particles coated with octadecyl silane), 4 mm (inside diameter) x 30 cm (Part 27324; Waters Associates, Milford, Mass.), and, for all other chemicals, a Lichrosorb silica column, 4.5 mm (inside diameter) x 25 cm,
packed with 10-μm Lichrosorb Si60 particles (Part 254-04; Altex Scientific Co., Berkeley, Calif.). Void volumes of both columns were about 2.5 ml.

**Chromatography.** Chromatography was at room temperature (~23°C). In most experiments we used a Spectra-Physics high-pressure liquid chromatograph, Model 3500B (Spectra-Physics Co., Santa Clara, Calif.). In a few cases a recently acquired Waters Associates Model ALC/GPC 244 high-pressure liquid chromatograph equipped with a Model 6000A solvent delivery system was used. Absorbance was at either 254 or 280 nm using a Spectra-Physics Model 230 dual-channel absorbance detector or a Waters Associates Model 440 absorbance detector. Flow rates were 2 ml/min except for B(e)P (3 ml/min) and 7-MB(a)A (4 ml/min). Eluates were collected in glass vials and dried under a flow of nitrogen gas. Recovery of sample was essentially 100% from the column. This was determined spectrophotometrically in several cases by reinjecting collected material back onto the column.

Details of the conditions used for the chromatographic separations are in the legend to Chart 1. In general, for aromatic amines the running solvents were either isooctane (2,2,4-trimethylpentane)/methylene chloride or, for the more polar compounds such as 2-AAF, chloroform/methanol [Matsushima et al. (22) have used a similar solvent system for thin-layer chromatography of 2-AAF derivatives]. One disadvantage of chloroform/methanol is that the chemosterility of methanol (35), and thus one can run aqueous acetonitrile solutions at a faster flow rate without exceeding the pressure limit of the column; and (b) acetonitrile has a greater ability to solubilize many polycyclic hydrocarbons than does methanol (35), and so greater amounts can be loaded into the system per injection.

**Mutagenesis Testing.** Mutagenesis testing was as previously described (4). All results reported are taken from the linear region of dose-response curves. Chemicals were protected from light with aluminum foil. Purified chemicals not immediately used for mutagenicity testing were dried and stored frozen, in sealed vials under nitrogen.

**RESULTS**

We have used HPLC to obtain highly purified samples of a number of carcinogens and noncarcinogens (8 aromatic amines and 3 polycyclic hydrocarbons) for mutagenesis testing. All chemicals were chromatographed under a variety of solvent conditions and retention times. HPLC tracings for the 11 chemicals are shown in Chart 1. The tracings shown are those from the preparative runs used to obtain the purified samples for which data are reported in Tables 1 and 2.

Before purification all 11 chemicals were weakly mutagenic in the Salmonella test, and for several reasons (see below) we suspected that mutagenic impurities might be responsible for all or part of the mutagenic activity.

Even though, in general, chemicals were of the highest purity commercially available, we found a number of impurities in all chemicals examined (see Chart 1) with only 1 exception, B(e)P. In almost all cases the impurities represented only a very small fraction of the total absorbing material. However, since ng amounts of the most potent mutagens will cause a significant mutagenic response in the Salmonella test (25-27), only very low levels of potent mutagenic impurities would, in theory, be required to cause a mutagenic response (see "Discussion").

We compared the mutagenicity of each of the 11 chemicals before and after purification on HPLC (Tables 1 and 2 and Chart 2), and in 4 of the 11 cases examined we found a significant change in the mutagenic activity after purification. In 1 case (7-OH-2-AAF, Table 1 and Chart 2), the purified chemical was virtually nonmutagenic; in 2 cases [bis-2,7-AAF and 7-MB(a)A, Table 2], direct mutagenic activity (not requiring the rat liver "S-9 Mix") was lost on
Impurities and the Salmonella/Microsome Test

Table 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Carcinogenicity</th>
<th>% impurity</th>
<th>Ratio</th>
<th>Revertants per:</th>
<th>Revertants per:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>µg</td>
<td>nmole</td>
<td>µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Before HPLC</td>
<td>After HPLC</td>
</tr>
<tr>
<td>2-Naphthylamine</td>
<td>+</td>
<td></td>
<td>19</td>
<td>252/4</td>
<td>9.01</td>
</tr>
<tr>
<td>1-NA</td>
<td>c0</td>
<td>0.2</td>
<td>133/40</td>
<td>121/40</td>
<td>0.43</td>
</tr>
<tr>
<td>4-Aminobiphenyl</td>
<td>+</td>
<td></td>
<td>25</td>
<td>5069/50</td>
<td>17.1</td>
</tr>
<tr>
<td>2-AB</td>
<td>?</td>
<td>1.49</td>
<td>202/50</td>
<td>239/200</td>
<td>0.27</td>
</tr>
<tr>
<td>2-AAF</td>
<td>+</td>
<td></td>
<td>4802/10</td>
<td>47/500</td>
<td>0.02</td>
</tr>
<tr>
<td>4-AAF</td>
<td>0</td>
<td>ND</td>
<td>357</td>
<td>272/200</td>
<td>0.30</td>
</tr>
<tr>
<td>5-OH-2-AAF</td>
<td>0</td>
<td>0.08</td>
<td>119/40</td>
<td>59/460</td>
<td>0.03</td>
</tr>
<tr>
<td>7-OH-2-AAF</td>
<td>c0</td>
<td>0.25</td>
<td>436/250</td>
<td>4/10</td>
<td>&lt;0.12</td>
</tr>
<tr>
<td>2-Aminoaanthracene</td>
<td>+</td>
<td></td>
<td>5251/20</td>
<td>21.2</td>
<td>22.8</td>
</tr>
<tr>
<td>1-AA</td>
<td>w†</td>
<td>0.2</td>
<td>2290/20</td>
<td>22.1</td>
<td>2365/20</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>*</td>
<td></td>
<td>23985/121</td>
<td>22.1</td>
<td>2365/20</td>
</tr>
<tr>
<td>B(e)P</td>
<td>w</td>
<td></td>
<td>154/20</td>
<td>1.94</td>
<td>1.54</td>
</tr>
</tbody>
</table>

a Strain TA100 and S-9 Mix with 20 µl of Aroclor-induced S-9 were used except for the aminobiphenyls (50 µl of S-9), the acetylaminofluorene derivatives (TA1538; 150 µl of phenobarbital S-9), and the aminoaantracenes (TA98; 50 µl of phenobarbital-induced S-9). All results are single points from the linear region of dose-response curves; e.g., 252/4 = 252 revertants/4 µg. Spontaneous revertants have been subtracted and were approximately 160 (TA100), 25 (TA1538), and 40 (TA98) per Petri plate. The ratio compares the mutagenic activities of the potent carcinogens to those of each weak relative on a nmole basis; e.g., 4-AAF was 357 times less mutagenic than was 2-AAF before HPLC.

b, +, carcinogen; 0, noncarcinogen; w†, weak carcinogen; c0, noncarcinogen in most studies, with some reports of weak or marginal activity; ?, generally assumed a noncarcinogen, based on structural considerations, but has not been tested for carcinogenicity. Carcinogenicity references are cited in Ref. 27.

Percentage of impurity (by weight) was calculated based on the absorption of the suspected potent relative at 254 nm, determined in a parallel run. ND, not detected.

1-AA has been considered a borderline carcinogen (6).

A benzo(a)pyrene impurity in B(e)P would be less expected than a number of other potentially mutagenic relatives, because of the usual route of synthesis of B(e)P.

Table 2

<table>
<thead>
<tr>
<th>Chemical</th>
<th>S-9 Mix with 20 µl of Aroclor-induced S-9 was used except for bis-2,7-AAF (150 µl of phenobarbital-induced S-9). Strains were TA98 [7-MB(a)A without S-9 Mix, 3-OH-B(a)P], TA1538 (bis-2,7-AAF, 3-methoxy-4-aminoazobenzene), and TA100 [7-MB(a)A + S-9 Mix].</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before HPLC</td>
</tr>
<tr>
<td></td>
<td>µg</td>
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<tr>
<td></td>
<td>Revertants per:</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>bis-2,7-AAF</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7-MB(a)A</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>3-OH-B(a)P</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>+</td>
</tr>
<tr>
<td>3-Methoxy-4-aminoazobenzene</td>
<td>+</td>
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<td></td>
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</tbody>
</table>

a 7-MB(e)A from a second source did not appear to contain the impurity.

b Results were variable from experiment to experiment.

c Dose-response curves for 3-methoxy-4-aminoazobenzene were nonlinear (27).

purification, while activity in the presence of the S-9 Mix was retained; in 1 case (2-AB) the mutagenic potency decreased, but did not disappear. Each of these cases is discussed briefly below.

Close Relatives of Potent Mutagens [2-AB, 1-NA, 7-OH-2-AAF, 5-OH-2-AAF, 4-AAF, 1-AA, and B(e)P]. We were particularly interested in Chemicals A to G in Chart 1 because these had a combination of properties that suggested, not only that mutagenic impurities might be present, but that in many cases we might be able to predict what the impurities were: (a) they had all been reported to be either very weakly carcinogenic or noncarcinogenic in the standard animal carcinogenicity tests; (b) they were all close relatives (in most cases isomers) of chemicals that are much more potent carcinogens in animal carcinogenicity tests (these are listed in Table 1 in italics) and are also potent mutagens in the Salmonella test; and (c) they were all very weakly mutagenic in the Salmonella test compared to their potent relatives (Table 1).

Since purification procedures commonly employed in the preparation of chemicals may not efficiently resolve substances that are isomeric or that differ only slightly in
structure, it seemed probable that at least some of the mutagenic activity of these weak mutagens could be due to the potent relative, present as an impurity. Also, even though in almost every case the chemicals used were quite pure, in most cases only a very small amount of impurity of the potent relative would be necessary to cause the weak mutagenic activity.

In all but 2 [4-AAF and B(e)P] of the 7 chemicals examined, we detected some impurity with the retention time expected for the potent relative [marked with an arrow on each tracing (A to G) in Chart 1]. Although no 2-AAF impurity peak was detected in 4-AAF, the minimum amount of 2-AAF required to account for the observed mutagenicity of 4-AAF is so small that it would not have been visible in the trailing edge of the main peak. The purified sample did not contain the trailing edge. Although we did not chemically characterize the impurities, chromatographic runs were carried out under conditions that varied the retention times, and in all cases each impurity had the retention time expected for the potent relative.

In each case the amount of potent relative was estimated from the tracing, based on its known absorption, and the percentage of impurity was calculated (Table 1). This rough estimate seemed to exclude the potent relative as responsible for the mutagenic activity of 1-NA, 1-AA, and B(e)P, since not enough of the potent relative was present to account for the observed mutagenicity of the weak relative (Table 1). However, the amount of the potent relative present in 7-OH-2-AAF, 5-OH-2-AAF, and 2-AB was of the correct order of magnitude to account for the activity of these compounds.

For all 7 weak relatives, we collected a sufficient amount of the purified chemical for a mutagenicity test and compared directly, in each case, the impure and pure weak relatives. In 2 of the 3 cases in which the amount of potent relative had appeared, from the tracing, to be sufficient to account for the mutagenicity, we found the purified weak relative significantly less mutagenic. (5-OH-2-AAF is difficult to interpret because the mutagenic activity of the impure sample is so weak.) For the other 4 weak relatives, there was no substantial change in activity upon purification.

These results are shown in Table 1.

7-OH-2-AAF was the most dramatic example, losing almost all mutagenic activity upon purification (Chart 2), and it serves as an illustration. If one assumes that the 0.25% impurity (Chart 1; Table 1) is all due to 2-AAF, then there was about 0.25 μg of 2-AAF present in each 100 μg of unpurified 7-OH-2-AAF tested. From the data shown in Table 1, one can then calculate that a 2-AAF impurity could have accounted for about (0.0025)(250)(4802/10) = 300 revertants, which is very close, given that this calculation is only an estimate, to the 436 revertants actually observed for 250 μg of unpurified 7-OH-2-AAF.4 As additional evidence that the impurity was most probably 2-AAF, we collected pure 7-OH-2-AAF and the impurity peak separately. We found that the mutagenic activity of impure 7-OH-2-AAF was restored by addition of the pure impurity peak to pure 7-OH-2-AAF (data not shown), although limited amounts of 7-OH-2-AAF prevented a repetition of this experiment.

The same kind of calculation may be made for 2-AB. It appears probable that about half of the mutagenic activity of unpurified 2-AB was due to a mutagenic impurity, which is very probably 4-aminobiphenyl. Pure 2-AB retained a significant amount of mutagenicity, and we believe that it is a weak mutagen.

Chemicals Thought to Require Metabolic Activation for Mutagenic Activity, Which Were Active Directly [bis-2,7-AAF, 7-MB(a)A, and 3-OH-B(a)P]. These 3 chemicals are carcinogens (6, 12, 17, 27) and either require metabolic activation as carcinogens or are closely related to carcinogens and mutagens that must be activated (1). All 3 chemicals showed a significant amount of mutagenic activity without activation (Table 2), especially bis-2,7-AAF, which was extremely active even after recrystallization from ethanol (2, 3). They also contained several impurities (Chart 1). 3-OH-B(a)P, used as a standard in enzyme assays for aryl hydrocarbon hydroxylase, contained at least 14 impurity peaks, including 2 major impurities (Chart 1). At higher sensitivity settings we could detect as many as 22 impurities. After purification on HPLC bis-2,7-AAF and 7-MB(a)A lost all direct mutagenic activity. Not surprisingly, since both are carcinogens (6), they retained mutagenic activity in the presence of S-9 Mix.

The mutagenic impurity in bis-2,7-AAF is of some interest because it must be a quite potent mutagen. Since none of the impurities accounted for more than 1% of the total UV-absorbing material (assuming that the impurities have a similar extinction coefficient to that of bis-2,7-AAF), only on the order of 0.1 μg of a single mutagenic impurity would have caused the almost 5000 revertants recorded in Table 2. Such a potent mutagen may be of theoretical interest, and in fact new classes of unusually potent mutagens could be discovered initially as mutagenic impurities.

Because it seemed possible that such a potent impurity might have also influenced the results of animal carcinogenicity studies done with this compound (see "Discussion"), we also tested a sample of bis-2,7-AAF (kindly

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4 This calculation assumes a linear dose-response curve in the low-dose region, which is in general correct (27) and appeared so in this case in 1 experiment with small amounts of 2-AAF in the presence of excess 7-OH-2-AAF, although limited material prevented more thorough analysis. In any case, the calculation is an estimate of the maximum reversion expected.
supplied by E. Weisburger) used by Morris et al. (30) in extensive animal carcinogenicity studies. This material showed no direct mutagenic activity (data not shown) at dose levels (up to 20 μg) at which the material from Schuchardt was strongly mutagenic (Table 2).

Results for 3-OH-B(a)P were quite erratic from experiment to experiment, and we cannot conclude whether the mutagenic activity is due to impurities or to 3-OH-B(a)P itself. The pattern of impurity peaks on HPLC changed markedly as the injection solution aged over 1 to 2 hr (data not shown). This suggests that the variable mutagenic activity could have been caused by oxidation or breakdown products of 3-OH-B(a)P or by a co-eluting impurity. The known oxidation products of 3-OH-B(a)P (40) do not appear to be mutagenic (41).

3-Methoxy-4-aminoazobenzene, a carcinogen (6), was purified on HPLC because of its unusually great mutagenic potency (467 revertants/nmole) compared to its close relative 4-aminoazobenzene (0.29 revertants/nmole) (27). The dose-response curve for 3-methoxy-4-aminoazobenzene was also extremely nonlinear (data not shown) (27). Purification on HPLC confirmed that essentially all of the mutagenic activity was due to 3-methoxy-4-aminoazobenzene. The HPLC tracing is shown in Chart 1, and results of the mutagenicity test are shown in Table 2.

DISCUSSION

We have reported here on the combined use of HPLC and the Salmonella/microsome test for rapid purification and detection of mutagenic impurities in 11 chemicals. These (most are quite pure already) were selected from almost 200 mutagens (27), as especially likely (see "Results" for discussion) to contain impurities. We have shown that 6 of these did in fact contain mutagenic impurities, and we have discussed each of these under "Results."

Impurities and Mutagenic Potency

We detected mutagenic impurities present in as small amounts as about 0.1% (Table 1). We also detected an extremely potent mutagenic impurity (present at about 1%) when only 10 μg of the impure chemical were tested (Table 2). That mutagenic impurities present in such very low amounts are detected in the Salmonella test is not surprising in view of the extremely wide range of the mutagenic potency of chemicals [over a million-fold (25-28)] in this test. As an extreme example, mg dose levels are required to detect the weakest mutagens, and this defines the usual upper limit of the amount of a chemical tested in a standard test protocol (about 10 mg). On the other hand, the most potent mutagens can be detected at doses as great as 1,000,000 times less, in the ng range. Thus, in an extreme hypothetical case, a very potent mutagenic impurity, present in a nonmutagen at as little as 0.0001%, could conceivably cause a mutagenic response. And if this were present as a 1% impurity (not an unusual amount in most commercial-grade chemicals), it could cause a mutagenic response when 1 μg of the nonmutagenic, unpurified chemical was tested.

The Problem of Mutagenic Impurities in Mutagenesis Testing

Obviously, in evaluating the role that mutagenic impurities will actually play in mutagenicity testing, of primary concern is the frequency with which they are likely to occur and how to recognize the problem when it does occur. Mutagenic impurities are probably relatively uncommon in highly purified commercial chemicals for 2 reasons. (Industrial chemicals may present more problems and are discussed separately.) (a) Most of the 136 mutagens for which we have reported quantitative mutagenicity data (27) were readily detected at dose levels of less than 10 μg, and almost all (86%) were detected at doses of less than 100 μg. Only 8 mutagens required doses of 1 mg or more for detection. Thus, one does not usually require high doses (where impurities would most probably present the greatest problem) to detect mutagenic activity. This strongly suggests that there were very few cases of mutagenic impurities among these 136 mutagens. (b) A mutagenic impurity must be quite a potent mutagen to be detected in the test. In our experience, testing a large number of mutagens, less than 5% of all the mutagens that we have detected are potent enough to be detected at the 0.1-μg level (1% of a 10-μg sample). However, about one-third can be detected at or below the 1-μg range. If this distribution of mutagens along the potency scale approximates the distribution in the real world, then when a mutagenic impurity is present (a rarity to begin with) about 30% of the time it would be detectable when 100 μg (for a 1% impurity) of the impure chemical are tested.

Mutagenic Impurities in Industrial Chemicals

The presence of mutagenic impurities in substances of high-grade purity is not likely to be a common occurrence. For industrial chemicals, which often contain fairly large amounts of impurities, mutagenic impurities may be more common. The Salmonella test can be used in the design of industrial syntheses and as a batch process monitor, to minimize the introduction of mutagenic impurities. At the Agricultural Division of American Cyanamid Co., Princeton, N. J., a research compound with potential utility in animals was identified as weakly mutagenic in Salmonella on TA1538 and TA98 (R. Gustafson, personal communication). About 30 samples from a variety of batches were tested, and a mutagenic impurity was suspected because a few of the batches were nonmutagenic. It was subsequently found that the mutagenic activity could be removed from the active batches by recrystallization. By testing of various process intermediates, the synthetic step that introduced the mutagenic impurity was also identified. Introduction of a charcoal adsorption and filtration step in the synthetic process removed the impurity. The Salmonella test was also used to determine that the final batch process chosen to synthesize this potentially useful chemical did not introduce any mutagenic impurities.

At Merck Sharp and Dohme, Rahway, N. J. (P. R. Vagelos, personal communication), certain batches of an important chemical were very weakly mutagenic (about 0.007 revertants/nmole), and the mutagenic activity was found to be due to an impurity. The Salmonella test is now being used...
The Detection of the Carcinogen 2-AAF as an Impurity in 7-OH-2-AAF by Salmonella but Not by Animal Cancer Tests

We have shown and have discussed under "Results" that the weak mutagenicity of a highly purified sample of 7-OH-2-AAF is probably due to a small amount of 2-AAF impurity, which is itself a potent carcinogen. The sample of 7-OH-2-AAF that we tested was actually used in thorough animal tests, which were substantially negative (Ref. 29; E. Weisburger, personal communication). Thus, this may be an example of the bacterial test being more efficient than the animal carcinogenicity tests in detecting small amounts of a potent carcinogen.

A comparison between the amounts of 2-AAF detected as mutagenic in the bacterial test with the minimum amount detectable by standard animal cancer tests is instructive. The amount of 2-AAF in the sample of 7-OH-2-AAF that we examined here was about 0.25% (Table 1). The carcinogenicity studies on 7-OH-2-AAF (8, 20, 29) were feeding studies in which the chemical was fed at concentrations on the order of 0.06%. Thus, in these studies the 2-AAF impurity was being fed at about 0.0002% in the diet. This is far below the amount usually administered in 2-AAF carcinogenicity studies [about 0.03% in the diet (6)]. The size of a rodent bioassay required to detect the much lower incidence of tumors that might be expected from a 0.0002% dose of 2-AAF would be impractically large. The size of the "megamouse" experiments currently in progress on 2-AAF at the National Center for Toxicological Research, Jefferson, Ark., (32) are illustrative. These studies have been designed to detect carcinogenicity of 2-AAF in the low-dose range (0.003 to 0.015% in the diet), and more than 24,000 mice are being used, with about 3,000 mice/dose level tested. The study has been designed to detect a 1% tumor incidence.

If this kind of sensitivity difference between the bacterial and animal tests carries over to the detection of weak carcinogenic activity in general, then this kind of explanation may also apply to some other "false positives" in the Salmonella test. This may actually be the case for some of the "noncarcinogens" that are weak mutagens in the bacterial test. These could be weak carcinogens difficult for the animal carcinogenicity tests to detect. [We have discussed this elsewhere (25, 26).]

Impurities and Animal Carcinogenicity Tests

As discussed above, the bacterial test may be more sensitive to the presence of mutagenic (or carcinogenic) impurities than are the animal cancer tests. Although we know of no case in which a carcinogenic impurity caused a false positive response in an animal cancer test, there are some examples where it seems a possibility.

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When Highly Impure Chemicals Have Been Tested.

We have discussed the highly impure dye auramine in this respect (25). Recently, auramine has been found to be weakly mutagenic for Salmonella (26). It would be of interest to determine whether the mutagenicity could be due to an impurity.

When a Chemical Contains an Unusually Potent Impurity.

bis-2,7-AAF, compared to 2-AAF and its other relatives, has a very broad tissue specificity and has been referred to as a ubiquitous carcinogen (6). This is a characteristic more often associated with direct-acting carcinogens, such as nitrosomethylurea, than with those requiring metabolic activation, as in the case of bis-2,7-AAF. We reported here (Table 2) the presence of a very potent direct-acting mutagenic impurity in 1 sample of bis-2,7-AAF (see "Results" for further discussion), which could conceivably have influenced the cancer tests. As discussed in "Results," another sample actually used in 1 of the many animal cancer tests on bis-2,7-AAF did not contain the impurity.

When a Chemical Has Been Administered to Animals in Unusually Large Amounts.

On a per kg of body weight basis, most carcinogens are active when given to rodents in daily p.o. doses on the order of mg/day, and some potent carcinogens can be detected in the μg/kg dose range. The weakest carcinogens that have been detected in animals have been given in daily doses on the order of g/kg. In such cases a potent carcinogen, present as an impurity, could conceivably cause a carcinogenic response. TCE and saccharin are possible examples, and we discuss these below. [We have discussed 2 other possible examples, thiourea and thioacetamide, elsewhere (25, 26).]

TCE, a major industrial solvent, is also used in an extraction step in the production of decaffeinated coffee, and the Food and Drug Administration has recently proposed that this use be discontinued because of a report that it is a weak carcinogen in mice (10). In this study daily doses of over 1 g/kg were administered by stomach tube for most of the lifetime of the animals. The lowest total dose that produced a response was almost 1 kg. TCE used in the carcinogenicity tests, although quite pure (>99%), contained a number of low-level impurities, including the carcinogen (19) epichlorohydrin (0.09%) and the possible carcinogen 1,2-epoxybutane (0.19%). The daily doses of these 2 impurities in the TCE carcinogenicity study were 2.2 to 4.4 mg of epoxybutane and 1 to 2 mg of epichlorohydrin per kg. These doses do not seem insignificant when compared to daily doses of known carcinogens that elicit a significant tumor response in rodents when administered p.o. [e.g., propane sultone (<1 mg/kg), nitrosomethylurea or MNNG (4 mg/kg), and a single 10-mg/kg dose of nitrosoethylurea (22)].

Saccharin, the artificial sweetener, causes bladder can-

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4 Two other studies (8, 20) also were substantially negative. These used preparations of 7-OH-2-AAF that, because of their routes of synthesis, were likely to contain a greater amount of 2-AAF as an impurity. One of these (20) actually reported a weak carcinogenic response. However, the increase in tumors occurred very late in the study, and all controls were killed earlier. During the time that the controls were alive, the study was negative.

* A role for epoxybutane is unlikely. Epoxybutane, although its structure and weak mutagenicity (27) suggest that it could be a carcinogen, was negative in 2 quite limited carcinogenicity tests (19). The only conclusion possible from such limited studies is that, if epoxybutane is a carcinogen, it is not likely to be very potent and, therefore, would probably not have been a factor in the TCE carcinogenicity test. Epichlorohydrin, on the other hand, is a known carcinogen (19) and is a potent mutagen (Ref. 31; V. Simon, manuscript in preparation). It cannot be ruled out as a contributor to the TCE carcinogenicity test result. TCE (containing epichlorohydrin and epoxybutane impurities) has recently been reported to be weakly mutagenic in the Salmonella test (36), and it would be of interest to determine whether highly purified TCE is mutagenic.
cer in rats ingesting large doses (more than 2 g/kg/day) over their lifetimes (7). Based on this finding the Food and Drug Administration has recommended that saccharin be banned as a food additive. Saccharin used in these most recent animal cancer tests, although quite pure, contained small amounts (about 20 ppm) of as yet unidentified impurities. The daily dose of impurities to the rats was about 50 μg/kg/day. This is quite a low dose, but some of the more potent rodent carcinogens (such as aflatoxin and 3-methylcholanthrene) can be detected in the standard animal cancer tests at doses in this range (22). Thus, it is possible that the carcinogenicity of saccharin could be due to a potent carcinogenic impurity. This possibility has recently been given more credence by the finding that impurities in saccharin are mutagenic in the Salmonella test (Ref. 37; also see discussion in Ref. 24).

Other Examples of Mutagenic Impurities

Several other chemicals of importance in terms of human exposure have been reported to contain mutagenic or carcinogenic impurities. (a) Quinolinate, a tryptophan metabolite, commercially obtained at 98% purity, was rather strongly mutagenic (26 revertants/nmole; M. Hollstein, R. Talcott, and E. Wei, personal communication, and Ref. 5), but after purification it was nonmutagenic (5). (b) The flame retardant Tris (9) contains the mutagen and known carcinogen 1,2-dibromo-3-chloropropane. (c) Chlorodane, a pesticide banned by the Environmental Protection Agency for most uses, but retained for use in termite control, contains mutagenic impurities (38). (d) Methoxychlor, suggested as a substitute for dichlorodiphenyltrichloroethylene, dieldrin, and chlordane (11), contains a weakly mutagenic impurity (16). It is currently under test for carcinogenicity at the National Cancer Institute.

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Detection of Mutagenic Impurities in Carcinogens and Noncarcinogens by High-Pressure Liquid Chromatography and the \textit{Salmonella}/Microsome Test

E. Victor Donahue, Joyce McCann and Bruce N. Ames


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