Studies on the N-Hydroxylation and Carcinogenicity of 4-Aminoazobenzene and Related Compounds

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Summary

N-Hydroxy-N-acetyl-4-aminazobenzene (N-hydroxy-AAB) was excreted in conjugate form in the urine of rats, mice, or hamsters given injections of 4-aminazobenzene (AB) or N-acetyl-4-aminazobenzene (AAB) and in the urine of rats given injections of N,N-dimethyl- or N-methyl-4-aminazobenzene. AAB and 3- and 4'-hydroxy-AAB were also excreted under these conditions.

Like AAB and AB, neither N-hydroxy-AAB nor N-hydroxy-AB was carcinogenic for rats under the conditions studied. The tests used were long-term feeding of N-hydroxy-AAB in the diet of rats, repeated i.p. injections of N-hydroxy-AB, N-hydroxy-AAB, or N-acetoxy-AAB into young female rats, and repeated s.c. injections of the cupric chelate of N-hydroxy-AAB into young female rats.

The syntheses of the following new compounds are described: N-hydroxy-AB, N-acetoxy-AAB, and N-hydroxy-AAB.

Introduction

Studies on 2-acetylaminofluorene (11-14, 21), 4-acetylaminobiphenyl (18), 2-naphthylamine (5), 4-acetylaminostilbene (1, 3), 7-fluoro-2-acetylaminofluorene (10), and 2-acetylaminophenanthrene (10) have shown that these compounds are metabolized in susceptible species in vivo to N-hydroxy derivatives and that these N-hydroxy metabolites are generally more carcinogenic than the parent compounds, especially at sites of application. Thus the N-hydroxy derivatives appear to be proximate carcinogens in the neoplastic processes induced by these compounds. The fact that the aromatic aminoazo dyes are carcinogenic primarily in the liver and generally not at sites of local application (16) has suggested that the aminoazo dyes are likewise active through the formation of reactive metabolites. The structural similarity between the aminoazo dyes and the carcinogenic aromatic amines and amides suggested that these reactive metabolites might be N-hydroxy derivatives.

Materials and Methods

Preparation of Compounds

AB (m.p. 124°-126°C) and DAB (m.p. 117°-118°C), purchased from Eastman Organic Chemicals, were purified by treatment with Norit and chromatography on alumina. AAB (m.p. 146°-147°C) (12) were synthesized as described in the respective literature citations. 10-Methyl-1,2-benzanthracene was a gift from Dr. Melvin Newman of Ohio State University. The syntheses of the other compounds are described below.

4-NITROAZOBENZENE. A mixture of 93 gm of nitrosobenzene and 125 gm of p-nitroaniline in 600 ml of glacial acetic acid was gently refluxed for 6 hr. The warm solution was mixed with 1
liter of 6 N HCl, and the mixture was kept at 5°C overnight. The black precipitate that formed was filtered, washed well with water, and stirred for 1 hr with 1 liter of benzene and 30 gm of NaHCO₃. The mixture was filtered, and the aqueous layer was separated from the benzene extract. The latter solution was taken into a 2-liter round-bottom flask and 32 gm of 4-nitroazobenzene, 35 gm of ascorbic acid, and 400 ml of ethanol were mixed well by shaking. One hundred ml of 8 N ammonium hydroxide were added with stirring, and the mixture was kept at 5°C overnight. The reduced mixture was concentrated on the water bath. A:-Hydroxy-AB (27 gm) was added in small portions over a 0.5-hr period, and the mixture was stirred until all of the compound dissolved, at which point the acetylation was complete. The precipitate was filtered and dried in vacuo over anhydrous calcium chloride at room temperature. The yield of N'-hydroxy-AB was 27 gm (90% of theory), and element analytical analyses gave: C, 66.22; H, 5.15; N, 19.71. The compound gave an immediate strong test with Tollen's reagent and a strong red color in ethanolic HCl. In alkaline ethanol the compound gave a brown solution which turned yellow; it gave only a faint red color upon acetylation.

N'-ACETOXY-N'-ACETYL-4-AMINOAZOBENZENE (N'-ACETOXY-AB) AND N'-HYDROXY-N'-ACETYL-4-AMINOAZOBENZENE (N'-HYDROXY-AB). A mixture of 160 ml of acetic anhydride and 5 ml of pyridine was stirred magnetically while cooled in an ice bath for 2 days. The yield of N'-hydroxy-AB was 27 gm (90% of theory), and the material was analytically pure. It melted with decomposition at 195°–197°C. Elemental analyses gave: C, 66.92; H, 5.13; N, 19.81 (theory for C₁₁H₁₃N₂O = C, 67.59; H, 5.20; N, 19.71). The compound gave an immediate strong test with Tollen's reagent and a strong red color in ethanolic HCl. In alkaline ethanol the compound gave a brown solution which turned yellow; it gave only a faint red color upon acetylation.

CUPRIC CHELATE OF N'-HYDROXY-4-AMINOAZOBENZENE (N'-HYDROXY-AB). A filtered (glass frit), saturated solution of cupric acetate (20 ml) in 95% ethanol was added to a solution of 500 mg of N'-hydroxy-AB in 10 ml of ice-cold 95% ethanol in the same manner as for the preparation of the chelate of N'-hydroxy-AAF (14). The precipitate was filtered by vacuum, washed successively with water and ethanol until the washes contained no yellow color, and dried in vacuo over anhydrous calcium chloride at room temperature. The yield of 356 mg was 59% of theory, and elemental analyses gave: C, 58.65; H, 4.22; N, 14.68 (theory for C₁₁H₁₃N₂O₄Cu = C, 58.71; H, 4.42; N, 14.48).

N'-ACETYL - 3 - HYDROXY - 4 - AMINOAZOBENZENE (3 - HYDROXY-AB). 3-Hydroxy-AB (7), 90 mg, was dissolved in 4 ml of pyridine, 0.7 ml of acetic anhydride was added, and the solution was set in an ice bath for 1 hr, after which it was left at 25°C for 12 hr. The product, 3-acetoxy-AB, was precipitated by the addition of 40 ml of water, collected by centrifugation, and washed once with 10 ml of water. The precipitate was then dis-
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solved in 0.8 ml of 70% ethanol, 1 ml of 15 M NH₄OH was added, and nitrogen was gently bubbled through the solution for 5 min. After the solvent was removed in vacuo with a flash evaporator, the residue was crystallized from ethanol-water to give 51 mg (47% of theory) of compound melting at 200°-210°C.

N-acetyl-4'-hydroxy-4-aminooazobenzene (4'-hydroxy-AB). 4'-Hydroxy-AB (15) was acetylated by the procedure used for 3-hydroxy-AB. The melting point of the N-acetyl derivative was 201°-202°C.

Metabolism Studies

Administration of Compounds. For injection into 55-75-gm male rats (Holtzman Rat Co., Madison, Wis.), 26.5 mg of AAB or equimolar amounts of N-hydroxy-AAB, AB, MAB, DAB, or 3'-methyl-MAB were suspended per ml of a 0.9% solution of sodium chloride by homogenization. An amount of dye equimolar to 10 mg of AAB/100 gm body weight was injected i.p. both at 0 and 6 hr. Female rats (100-110 gm) received one s.c. injection of 10 mg of AAB in 0.2 ml of triacrylpron (triethanamin, Eastman Organic Chemicals). For i.p. injection into 18-20-gm female mice (ICR/He, A. R. Schmidt Co., Madison, Wis.), the finely ground compounds were suspended in triacrylpron at a concentration equimolar to 10 mg of AAB/ml, and 0.2 ml was injected once. For i.p. injection into male hamsters (40-50 gm, Con Olson Co., Madison, Wis.), the compounds were suspended in triacrylpron at a concentration equivalent to 5.0 mg/ml of AAB, and 0.25 ml was injected once. Because of the acute toxicity of N-hydroxy-AB when it was injected i.p., 0.33 or 0.10 ml, respectively, of a triacrylpron solution (17.8 mg/ml) was injected s.c. into the left hind leg of rats or mice. Immediately following injection of the compounds the animals were placed in stainless steel wire-mesh cages with stainless steel funnels. The urine samples were collected for 24 hr, after the initial injection of dye, in 100-ml centrifuge tubes set in Dewar flasks filled with Dry ice.

With the exception of the experiment in which the metabolism of several dyes was compared in male rats which had been maintained from weaning on the basal diet with or without 1 mg of riboflavin/kg of diet for 17 days prior to the injection of dye, all of the animals were fed the semipurified diet (1) for 2 days prior to the administration of the compounds. Water was available ad libitum during the period of urine collection, but no food was administered.

Analysis of urine samples. Urine samples from 2-9 rats, 6-10 mice, or 2-8 hamsters were pooled for each analysis. Each pooled urine sample was melted at room temperature, adjusted to pH 6 with a few drops of glacial acetic acid, and diluted to 75 ml with water. Sixty ml of 1 M pH 6 sodium acetate buffer, 100 mg each of Taka-Diastase and bacterial β-glucuronidase (Sigma Chemical Co., St. Louis, Mo., 32 units/mg) in 10 ml of water, and 0.5 ml of chloroform were added. After incubation at 37°C for 12 hr, the sample was cooled to 5°C and extracted in a separatory funnel twice with 100 ml of ice-cold peroxide-free ethyl ether. (Extraction at 5°C minimized the formation of emulsions at the interface; those that did form were broken by the addition of a few drops of ethanol.) After being dried with anhydrous sodium sulfate, the combined ethereal extracts were evaporated to dryness in vacuo with a flash evaporator, and the residue was dissolved in 45 ml of petroleum ether (Skellysolve B) and 5 ml of ethyl ether. The petroleum ether-ethyl ether solution was then extracted 1 to 3 times with 15 ml of 4 N HCl to remove the free amines; 1 extraction was sufficient when an acetylated aminoazo dye had been administered while 3 were generally required when an amine or hydroxyamine had been injected. After the organic layer was washed with 15 ml of water, it was dried over sodium sulfate and divided into 2 equal portions, each of which was taken to dryness at room temperature under a gentle stream of nitrogen.

The residue from each portion was dissolved in 0.5 ml of ethyl ether and spotted on two to five 0.7 x 38-cm strips of Whatman No. 1 filter paper. After equilibration for 12 hr in jars which contained both the upper and lower phases of the solvent system of cyclohexane, t-butanol, glacial acetic acid, and water (18:2:2:1), ascending chromatograms were run with the paper dipped in the upper phase for 6-8 hr. In general the chromatographic strips obtained from 1 portion of the extract were used for spray reactions and the zones from the strips obtained from the 2nd portion were eluted and combined for spectral determinations.

The Rf's of the major acetylated azo dye metabolites and the colors of these metabolites after treatment of the chromatograms with 1 N HCl, 1% p-dimethylaminobenzaldehyde in 1 N HCl, or the Folin-Ciocalteu reagent (Fisher Scientific Co., Pittsburgh, Pa.) are given in Table 1. Unfortunately, the value of the spray reagents was limited because of the yellow color of the metabolites.

All of the corresponding zones on the strips prepared from a single aliquot of the extract were eluted together in 5.0 ml of 95% ethanol. The spectra of these eluates were either determined directly in a Beckman model DB-1 recording spectrophotometer or, in those cases in which the spectrum was to be determined both in neutral and in alkaline solution, the ethanolic extracts of the paper sections were taken to dryness in vacuo to remove all traces of acetic acid from the chromatography solvent, and the residue was then dissolved in 5.0 ml of 95% ethanol. After each spectrum had been determined in ethanol solution, 100 μl of 1.0 N KOH were added per 5 ml of ethanol, and the spectrum was determined immediately before any precipitation of potassium carbonate occurred. The wave lengths of maximum absorption of the major acetylated aminoazo dye metabolites and the molar absorption coefficients at these wave lengths are recorded in Table 1.

Carcinogenicity Studies

In Experiment 1 groups of 14-16 adult male rats (Holtzman Rat Co.) with an average initial weight of 200 gm were fed a semipurified diet (1) which contained 1 mg of riboflavin/kg and 0.057% of MAB for 16 weeks or equimolar amounts of AAB or N-hydroxy-AAB for 22-35 weeks. For Experiment 2, adult male rats (Charles River Breeding Laboratories, Wilmington, Mass.) were fed N',N-dimethyl-4'-ethyl-4-aminooazobenzene (17) for 16 weeks or N-hydroxy-AAB for 44 weeks at the same molar level as in Experiment 1. In both experiments the animals were maintained from the end of the dye-feeding periods on the same semipurified diet. In the 1st experiment the surviving animals were killed for examination at 54 weeks. In Experiment 2 the rats which had been fed N-hydroxy-AAB were laparotomized and the livers were examined carefully at 54 weeks. These animals and their controls are being maintained until death.

In Experiment 3 the test compounds were injected i.p. into...
groups of 20 weanling female rats (Charles River Breeding Laboratories) with an average initial weight of 50 gm. Each compound was suspended just prior to injection by homogenization in a solution which contained 1.75% of gum acacia and 0.9% of sodium chloride; the suspensions were maintained by mixing with a magnetic stirrer and 0.6 ml/100 gm body weight was injected. AB, MAB, N-hydroxy-AAB, and N-acetoxy-AAB were each injected at a level equimolar to 10.0 mg of AB/100 gm body weight for the first 6 injections; 9 injections were then made at a level equimolar to 7.5 mg of AB/100 gm. Because of its acute toxicity N-hydroxy-AAB was administered at a level of 3.6 mg/100 gm body weight for 15 injections; larger doses caused severe methemoglobinemia which caused death. As a control on the susceptibility of the rats to mammary tumor induction, 1 group of animals received 12 injections of N-hydroxy-AAF (4.0 mg/100 gm body weight for the 1st 6 injections and then 3.0 mg/100 gm). The rats were all fed a grain diet (21), and the experiment was terminated at 52 weeks. For Experiment 4 female rats from the Charles River Breeding Laboratories with initial weights of 90-100 gm were given injections s.c. in the right hind leg 12 times at weekly intervals with a suspension of 3.9 mg of the cupric chelate of N-hydroxy-AAB in tricaprylin. This experiment was controlled by a group of rats given injections once of 2.0 mg of 10-methyl-1,2-benzanthracene. Wayne Breeder Blox pellets (Allied Mills, Inc., Chicago, Illinois) were fed, and the experiment was terminated at 15 months.

In all cases the rats were given food and water ad libitum. The rats of Experiments 1 and 2 were weighed biweekly during the period of dye administration; those of Experiments 3 and 4 were weighed weekly during the injection periods. Thereafter, all of the rats were weighed monthly. All of the rats were subjected to routine gross autopsies which included examination of the injection site, mammary and ear duct gland tissues, and the organs of the abdominal and thoracic cavities. Pieces of all tumors or other abnormal tissues were fixed in 10% neutral formalin, sectioned at 5-6 μm, and stained with hematoxylin and eosin. We are indebted to Dr. Henry Pitot of this department who studied these sections and diagnosed the tumors.

### Results

Identification of N-hydroxy-AAB, 3-hydroxy-AAB, 4'-hydroxy-AAB, and AAB as metabolites of AB and AAB. Prior to the present work Ishidate and Hashimoto (8) had administered AB and DAB by stomach tube to rats and had identified AB and the ethereal sulfates of 3-hydroxy-AB, 4'-hydroxy-AB, and 4'-hydroxy-AAB in the urines of these rats in amounts similar to those noted below for the various N-acetylated derivatives. In the present work conjugates of N-hydroxy-AAB and 3-hydroxy-AAB were found in addition to the conjugated 4'-hydroxy-AAB noted by Ishidate and Hashimoto; in general the free amines were not investigated in these studies. Preliminary identification of the metabolites noted in the present studies was based on their Rf's on chromatography on paper and the reactions of the zones of the metabolites with the spray reagents (Table 1). Of these reagents the acidic p-dimethylaminobenzaldehyde reagent distinguished between N-hydroxy-AAB, the phenolic derivatives, and AAB while the Folin-Ciocalteu reagent did not react with AAB but reacted similarly with 3-hydroxy-AAB, 4'-hydroxy-AAB, and 4'-hydroxy-AAB. However, the yellow colors of the dyes at neutrality and their pink colors under acidic conditions made these reactions less definitive than has previously been observed with colorless amides and their derivatives (6, 13). In general the Rf's of the metabolites extracted from urine as described in the Materials and Methods section were 15-20% lower than those obtained on chromatography of the pure synthetic compounds. These lower Rf's were also obtained on addition of the synthetic compounds to extracts of normal urine. However, rechromatography of the metabolites, in the absence of the contaminants present in the original extracts, resulted in Rf's similar to those of the synthetic compounds.

More definitive identification of N-hydroxy-AAB, 3-hydroxy-AAB, and AAB, the metabolites of major interest in these studies, was obtained from comparison of the absorption spectra of the metabolites with those of the synthetic compounds in neutral and in alkaline ethanolic solutions (Chart 1). While the spectrum of AAB changed very little on addition of alkali, the spectra of N-hydroxy-AAB and 3-hydroxy-AAB showed marked changes; in
CHART 1. The ultraviolet absorption spectra of synthetic and metabolic N-acetyl-4-aminoazobenzene (AAB), N-hydroxy-AAB, and 3-hydroxy-AAB in 95% ethanol and in 0.02 N KOH in 95% ethanol.
all cases the spectra of the metabolites and synthetic compounds matched closely.

N-Hydroxy-AAB and 3-hydroxy-AAB were excreted by the rats primarily as water-soluble conjugates. When the urine from rats given injections of AAB was extracted with ethyl ether prior to treatment with the enzymes, no N-hydroxy- or 3-hydroxy-AAB could be detected, and only 0.07% of the injected AAB was recovered as urinary AAB. When the ether was removed from the extracted urine with nitrogen, and the urine was then incubated with \( \beta \)-glucuronidase and Takadiastase in the usual manner, 0.25, 0.26, and 0.31% of the injected AAB was recovered as N-hydroxy-AAB, 3-hydroxy-AAB, and AAB, respectively. The large increase in the amount of AAB after incubation with the hydrolytic enzymes indicated either that this compound was excreted as a conjugate or that it arose from some other conjugate. Some of the AAB was undoubtedly derived from N-hydroxy-AAB, but it is uncertain how much arose in this manner. On chromatography of 500 \( \mu \)g of synthetic N-hydroxy-AAB, in the same manner as for the urinary metabolite, 12% of the N-hydroxy-AAB was recovered as AAB and 78% as N-hydroxy-AAB. Likewise, when 500 \( \mu \)g of N-hydroxy-AAB were added to the pooled 24-hr urine samples from 4 rats and the urine was analyzed by the usual procedure, 17% of the N-hydroxy-AAB added was recovered as AAB. The ratios of N-hydroxy-AAB to AAB in the urines of the various animals administered AAB or its derivatives differed widely (Table 2) and did not furnish evidence that the major share of the AAB was derived by reduction of N-hydroxy-AAB after the urine was excreted. However, the possibility that reduction might occur to a greater extent with smaller amounts of N-hydroxy-AAB than with larger concentrations of the hydroxamic acid and the possible influences of urinary constituents on this reduction were not examined. For this reason no attempt was made to correct the metabolic data (Table 2) to account for any losses or alterations during analysis.

Quantitation of AAB and its N-, 3-, and 4'-hydroxy derivatives in the urines of animals treated with AAB and its derivatives. In this survey the urine was pooled from a number of animals for each analysis, but in many cases only 1 pooled urine sample was analyzed for each set of conditions. For this reason, only general conclusions can be drawn and no significance can be attached to small differences.

Of primary interest in these studies was the quantitation of the excretion of N-hydroxy-AAB as one approach to determining the extent to which the N-hydroxy derivative was formed and main-

**TABLE 2**

**The Urinary Excretion of N-Acetyl-4-aminoazobenzene and Its N-, 3-, and 4'-Hydroxy Derivatives by Rats, Mice, and Hamsters after Administration of Aminoazo Dyes**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Compound injected and route</th>
<th>Diet</th>
<th>No. of analyses</th>
<th>% of dose excreted as</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AAB</td>
</tr>
<tr>
<td>Rat</td>
<td>F</td>
<td>AAB (s.c.)</td>
<td>Control</td>
<td>1 (8)</td>
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<td></td>
<td>M</td>
<td>AAB (i.p.)</td>
<td>Control</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>AB (i.p.)</td>
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<td>0.09</td>
</tr>
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<td>Mouse</td>
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<tr>
<td></td>
<td>F</td>
<td>AB (i.p.)</td>
<td>Control</td>
<td>1 (10)</td>
<td>n.i.</td>
</tr>
<tr>
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* The abbreviations used are: AB, 4-aminazobenzene; MAB, N-methyl-4-aminazobenzene; DAB, N,N-dimethyl-4-aminazobenzene; AAB, N-acetyl-4-aminazobenzene; ribo.-def., riboflavin-deficient.

* Injection schedules: N-Hydroxy-AB was dissolved in tricaprylin (17.8 mg/ml), and 0.33 or 0.20 ml was injected s.c. into rats or mice, respectively. The other male rats were injected i.p. at 0 and 6 hr with an amount of dye equimolar to 10 mg of AAB/100 gm body weight; 26.5 mg of AAB or an equivalent amount of another dye were suspended per ml of 0.9% sodium chloride solution. The female rats received 10 mg of AAB in 0.2 ml of tricaprylin. The mice were injected once with 0.2 ml of a tricaprylin suspension (equivalent to 10 mg of AAB/ml), while the hamsters received 0.25 ml of a tricaprylin suspension which contained 5.0 mg of AAB or an equivalent amount of AB or N-hydroxy-AAB/ml.

* The numbers in parentheses denote the No. of animals whose urine was pooled for the analyses.

* Average ± the probable error.

* n.i. (not identified) signifies that no metabolite could be identified. The total absorption in these areas generally accounted for less than 0.01% of the dye administered. In the case of mice administered either AB or N-hydroxy-AB, however, an unidentified metabolite was found on the chromatograms in the position normally occupied by AAB, and no AAB could be detected. In these instances the unidentified metabolite may have accounted for about 0.1% of the administered compound.
tained under various conditions. Following the administration of AAB, rats excreted about 0.25% of the dose as N-hydroxy-AAB while mice and hamsters excreted nearly 10 times as much (Table 2). Hamsters and mice also excreted much more (20 and 5% of the dose, respectively) of administered N-hydroxy-AAB as N-hydroxy-AAB than did rats (0.7% of the dose); this fact suggested that mice and hamsters metabolized any N-hydroxy-AAB which was formed in vivo less readily than did rats. Hamsters appeared to excrete similar amounts of N-hydroxy-AAB whether AAB or AB was administered, while rats and mice excreted less N-hydroxy-AAB when AB was injected. Administration of MAB or DAB to rats also resulted in the excretion of small amounts of N-hydroxy-AAB, probably through the intermediate formation of AAB.

Rats, mice, and hamsters also excreted 3-hydroxy-AAB and 4'-hydroxy-AAB after administration of AAB or N-hydroxy-AAB or, with the exception of mice, after administration of AB. Rats given injections of MAB or DAB also excreted small amounts of these metabolites. The amounts of 4'-hydroxy-AAB were generally greater when the compounds were administered to mice or hamsters (0.5-1.5% of the dose) rather than to rats (generally less than 0.2% of the dose). With any of the species or compounds studied the amounts of 3-hydroxy-AAB in the urine ranged from 0.06 to 0.36% of the compound administered.

With rats and mice, but not with hamsters, the amounts of each of the acetylated metabolites in the urine was less when a nonacetylated compound (N-hydroxy-AB or AB) was administered as compared to the amounts excreted after injection of the N-acetyl derivatives. This difference was particularly marked in studies with mice, which excreted very little of the acetylated metabolites after administration of AB and none after injection of N-hydroxy-AB.

Some AAB was found in the urine from all of the animals studied except the mice injected with AB or N-hydroxy-AB. The amounts of AAB were generally greatest when AAB or N-hydroxy-AAB was injected; in these cases up to 1% of the dose was excreted as the amide. While some of the AAB presumably arose by reduction of N-hydroxy-AAB during analysis of the urine (see above), it seems likely that some was excreted as AAB. Thus, if the AAB arose entirely by reduction of N-hydroxy-AAB after excretion of the urine, one would expect some general correspondence between the amounts of these 2 compounds. Such a correspondence was not found, and the disparity was especially great when large amounts of N-hydroxy-AAB were excreted.

The fraction of the administered dose which was accounted for as the sum of the 4 acetylated aminoazo dye metabolites studied ranged from less than 1% in most of the experiments with rats to more than 20% when N-hydroxy-AAB was administered to hamsters. No other azo dye metabolites were found in identifiable amounts on the chromatograms. While the azo dyes with an unconjugated amino group were not examined in these studies, the color of the acidic extract of the ether-soluble metabolites and the results of other studies (8, 16) indicate that the amount of these dyes excreted in the urine accounts for only a small fraction of the administered dose. 4 Other studies have shown that a re-

4 In 1 experiment, the urine from mice given injections of AAB was fractionated in a manner which revealed 4'-hydroxy-AB in an amount equivalent to 1.2% of the administered dye. The
and 2 sebaceous gland carcinomas were found in the rats given injections of N-hydroxy-2-acetylaminofluorene (Table 4). Each of the dyes except N-hydroxy-AB was administered at 3 times the molar level of N-hydroxy-2-acetylaminofluorene and 15 injections, instead of 12, were given. Because of its toxicity at higher levels the amount of N-hydroxy-AB injected was only a little higher than that of N-hydroxy-2-acetylaminofluorene. Severe methemoglobinemia was observed in many of the rats al-

### Table 3
The Lack of Carcinogenic Activity of N-Acetyl-4-aminoazobenzene and Its N-Hydroxy Derivative on p.o. Administration to Male Rats

<table>
<thead>
<tr>
<th>Compound Fed</th>
<th>No. of Rats Started</th>
<th>Dye-Feeding Period (wk.)</th>
<th>Avg. Wt. Gain at 10 Wks. (gm)</th>
<th>Cumulative No. of Rats with Liver Carcinomas by:</th>
<th>Negative Survivors</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 wk.</td>
<td>40 wk.</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methyl-4-aminoazobenzene</td>
<td>15</td>
<td>16</td>
<td>53</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>N-Acetyl-4-aminoazobenzene</td>
<td>8</td>
<td>22</td>
<td>77</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-Hydroxy-N-acetyl-4-aminoazobenzene</td>
<td>8</td>
<td>35</td>
<td>69</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4'-Ethyl-N,N-dimethyl-4-aminoazobenzene</td>
<td>20</td>
<td>16</td>
<td>-2</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>N-Hydroxy-N-acetyl-4-aminoazobenzene</td>
<td>20</td>
<td>44</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None (basal diet)</td>
<td>20</td>
<td></td>
<td>132</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Male rats from the Holtzman Rat Co. (Experiment 1) or the Charles River Breeding Laboratories (Experiment 2) were fed the compounds at levels equimolar to 0.057% of N-methyl-4-aminoazobenzene for the times indicated. Both during and after the dye-feeding periods the rats were fed a semipurified diet (1) which contained 1 mg of riboflavin/kg.

### Table 4
The Lack of Carcinogenicity of 4-Aminoazobenzene, Its N-Hydroxy Derivatives, and of Certain Other Aminoazo Dyes on i.p. Injection into Young Female Rats

<table>
<thead>
<tr>
<th>Compound Injected</th>
<th>Avg. Wt. Gain at 5 Wks. (gm)</th>
<th>Cumulative No. of Rats with Mammary Tumors by:</th>
<th>Other Tumors</th>
<th>Negative Survivors 12 mo.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 mo.</td>
<td>9 mo.</td>
<td>12 mo.</td>
<td></td>
</tr>
<tr>
<td>4-Aminoazobenzene</td>
<td>124</td>
<td>0</td>
<td>0</td>
<td>1B</td>
</tr>
<tr>
<td>N-Methyl-4-aminoazobenzene</td>
<td>113</td>
<td>0</td>
<td>0</td>
<td>1M</td>
</tr>
<tr>
<td>N-Hydroxy-4-aminoazobenzene</td>
<td>113</td>
<td>0</td>
<td>0</td>
<td>1B</td>
</tr>
<tr>
<td>N-Hydroxy-N-acetyl-4-aminoazobenzene</td>
<td>114</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-Acetoxy-N-acetyl-4-aminoazobenzene</td>
<td>116</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-Hydroxy-2-acetylaminofluorene</td>
<td>84 (30)c</td>
<td>14 (52)</td>
<td>14 (52)</td>
<td>1 (Cholangioma)</td>
</tr>
<tr>
<td>None (solvent only)</td>
<td>121</td>
<td>0</td>
<td>0</td>
<td>1M</td>
</tr>
</tbody>
</table>

* Starting at weaning, groups of 20 female rats were given injections i.p. 3 times weekly for 6 injections with 10 mg of AB or equimolar amounts of the other aminoazo dyes (except N-hydroxy-AB)/100 gm body weight in 0.6 ml of a solution containing 1.75% of gum acacia and 0.9% of sodium chloride; then 9 injections were made at a level equimolar to 7.5 mg of AB/100 gm body weight. N-Hydroxy-AB was administered at a level of 3.6 mg/100 gm body weight for 15 injections. N-Hydroxy-2-acetylaminofluorene was injected at a level of 4 mg/100 gm body weight for the 1st 6 injections and then at a level of 3 mg/100 gm body weight for another 6 injections. M = malignant tumor, B = benign tumor. For other abbreviations, see footnote a, Table 2.

- Most of the deaths of the non-tumor-bearing animals occurred as a result of the methemoglobinemia which developed very soon after injection of the dyes. The following deaths occurred within the 1st 3 weeks from this cause: AB, 1; N-methyl-4-aminoazobenzene, 5; N-hydroxy-AB, 4; N-hydroxy-N-acetyl-4-aminoazobenzene, 2; and N-acetoxy-N-acetyl-4-aminoazobenzene, 5.
- The numbers in parentheses indicate the total number of mammary tumors in the group; 90% of these tumors were malignant.
- Arose near ear duct gland.
been demonstrated in vitro with rat and hamster liver microsomes. AAB, especially by the rat, it is not possible to estimate the %

Discussion

The identification of N-hydroxy-AAB in the urine of rats, mice, and hamsters administered AB or AAB and in the urine of rats administered MAB or DAB demonstrates that the aminoazo dyes, like a series of other amines and amides, are N-hydroxylated in vivo. In view of the extensive metabolism of N-hydroxy-AAB, especially by the rat, it is not possible to estimate the conversion of AAB to its N-hydroxy derivative from the present data. Furthermore, while it is possible that some of the N-hydroxy-AAB was formed by oxidation of AB, MAB, or DAB with subsequent acetylation or demethylation and acetylation, it seems most likely that the urinary N-hydroxy-AAB arose directly from N-hydroxylation of AAB. The latter reaction has been demonstrated in vivo with rat and hamster liver microsomes (P. D. Lotlikar, J. A. Miller, and E. C. Miller, unpublished data). Similarly, Matsumoto and Terayama (9) have inferred that N-hydroxy-3,2'-dimethyl-AB may be formed by mice from 3,2'-dimethyl-AB (o-aminoazotoluene) on the basis of their isolation of 4,4'-bis-(o-tolylazo)-2,2'-dimethylazobenzene from the livers of mice administered this carcinogenic dye; the triazo derivative could be formed by reduction of the azoxy reaction product of N-hydroxy-3,2'-dimethyl-AB and its oxidation product 4-nitroso-3,2'-dimethylazobenzene.

N-Hydroxylation have proved to be of particular importance in the metabolism and carcinogenicity of carcinogenic aromatic amines and amides (1, 3, 5, 6, 10-14, 18, 21). The high carcinogenic activities of the N-hydroxy metabolites of these carcinogens under a variety of conditions have led to the conclusion that N-hydroxylation represents a metabolic step by which these compounds are activated. On the other hand, AAB and AB are essentially nonecacinogenic for the rat (16, 19, 23), and N-hydroxylation did not convert these compounds into more active compounds. Thus, the long-term administration of N-hydroxy-AAB in the diet of rats in the manner conventionally used for the induction of liver tumors by aminoazo dyes failed to elicit any tumors or liver damage. Likewise, repeated i.p. injections of N-hydroxy-AAB, N-hydroxy-AB, and N-acetoxy-AAB in young female rats under conditions suitable for mammary tumor induction by other N-hydroxy derivatives (12, 18) and the repeated s.c. injection of the poorly soluble cupric chelate of N'-hydroxy-AAB, a sensitive test for the carcogenic action of N-hydroxy-2-acylaminoazofluorene (14, 21), did not result in tumors. Thus, although N-acylation in vivo is clearly an activation process for carcinogenic aromatic amines and amides, it is evident that the structure of a N-hydroxy metabolite must still largely govern its carcinogenicity.

In view of the studies on a variety of aminoazo dyes related to DAB which showed that the presence of at least 1 N-methyl group greatly enhances the carcogenic activity of these dyes (2, 16), the lack of carcogenicity of N-hydroxy-AAB and of N-hydroxy-AB was not unexpected. Recently Terayama and Orii (25) have shown that DAB-N-oxide is carcinogenic when administered to rats in the drinking water, and they have suggested that this N-methylated N-oxidation product may be a proximate carcogenic metabolite of DAB and MAB. This would require the methylation of MAB to DAB in vivo. We have recently shown that this reaction does not appear to occur in the rat (22). Furthermore, since DAB-N-oxide is readily converted to DAB and MAB (24, 25) and since it does not appear to have enhanced activity over that of these compounds...
(although no direct comparison was made), there is no evidence that the activity observed was due to the N-oxide rather than as a result of its conversion to DAB and MAB. The results in the present paper and other results recently obtained in this laboratory point to N-hydroxy-MAB as a probable carcinogenic metabolite of DAB and MAB. Although we have not been able to synthesize N-hydroxy-MAB, we have prepared its O-benzoxy derivative. This compound has strong carcinogenic activity at the site of s.c. injection in adult rats and causes liver and kidney damage when injected into new-born rats (L. A. Poirier, K. Sato, J. A. Miller, and E. C. Miller, manuscript in preparation). MAB was inactive in these tests.

Acknowledgment

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Studies on the $N$-Hydroxylation and Carcinogenicity of 4-Aminoazobenzene and Related Compounds

Kei Sato, Lionel A. Poirier, James A. Miller, et al.

*Cancer Res* 1966;26:1678-1687.

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