N-Benzoyloxy-N-methyl-4-aminoazobenzene: Its Carcinogenic Activity in the Rat and Its Reactions with Proteins and Nucleic Acids and Their Constituents in Vitro

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SUMMARY

N-Benzoyloxy-N-methyl-4-aminoazobenzene (N-benzoyloxy-MAB), a new derivative of the hepatocarcinogen N-methyl-4-aminoazobenzene (MAB), was synthesized. This toxic ester of N-hydroxy-MAB produced sarcomas at the site of repeated s.c. injections in rats; MAB was inactive under these conditions. Unlike MAB, N-benzoyloxy-MAB reacted readily in vitro at pH 7 with protein, RNA, and DNA to form macromolecular-bound dye. Five nucleophilic components of these macromolecules (methionine, cysteine, tryptophan, tyrosine, and guanosine) reacted with N-benzoyloxy-MAB under similar conditions to form polar dyes; other common amino acid and nucleoside components did not react. The reactions of N-benzoyloxy-MAB with proteins, nucleic acids, and their components may be useful prototypes in studies on the protein- and nucleic acid-bound dyes formed in vivo by the aminoazo dyes. The biologic and chemical properties of N-benzoyloxy-MAB parallel those of the carcinogenic aromatic N-acyloxy amides. These data, which form the subject of this report, and the recent studies from this laboratory on the similar reactivity and carcinogenicity of N-acetoxy-AAF (10, 14-16, 19) support the concept that N-hydroxy metabolites of carcinogenic aromatic amines and amides are activated in vivo by esterification of their N-hydroxy metabolites.

INTRODUCTION

N-Hydroxylation has been demonstrated as an important step in the metabolic activation of carcinogenic amides. Thus, AAF (9, 27) and its 7-fluoro derivative (20), 4-acetylamino-

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4 The following abbreviations are used (the Chemical Abstracts nomenclature is given in parentheses): AB, 4-aminoazobenzene (p-phenylazoaniline); AAB, N-acetyl-4-aminoazobenzene (p-phenylazoacetanilide); MAB, N-methyl-4-aminoazobenzene (N-methyl-p-phenylazoaniline); DAB, N,N-dimethyl-4-aminoazobenzene (N,N-dimethyl-p-phenylazoaniline); AAF, 2-acetylaminofluorene (N-2-fluorenlyacetamide).

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biphenyl (35), 4-acetylaminothylbene (2, 4), and 2-acetylaminophenanthrene (20) are each metabolized to N-hydroxy derivatives by species in which they are carcinogenic. Furthermore, when administered to rats, these N-hydroxy metabolites are more carcinogenic than the parent compounds at sites where the amides are active and produce tumors at sites of local application where the parent compounds have little or no activity (2, 5, 20, 23, 24, 28, 35, 39). The isolation of N-hydroxy-N-acetyl-4-aminoazobenzene from the urine of rats, mice, or hamsters administered AB or its carcinogenic N-methyl or N,N-dimethyl derivatives (MAB or DAB) showed that the aminoazo dyes are likewise N-hydroxylated in vivo (42). However, neither this metabolite nor N-hydroxy-AB was carcinogenic on administration to rats (42). The inactivity of these compounds is consistent with the apparent requirement of a N-methyl group for strong carcinogenicity in the series of aminoazo dyes structurally related to MAB and DAB (3, 6, 29). These data suggested that the proximate carcinogenic metabolite(s) of these dyes would contain both a N-methyl group and a N-hydroxy group.

When repeated attempts to synthesize N-hydroxy-N-methyl-4-aminoazobenzene (N-hydroxy-MAB) failed, its O-benzoyl derivative, N-benzoyloxy-MAB, was prepared on the premise that it might release the presumed carcinogenic metabolite in vivo. This ester induced a high incidence of sarcomas in rats on repeated subcutaneous injection. Furthermore, N-benzoyloxy-MAB reacted nonenzymatically in neutral solution with certain amino acids or proteins and with guanosine, DNA, and RNA. These data, which form the subject of this report, and the recent studies from this laboratory on the similar reactivity and carcinogenicity of N-acetoxy-AAF (10, 14-16, 19) support the concept that N-hydroxy metabolites of carcinogenic aromatic amines and amides are activated for carcinogenesis by esterification of the N-hydroxy group (32).

MATERIALS AND METHODS

Preparation of Compounds

Synthesis of N-Benzoyloxy-MAB. N-Benzoyloxy-MAB was synthesized by a modification of the procedure used by Horner and Steppan (13) for the synthesis of N-benzoyloxy-N-ethylaniline. It was found necessary to perform all operations between -2°C and 5°C, and all solutions were also maintained within this temperature range. MAB (20 gm) dissolved in 70 ml
of chloroform and an equimolar quantity of benzoyl peroxide (22.8 gm) in 100 ml of chloroform were mixed in a 2-liter flask. After 1 hr, 800 ml of hexane (Skellosolve II) were added, and a tarry residue precipitated and adhered to the sides of the flask. The supernatant solution was transferred to another flask, together with 2 washings of the tarry residue with 200-ml volumes of hexane. After a few minutes to permit further precipitation of tar the hexane-chloroform solution was decanted into a separatory funnel. The organic layer was then extracted as rapidly as possible 4 times with 250 ml of 2 n HCl, twice with 250 ml of 1 n NaOH, twice with 250 ml of 2 n HCl, twice with 200 ml of saturated sodium bicarbonate solution, and twice with 200 ml of water. Little dye was extracted by the alkaline solutions, but large amounts were removed with the acid extractions. The extracts with HCl following the extractions with NaOH appeared to remove aminozao dye contaminants which developed on contact with alkali but were not soluble in it. The aqueous extracts were discarded, and the organic phase was dried over anhydrous sodium sulfate.

The resultant clear orange-yellow solution was taken to dryness in vacuo in a rotary flash evaporator, in which the evaporating flask was immersed in an ice-water mixture, and the condensing flask was set in a Dry Ice-acetone bath. The red-brown oily residue was dissolved in 40 ml of acetic and 325 ml of absolute ethanol, after which 1.2 liters of water were added. The resulting suspension was left at 3°C for 3-4 days to facilitate precipitation of the N-benzyloxy-MAB, which was then collected by suction filtration on Whatman No. 40 paper and dried over CaCl₂ in a vacuum desiccator for 3-4 days at 3°C. The yellow-orange precipitate (3.5 gm, 11% of theory) melted at 89-91°C, with decomposition marked by darkening and a rapid evolution of gas. Elementary analyses (carried out by Huffman Laboratories, Wheatridge, Colorado) gave the following data:

Calculated for C₂₉H₁₇N₄O₂: C, 72.49; H, 5.17; N, 12.68

Found: C, 72.44; H, 5.62; N, 12.34

Verification that the product was N-benzyloxy-MAB was obtained by reduction to MAB. For this purpose, 11 mg were dissolved in 10 ml of ethyl ether, and 50 mg of finely powdered lithium aluminum hydride were added. The mixture was stirred for 6 hr with a magnetic stirrer at room temperature, after which 2 ml of ethyl acetate were added dropwise to destroy excess lithium aluminum hydride and 1 ml of water was added dropwise to destroy the lithium aluminum hydride-dye complex. After 2 extractions with 20 ml of water, the ether was removed in vacuo, and the residue was chromatographed on alumina with benzene. Sixty % of the starting material was recovered as a product which was identical with MAB as determined by its spectrum in ethanol and acidic ethanol, its melting point, and the lack of a melting point depression when mixed with authentic MAB.

The infrared spectrum of N-benzyloxy-MAB (CCl₄, 80 mg/ml) obtained on a Beckman IR-10 recording spectrophotometer showed no N-H peak between 3100 and 3500 cm⁻¹, but did show a carbonyl peak at 1760 cm⁻¹. This peak is consistent with the presence of an ester linkage, but would not be exhibited by an amide linkage. A nuclear magnetic resonance spectrum of a carbon tetrachloride solution showed the theoretical ratio of 14 aromatic hydrogens to 3 aliphatic hydrogens. As determined with a Beckman DB recording spectrophotometer, N-benzyloxy-MAB had an absorption maximum at 357 mμ in 95% ethanol and at 507 mμ in 2.5 n HCl in 80% ethanol.

When stored at -10°C in the dark, N-benzyloxy-MAB is fairly stable; no change in physical appearance or melting point was detected after storage for 1 year. However, when the compound was left at room temperature in daylight for 4-6 days, extensive decomposition occurred.

Other 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 recrystallization from benzene-hexane. Benzoyl peroxide (Eastman Organic Chemicals) was used without further purification. N-Hydroxy-AB (m.p. 195-197°C, with decomposition) (42), N-hydroxy-AAB (m.p. 136-138°C) (42), DAB-N-oxide (124-125°C) (8, 37), MAB (87-88°C) (26), and N-hydroxy-AAF (m.p. 146-148°C) (24, 38) were prepared by published methods. N-Benzoyl-MAB (m.p. 95-96°C) was prepared by benzyolation of MAB.

Carcinogenicity Studies

Subcutaneous Injections. Charles River CD random-bred rats (Charles River Breeding Laboratories, Wilmington, Mass.) were housed singly or in groups of 2 in wire-bottomed cages and were given food and water ad libitum. Each group in Experiment 1 was composed of 10 male and 10 female rats with initial weights of 90-110 gm; each group in Experiment 2 contained 20 males that weighed 125-150 gm. In both experiments each rat received by s.c. injection into the right hind leg an amount of compound equimolar to 2.5 mg of MAB in 0.2 ml of trioctanoin (tricaprylin, Eastman Organic Chemicals) twice weekly for 12 weeks, except that the rats treated with N-hydroxy-AAF received 3.0 mg twice weekly for 8 or 9 weeks. Each compound, finely ground in a Mullite mortar, was suspended or dissolved without heat with the aid of a magnetic stirrer immediately prior to its injection. For the first 12 weeks of each experiment the rats were fed an 18% casein, semipurified diet (2) which contained 0.5 mg (Experiment 1) or 1.0 mg (Experiment 2) of riboflavine/kg in an effort to minimize the reductive cleavage of the dyes by the tissues (36). Thereafter, all of the animals were fed Wayne Breeder Blox pellets (Allied Mills, Inc., Chicago, Illinois). The rats were weighed every 2nd week during the injection periods and monthly thereafter. The surviving rats of Experiments 1 and 2 were killed at 20 and 14 months, respectively.

Intraperitoneal Injections into Neonatal Rats. Adult male and female Charles River CD random-bred rats were mated, and the pregnant females were housed singly in solid-bottomed cages. Within 24 hr after birth the young of each litter were divided randomly into 2 groups (distinguished by cutting the tails of 1 group). In Experiment 1, each rat received 0.05 ml of heat-sterilized corn oil alone or containing 200 µg of N-benzyloxy-MAB or an equivalent amount of MAB on Days 1, 2, and 3 and twice as much of the solution on Day 4. In Experiment 2, each rat received 0.05 ml of heat-sterilized trioctanoin alone or containing 160 µg of N-benzo-
The values were corrected by the absorption obtained when
thereafter the rats received no further treatment, except that
analysis.
Solvent was removed in vacuo, and the residue was dissolved
7-ml portions of peroxide-free ethyl ether: ethanol (5:1); this
spectrophotometrically after addition of 1 ml of the aqueous
reaction mixture and, after 90 min at room temperature, the
reaction with methionine, 1 ml of 11 N KOH was added to the
the reaction mixture with the dye but without an amino acid was
carried through the same procedure.

For both Experiments 3 and 4, 1.7 µmoles of guanosine-8-14C
or -3H, 1 µmole of N-benzoyloxy-MAB, and 5 µmoles of
sodium citrate buffer, pH 7, were incubated for 20 hr. This incu-
bation system was also carried out on a 40-fold scale without
radioactive guanosine to obtain sufficient of the product for
spectrophotometric estimation. In this case, the unreacted dye
was removed by 4 extractions with 0.7 volume of water-saturated
ethyl ether, the aqueous layer was evaporated to dryness in
toluene at 32°C, and the product, after extraction into ethanol,
was chromatographed on cellulose thin layers with the butanol:
acetic acid:water system. The yellow band (RF of 0.80-0.95)
was eluted with ethanol; after concentration the product was
chromatographed on cellulose thin layers with the butanol:
water 1:18:4:16.6) (11), and those with
guanosine were chromatographed in the latter system and in
the n-butanol:glacial acetic acid:water system. With the sys-
tems used, the nucleosides moved with Rf's of less than 0.4.
The product formed from N-benzoyloxy-MAB and guanosine
had a Rf of 0.85-0.9 in both solvent systems used for its
separation.

4 The scintillating fluid was composed of naphthalene, 738
gram; diphenyloxazole, 46 gm; α-naphthylphenyloxazole, 0.46 gm;
xylene, 3500 ml; dioxane, 3500 ml; and absolute ethanol, 2100 ml.

In Vitro Reactivity of N-Benzoyloxy-MAB

Reaction with Protein. An amount of dye equimolar to
1.0 mg of N-benzoyloxy-MAB dissolved in 1.0 ml of 95%
ethanol was added to 4.0 ml of 0.003 M sodium citrate buffer,
pH 7, containing 125 mg of bovine serum albumin (Sigma
Chemical Co.); no precipitation of the dyes was evident. The
solutions were incubated at 37°C for 4 hours, and the protein
was then precipitated by the addition of 20 ml of acetone. All
of the manipulations up to this stage were carried out in a
nitrogen atmosphere in a glove box. The protein was recovered
by centrifugation, washed 3 times at room temperature with
10-ml aliquots of acetone, and twice extracted at 70°C for
30 min in 10-ml portions of ethanol. The protein samples were
then dried in vacuo, and 50-µg aliquots were analyzed for bound
dye (21, 25). After the alkaline hydrolysis, the nonpolar dyes
were extracted into 20 ml of hexane:benzene (6:1), from
which they were extracted into 1.5 ml of 7 N HCl for spectro-
photometric analysis with a Beckman DB recording spectro-
photometer. The polar dye was then extracted twice into
7-ml portions of peroxide-free ethyl ether:ethanol (5:1); this
solvent was removed in vacuo, and the residue was dissolved
in a mixture of 0.6 ml of ethanol and 0.8 ml of 7 N HCl for
analysis.

Reaction with Amino Acids. The dyes (1,5 µmoles) dissolved
in 0.5 ml of ethanol were added to 2.0 ml of 0.1 M sodium
phosphate buffer, pH 7, which contained 50 µmoles of amino
acid (as a suspension for the reactions with tyrosine and cyst-
tine). The mixture was incubated 1.5 or 20 hr at 37°C and
then extracted 3 times with 5 ml of 15% benzene in hexane.
The steps to this point were carried out in a nitrogen atmos-
phere (glove box). The water-soluble dye was then estimated
spectrophotometrically after addition of 1 ml of the aqueous
layer to 1.5 ml of a 2:1 mixture of 12 N HCl and 95% ethanol.
The values were corrected by the absorption obtained when
the reaction mixture with the dye but without an amino acid was
carried through the same procedure.

For the determination of 3-methylmercapto-MAB after
reaction with methionine, 1 ml of 11 N KOH was added to the
reaction mixture and, after 90 min at room temperature, the
solution was extracted 3 times with 5 ml of 15% benzene in
hexane. The benzene-hexane solution was washed once by
extraction with water, evaporated to dryness in vacuo at 32°C,
and dissolved in ethyl acetate for gas chromatography (43).

Reaction with Guanosine and Other Nucleosides. The
standard procedure for all of the experiments included incu-
bation of a nucleoside containing 14C or 3H with N-benzoyloxy-
MAB or another dye at pH 7 in 28% ethanol in a nitrogen
atmosphere (glove box). At the end of the incubation, aliquots of
the reaction mixtures, with or without dye, were chromatog-
graphed on cellulose (Brinkmann MN 300µp) in a solvent
system containing n-butanol:glacial acetic acid:water (50:
11:25) (unless otherwise specified). After the chromatograms
were dried in a stream of warm air, successive fractions were
scraped off and transferred to vials of scintillating fluid for
radioactivity determinations in a Packard scintillation counter.
In each case, the counts in the area occupied by the product
were corrected by subtraction of the low counts in the similar
area of a chromatogram of a reaction mixture without dye. The
% reaction was calculated on the basis of the limiting reactant.

In Experiment 1, 0.02 µmole of guanosine-14C, 0.5 µmole
of dye, and 2 µmoles of sodium citrate buffer were incubated for
3.5 hr. In the 2nd experiment 0.08 µmole of a nucleoside, 0.5
µmole of N-benzoyloxy-MAB, and 2 µmoles of sodium citrate
buffer, pH 7, were incubated for 3 hr. The reaction mixtures
with thymidine-2-3H, uridine-2-14C, and cytidine-2-14C were
chromatographed in the upper phase from a mixture of ethyl acetate:
formic acid:water (12:1:7) (11); the reaction mixtures contain-
ing adenosine-8-14C were chromatographed in isopropanol:
water:concentrated HCl (65:18.4:16.6) (11), and those with
guanosine were chromatographed in the latter system and in
the n-butanol:glacial acetic acid:water system. With the sys-
tems used, the nucleosides moved with Rf's of less than 0.4.
The product formed from N-benzoyloxy-MAB and guanosine
had a Rf of 0.85-0.9 in both solvent systems used for its
separation.
atmosphere for 4 hr and then extracted under nitrogen 3 times with an equal volume of peroxide-free ethyl ether. The RNA was precipitated by the addition of 2.5 volumes of ethanol and NaCl to a final concentration of 0.05 M, collected by centrifugation, and dissolved in 0.002 M sodium citrate buffer, pH 7.0, for spectral analysis. Spectra of the RNA were also obtained in the pH 7.0 buffer and in 88% formic acid after the RNA had been reprecipitated by the addition of ethanol and NaCl. The twice precipitated RNA (8.5 mg) was dissolved in 9 ml of 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.9; 1 mg each of venom phosphodiesterase (Sigma) and bacterial alkaline phosphatase (Sigma, Type III-s) were added, and the solution was incubated at 37°C for 60 hr. The solution was neutralized and evaporated to 3 ml (bath temperature of 40°C) and chromatographed on Whatman No. 3MM paper in the butanol-acetic acid-water system. After the paper was dry, the major yellow band (Rf of 0.85) was eluted with ethanol; minor yellow bands with Rf's of 0.3 and 0.27 were not studied.

Calf thymus DNA (Sigma) was dissolved (0.25 mg/ml) in 0.001 M sodium citrate buffer, pH 8, denatured by heating at 100°C for 10 min, and then rapidly cooled. This sample and another sample which had not been heated were titrated to pH 7 with citric acid, and 4.5-mg portions (18 ml) of the native or denatured DNA were combined with 4.5 ml of ethanol (alone or containing 0.08 mmole of N-benzoyloxy-MAB or MAB), and incubated at 37°C in a nitrogen atmosphere. After extraction of the excess dye 3 times with equal volumes of ethyl ether, the DNA was precipitated by the addition of 2.5 volumes of ethanol and NaCl to a final concentration of 0.05 M. After standing overnight at 3°C the DNA was collected by centrifugation, washed once with ethanol, and redissolved in 0.001 M sodium citrate buffer, pH 7.0. After spectrophotometric estimation, the DNA was reprecipitated by addition of ethanol and NaCl, collected, and again dissolved in pH 7 buffer for spectral study. A spectrum was obtained of an aliquot which was evaporated to dryness in vacuo and dissolved in 88% formic acid.

RESULTS

Carcinogenicity and Toxicity of N-Benzoyloxy-MAB

Subcutaneous Injection Experiments. When administered by repeated s.c. injections, N-benzoyloxy-MAB (total dose of 94 mg) induced sarcomas at the injection site in all of the rats which survived for 4 months or longer. At 9 months, the incidence of sarcoma-bearing rats was 55% in Experiment 1 and 100% in Experiment 2 (Table 1). By contrast, only 1 sarcoma developed at the site of s.c. injections of an equimolar amount of DAB-N-oxide, and no tumors developed at the sites of injection of MAB, N-benzoyl-MAB, N-hydroxy-AB, AB, DAB, benzoyl peroxide, or the solvent (trioctanoin) alone by the termination of Experiments 1 and 2 at 20 and 14 months, respectively. Few tumors were found at sites distant from the injections. Of the rats injected with MAB in Experiment 1, 2 had carcinomas of the liver and 1 had a carcinoma of the earduct gland when the surviving rats were killed at 20 months. One rat injected with DAB-N-oxide developed an adenocarcinoma of the small intestine.

As a positive control, each experiment included a group of rats which received 16 or 18 injections of N-hydroxy-AAF (total dose of 48 or 54 mg). In accordance with previous experience (39), moderate numbers of sarcomas developed at the injection site, but, because some of the animals died with tumors at other sites (especially the mammary and earduct glands), the final incidences of sarcomas were lower than when N-benzoyloxy-MAB was injected.

Intraperitoneal Injections into Neonatal Rats. Groups of approximately 150 rats received i.p. injections of MAB in oil solution (corn oil or trioctanoin) or the solvents alone during the 1st 3 or 4 days of life, and 200 rats received similar injections of N-benzoyloxy-MAB. In the 1st experiment, the compounds were injected in corn oil on each of the 1st 4 days; this schedule permitted a survival at weaning of 64-75% of the rats that received MAB or corn oil, but only 24% of the rats treated with N-benzoyloxy-MAB (total dose of 1 mg). Accordingly, in Experiment 2 the rats were injected only on each of the 1st 3 days of life (total dose of N-benzoyloxy-MAB of 0.48 mg). Under these conditions, 60-72% of the rats in each of the 3 groups survived to weaning. Of the rats weaned in both experiments, 64, 90, and 98% of those injected with N-benzoyloxy-MAB, MAB, or the solvents alone were alive at 13 months and 20, 68, and 75%, respectively, survived until the experiments were terminated at 19 months (Table 2).

Of the rats injected with N-benzoyloxy-MAB, 3 died with embryonal renal carcinomas (bilateral in 2 cases) at 7-12 months, 1 had a carcinoma of the earduct glands, and 2 developed sarcomas at or near the injection site. No tumors of these types were found in the rats which received injections of MAB or the solvents alone. In each of the groups, mammary tumors developed in some female rats which survived more than 1 year, and a few other tumors were also noted (Table 2).

The majority of the deaths in the rats treated with N-benzoyloxy-MAB were associated with renal damage which was diagnosed histologically as a severe glomerulonephritis. Calcification of the aorta and thrombus formation in the atrium of the heart were also observed in the old rats. Several rats died as a result of abdominal hemorrhage which was secondary to an engorgement and rupture of blood vessels in the mesentry. While each of these lesions was seen in a few rats that received MAB or the vehicles alone, they were much more frequent in the rats treated with N-benzoyloxy-MAB. Many of the rats treated with N-benzoyloxy-MAB also showed some hepatic damage, especially when death occurred within the 1st few months after treatment. Microscopically, these lesions included slight to severe fibrosis with or without septal formation, areas of cholangiobrosis, bile duct proliferation, fatty metamorphosis, and regenerating nodules. In a few cases the hepatic changes were compatible histologically with hepatic carcinoma; however, gross malignant liver tumors were not found. Two cholangiommas (less than 0.5 mm in diameter) were noted. The major pathologic change noted in the livers of the N-benzoyloxy-MAB-treated rats which survived more than 1 year was congestion, which was apparently secondary to circulatory insufficiency. The livers of the rats treated with MAB were normal in gross appearance; 1 small cholangioma was found in this group.

Reactions of N-Benzoyloxy-MAB in Vivo and in Vitro

Proteins and Amino Acids. In preliminary experiments, i.p. injections of trioctanoin suspensions of N-benzoyloxy-MAB
TABLE 1
Carcinogenic Activities of \( \text{N-Benzoyloxy-}, N, N'-\text{dimethyl-4-aminoazobenzene and Related Compounds Administered by Repeated s.c. Injections in Rats} \)

Each rat was injected s.c. in the right hind leg twice weekly with 0.2 ml of trioctanoin in which the test compound had been dissolved or suspended without heat immediately prior to injection.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>No. of rats and sex</th>
<th>Average weight gain lst 8 weeks (gm)</th>
<th>Cumulative No. of rats with sarcomas at injection site</th>
<th>No. of rats with other tumors</th>
<th>Negative survivors*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 mo.</td>
<td>9 mo.</td>
<td>12 mo.</td>
<td>14-20 mo.</td>
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<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>( N)-Benzoyloxy-MAB(^\text{a})</td>
<td>24 × 3.9 mg</td>
<td>10 M</td>
<td>62</td>
<td>2</td>
<td>6</td>
<td>9</td>
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<tr>
<td></td>
<td>24 × 2.5 mg</td>
<td>10 M</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td></td>
<td></td>
<td>10 F</td>
<td>48</td>
<td>2</td>
<td>5</td>
<td>10</td>
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<tr>
<td>( N)-Hydroxy-2-acetylaminofluorene</td>
<td>16 × 3.0 mg</td>
<td>10 M</td>
<td>26</td>
<td>0</td>
<td>3</td>
<td>5</td>
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<td></td>
<td></td>
<td>10 F</td>
<td>27</td>
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<td>4</td>
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<tr>
<td>None (vehicle only)</td>
<td>24 × 0.2 ml</td>
<td>10 M</td>
<td>69</td>
<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td></td>
<td>10 F</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Experiment 2</td>
<td></td>
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<tr>
<td>( N)-Benzoyloxy-MAB(^\text{a})</td>
<td>24 × 3.9 mg</td>
<td>20 M</td>
<td>159</td>
<td>9</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>24 × 2.5 mg</td>
<td>20 M</td>
<td>158</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( N)-Benzoyl-MAB</td>
<td>24 × 3.7 mg</td>
<td>20 M</td>
<td>162</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( N)-Hydroxy-AB</td>
<td>24 × 2.5 mg</td>
<td>20 M</td>
<td>167</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>( N)-Hydroxy-AB</td>
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<td>0</td>
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<tr>
<td>DAB-N-oxide</td>
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<td>20 M</td>
<td>166</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DAB</td>
<td>24 × 2.7 mg</td>
<td>20 M</td>
<td>156</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Benzoylperoxide</td>
<td>24 × 2.9 mg</td>
<td>20 M</td>
<td>182</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( N)-Hydroxy-2-acetylamino fluorene</td>
<td>18 × 3.0 mg</td>
<td>20 M</td>
<td>164</td>
<td>8</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (vehicle only)</td>
<td>24 × 0.2 ml</td>
<td>20 M</td>
<td>164</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Rats killed tumor-free at the termination of Experiments 1 and 2 at 20 and 14 months, respectively.

The abbreviations used are: AB, 4-aminoazobenzene; MAB, \( N\)-methyl-4-aminoazobenzene; DAB, \( N\),\( N\)-dimethyl-4-aminoazobenzene.


### Table 2

Survival of Rats and Occurrence of Tumors after i.p. Injections of N-Methyl-4-aminoazobenzene (MAB) or Its N-Benzoyloxy Derivative into Neonatal Rats

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total dose (mg)</th>
<th>No. injected</th>
<th>No. alive at 23 days</th>
<th>No. weaned and sex</th>
<th>No. of survivors at 7 mo.</th>
<th>No. of survivors at 10 mo.</th>
<th>No. of survivors at 13 mo.</th>
<th>No. of survivors at 19 mo.</th>
<th>No. of rats with gross tumors by 19 mo.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Benzoyloxy-MAB</td>
<td>1.00</td>
<td>46</td>
<td>11</td>
<td>7 M</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1, bilateral renal carcinomas (7 mo.) 1, multiple papillomas (urinary bladder) (19 mo.) 1, bilateral renal carcinomas (12 mo.) 1, mammary gland carcinoma (19 mo.)</td>
</tr>
<tr>
<td>Corn oil only</td>
<td></td>
<td>33</td>
<td>25</td>
<td>8 M&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>2, mammary gland adenomas (19 mo.) 1, carcinoma in situ (skin) (19 mo.) 1, pulmonary adenoma (17 mo.)</td>
</tr>
<tr>
<td>Experiment 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>162</td>
<td>98</td>
<td>56 M</td>
<td>55</td>
<td>48</td>
<td>28</td>
<td>8</td>
<td>3, pancreatic adenomas (13-15 mo.) 2, sarcomas (injection site) (13 and 14 mo.) 1, renal carcinoma (7 mo.) 1, basal cell carcinoma of lip (19 mo.) 2, mammary gland adenomas (19 mo.) 1, carcinoma of ear-duct gland (13 mo.) 1, cholangioma (19 mo.) 1, leiomyoma (small intestine) (19 mo.) 2, cutaneous papillomas (19 mo.) 1, malignant lymphoma (14 mo.) 6, mammary gland adenomas (17-19 mo.) 1, mammary gland carcinoma (15 mo.) 1, cholangioma (17 mo.) 1, sarcoma (foot) (17 mo.) 1, cutaneous papilloma (19 mo.) 1, mammary gland carcinoma (8 mo.) 7, mammary gland fibroadenomas (15-19 mo.)</td>
</tr>
<tr>
<td>MAB</td>
<td>0.65</td>
<td>47</td>
<td>30</td>
<td>8 M&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Corn oil only</td>
<td></td>
<td>33</td>
<td>25</td>
<td>8 M&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Trioctanoin only</td>
<td>0.48</td>
<td>162</td>
<td>98</td>
<td>56 M</td>
<td>55</td>
<td>48</td>
<td>28</td>
<td>8</td>
<td>3, pancreatic adenomas (13-15 mo.) 2, sarcomas (injection site) (13 and 14 mo.) 1, renal carcinoma (7 mo.) 1, basal cell carcinoma of lip (19 mo.) 2, mammary gland adenomas (19 mo.) 1, carcinoma of ear-duct gland (13 mo.) 1, cholangioma (19 mo.) 1, leiomyoma (small intestine) (19 mo.) 2, cutaneous papillomas (19 mo.) 1, malignant lymphoma (14 mo.) 6, mammary gland adenomas (17-19 mo.) 1, mammary gland carcinoma (15 mo.) 1, cholangioma (17 mo.) 1, sarcoma (foot) (17 mo.) 1, cutaneous papilloma (19 mo.) 1, mammary gland carcinoma (8 mo.) 7, mammary gland fibroadenomas (15-19 mo.)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each rat was injected i.p. with 0.05 ml of sterile corn oil alone or containing 0.2 mg of N-benzoyloxy-MAB or 0.13 mg of MAB within 24 hr after birth and on each of the succeeding 2 days; on the 4th day the rats were injected with 0.1 ml of the same solutions.

<sup>b</sup> Because of the poor survival of the rats which received injections of N-benzoyloxy-MAB in this preliminary experiment, only 8 male and 8 female rats of the 30 and 25 rats injected with MAB or corn oil were kept at weaning.

Each rat was injected i.p. with 0.05 ml of sterile trioctanoin alone or containing 0.16 mg of N-benzoyloxy-MAB or 0.10 mg of MAB within 24 hr after birth and on each of the succeeding 2 days.
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(24 mg/100 gm body weight) into adult male rats resulted 24 hours later in levels of hepatic protein-bound dye similar to those observed after administration of an equimolar amount of MAB. However, while formalin-fixed, 2-mm-thick slices of acetone-washed perfused livers from rats which had been injected i.p. with MAB were evenly pink after a few minutes' immersion in 10% trichloroacetic acid (25), similar slices from the livers of rats treated with N-benzoyloxy-MAB had a deep pink surface rim around a much lighter pink interior. Furthermore, slices of the acetone-washed kidneys of N-benzoyloxy-MAB-injected rats, after formalin fixation and immersion in 10% trichloroacetic acid, similarly showed a thin shell of deep pink; the kidneys of MAB-injected rats did not show this reaction. A red color in acidic media is characteristic of the aminoazo dyes related to MAB, and the much deeper color of the exterior of the organs from the rats injected with N-benzoyloxy-MAB suggested that this compound might be reacting nonenzymatically with tissue constituents with which it came into contact.

In vitro studies showed that N-benzoyloxy-MAB reacted readily at pH 7 and 37°C with bovine serum albumin to yield protein-bound derivatives that could not be extracted with solvents. Hydrolysis of the protein with alkali liberated about 25% of the dye in a hexane-benzene extractable form (nonpolar dye), and the remainder of the dye was extracted from the alkaline solution with ethanolic ethyl ether (polar dye) (Table 3).

### TABLE 3

<table>
<thead>
<tr>
<th>Dye</th>
<th>Amino acid</th>
<th>% reaction at 90 min†</th>
<th>Absorption maximum of water-soluble dye (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Benzoyloxy-MAB</td>
<td>Tryptophan</td>
<td>25</td>
<td>525</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>6</td>
<td>519</td>
</tr>
<tr>
<td></td>
<td>Cysteine</td>
<td>6</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>25</td>
<td>518</td>
</tr>
<tr>
<td></td>
<td>Water-soluble dye</td>
<td>1.4</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>Total 3-methylmercaptopro-MAB released</td>
<td>&lt;0.7</td>
<td></td>
</tr>
<tr>
<td>N-Methyl-4-aminoazo-benzene</td>
<td>Alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxypoline, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, or valine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N,N-Dimethyl-4-aminoazo-benzene</td>
<td>Tryptophan, tyrosine, cysteine, or methionine</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>4-Aminoazo-benzene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N,N-Dimethyl-4-aminoazo-benzene-N-oxide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-4-aminoazo-benzene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-N-acetyl-4-aminoazo-benzene</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bovine serum albumin (125 mg) was incubated at pH 7 with 1.0 mg of N-benzoyloxy-N-methyl-4-aminoazo-benzene or an equivalent amount of another dye for 4 hours at 37°C in a nitrogen atmosphere. The analytic procedure is described in the Materials and Methods section. The analyses for each of the polar and nonpolar fractions were corrected for blank values of 0.02 which were obtained when serum albumin was carried through the same procedure in the absence of any dye.

† The % reaction was calculated on the assumption that the products had the same molar absorption coefficients as MAB. A blank equivalent to a reaction of 1.5% was subtracted from all values based on water-soluble dye when the amino acids were incubated with N-benzoyloxy-MAB. A blank of 0.7% was obtained when MAB was incubated and extracted as described.

‡ The sulfonium derivative formed from methionine gradually decomposes even at neutrality (see Chart 1). Therefore, the amount of 3-methylmercaptopro-MAB formed spontaneously plus that formed after the addition of alkali is a better estimate of the extent of reaction than the amount of water-soluble dye.

No protein-bound derivatives were found after similar incubations of bovine serum albumin with MAB, DAB, AB, DAB-N-oxide, N-hydroxy-AB, or N-hydroxy-AAB.

On incubation of N-benzoyloxy-MAB separately with each of the common amino acids methionine, tryptophan, tyrosine, and cysteine each yielded a water-soluble dye (Table 4). No reaction was detected with any of the other amino acids, but the blank value obtained in the absence of any amino acid would have obscured reactions of less than about 0.7%. No reaction was obtained on incubation of MAB with methionine, tryptophan, tyrosine, or cysteine. Table 4 shows the % reactions obtained at 90-min incubations; the extent of reaction of N-benzoyloxy-MAB with any of the amino acids was not increased when the incubation period was extended to 20 hr.

In other work from this laboratory (16), the dye formed on reaction of N-benzoyloxy-MAB with methionine has been presumptively characterized as 3-(S-methionyl)-MAB, which gradually decomposes even in neutral solution to a dye which

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has been characterized as 3-methylmercapto-MAB and α-aminobutyrolactone (Charts 1, 2). 3-Methylmercapto-MAB is extracted from the reaction mixture with the benzene-hexane mixture used to remove unreacted N-benzoyloxy-MAB and its degradation products. Hence, the values obtained for reaction of N-benzoyloxy-MAB with methionine on the basis of water-soluble dye are minimal values; analyses for 3-methylmercapto-MAB by gas chromatography after addition of the aqueous layer was estimated spectrophotometrically after addition of 1.5 ml of 12 N HCl:ethanol (2:1) ml.

Studies are in progress on the structures of the products formed in vitro from N-benzoyloxy-MAB and protein, cysteine, tryptophan, and tyrosine.

Reactions with Nucleic Acids and Nucleosides. After incubation of RNA or DNA with N-benzoyloxy-N-methyl-4-aminoozobenzene (N-benzoyloxy-MAB) to 3-methylmercapto-MAB. Methionine (300 µmoles), 9 µmoles of N-benzoyloxy-MAB, and 460 µmoles of phosphate buffer, pH 7, in 15 ml of 20% ethanol were incubated 40 min under nitrogen and then extracted immediately 3 times with an equal volume of 15% benzene in hexane. The aqueous phase was then incubated at 37°C, and at the times indicated in the chart 2.5-ml aliquots were extracted 3 times with an equal volume of 15% benzene in hexane. The 3-methylmercapto-MAB was determined in the organic phase by gas chromatography and the polar dye remaining in the aqueous layer was estimated spectrophotometrically after addition of 1.5 ml of 12 N HCl:ethanol (2:1) ml.

To determine with which components of the nucleic acids the N-benzoyloxy-MAB had reacted, 14C-labeled nucleosides were incubated at pH 7 and 37°C with the dye, and the products were chromatographed on cellulose thin-layer plates. With a 6-fold ratio of N-benzoyloxy-MAB to guanosine, about 3% of the radioactivity from guanosine-8-14C was converted to a product which migrated in the butanol:acetic acid:water system with an Rf of about 0.9 (Table 5). Under the same conditions, there was no evidence for reaction of N-benzoyloxy-MAB with radioactive adenosine, uridine, cytidine, or thymidine. Conversion of 0.02% of these nucleosides to a product with a different Rf could have been detected. In each case, these reaction mixtures were chromatographed in systems in which the parent nucleosides migrated with Rf's of less than 0.4 to facilitate the detection of less polar products which would be expected to migrate faster in the systems used.

Comparative studies showed that MAB had no detectable reaction with guanosine-8-14C, while N-hydroxy-AB gave evidence of a slight reaction (Table 5). The chemical nature of the reaction between guanosine and N-benzoyloxy-MAB has received some preliminary investigation. Recent studies from this laboratory showed that N-acetoxy-AAF reacted with guanosine to form N-(guanosine-8-yl)-2-acetylaminofluorene (14). N-Benzoyloxy-MAB apparently does not react in an analogous fashion, since incubation of this dye with either guanosine-8-14C or guanosine-8-3H results in incorporation of equivalent amounts of radioactivity from each guanosine derivative in the product (Table 5). The para aminoazo dyes characteristically show strong absorption at 500-530 µm and 520-532, 330, and 262 µm. These spectra, as well as those of MAB and N-benzoyloxy-MAB for comparison, are shown in Charts 3 and 4. The extent of reaction was about 0.3-0.5 times as great with native as with heat-denatured DNA.

No nucleic acid-bound dye could be detected spectrophotometrically when MAB was incubated with denatured DNA or rRNA under the conditions used for reaction of N-benzoyloxy-MAB with these nucleic acids.

Digestion of the N-benzoyloxy-MAB-reacted RNA with phosphodiesterase and alkaline phosphatase and chromatography of the digest on paper in the butanol:acetic acid:water system yielded 3 bands. The most intensely colored band moved with an Rf of 0.85; elution of this product with ethanol yielded a solution with an absorption maximum at 330 µm (Chart 3). In 88% formic acid, this material had a weak maximum at 515 µm in addition to the 330 µm peak (Chart 4). Below 300 µm the details of the absorption spectra were obscured, probably by impurities extracted from the chromatographic paper. The less prominent bands on the paper chromatogram of the RNA digest had Rf's of 0.39 and 0.27; on the basis of their Rf's these yellow derivatives may be the corresponding dye-nucleotide and dipyridylpolynucleotides released by the enzymes.

To determine the components of the nucleic acids the N-benzoyloxy-MAB had reacted, 14C-labeled nucleosides were incubated at pH 7 and 37°C with the dye, and the products were chromatographed on cellulose thin-layer plates. With a 6-fold ratio of N-benzoyloxy-MAB to guanosine, about 3% of the radioactivity from guanosine-8-14C was converted to a product which migrated in the butanol:acetic acid:water system with an Rf of about 0.9 (Table 5). Under the same conditions, there was no evidence for reaction of N-benzoyloxy-MAB with radioactive adenosine, uridine, cytidine, or thymidine. Conversion of 0.02% of these nucleosides to a product with a different Rf could have been detected. In each case, these reaction mixtures were chromatographed in systems in which the parent nucleosides migrated with Rf's of less than 0.4 to facilitate the detection of less polar products which would be expected to migrate faster in the systems used.

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MAB with guanosine, as compared to the lack of observable reaction with other nucleosides, the reaction with nucleic acids would be expected to involve the guanine bases. A puzzling feature, however, is the fact that in neutral solution the absorption maxima of the dye-containing products from DNA, RNA, and the nucleoside derived from RNA are all at 330 m\(\mu\), while under comparable conditions the product derived from guanosine has a maximum at 375 m\(\mu\) (Chart 3). Further studies on the structures of these products are in progress.

**DISCUSSION**

The present studies with the aminoazo dyes MAB and N-benzoyloxy-MAB clearly parallel the recent studies with AAF in this laboratory. In each case an N-acyloxy derivative, N-benzoyloxy-MAB or N-acetoxy-AAF (10), has induced high incidences of sarcomas at the sites of repeated s.c. injections. The parent compounds, MAB and AAF, are not carcinogenic at sites of repeated injection, although each is hepatocarcinogenic when administered orally and AAF also induces tumors at other sites. N-Hydroxy-AAF, a proximate carcinogenic metabolite of AAF, also produces tumors at sites of repeated injections (3) but appears to be less active than N-acetoxy-AAF (10); N-hydroxy-MAB has not been available for similar comparisons with N-benzoyloxy-MAB. To continue the parallel, each of the N-acyloxy derivatives reacts in vitro at neutrality with cellular nucleophiles, while MAB and AAF (and also N-
N-Benzoyloxy-N-methyl-4-aminoazobenzene

CHART 3. Representative spectra in neutral solvents of the reaction products of native and denatured DNA, sRNA, and guanosine with N-benzoyloxy-N-methyl-4-aminoazobenzene (N-benzoyloxy-MAB). The nucleic acids, their reaction products, and guanosine were dissolved in 0.002 M sodium citrate buffer, pH 7; the nucleoside obtained from the sRNA product and from guanosine were dissolved in ethanol. The spectra of N-benzoyloxy-MAB and MAB in ethanol are given for comparison. Note that the scale for the absorbance of the nucleic acid products has been expanded 5-fold from 300 to 540 μ to show the detail.

hydroxy-AAF) are not reactive in vitro in these systems. The nucleophiles attacked by N-benzoyloxy-MAB and N-acetoxy-AAF (14–16, 19, 47) are the same, viz., guanosine, methionine, cysteine, tryptophan, and tyrosine as well as DNA, RNA, and protein. In each case the reaction products with methionine in vitro have been characterized as the 3-methylmercapto derivatives (16). 3-Methylmercapto-MAB and 3-methylmercapto-AAF have also each been identified as alkaline degradation products of liver protein from rats administered MAB or AAF (10, 43). This formation of sulfonium precursors of 3-methylmercapto-MAB and 3-methylmercapto-AAF in vitro thus provides evidence that MAB and AAF are metabolized in the rat to esters or related compounds with the reactive properties of N-benzoyloxy-MAB and N-acetoxy-AAF. These reactive metabolites may include the sulfate, phosphate, and carboxylic acid esters and the O-glucuronides of N-hydroxy-MAB and N-hydroxy-AAF (15, 32). Thus, these ester or ester-like derivatives (or the aminonium or amidonium cations derived therefrom; see...
Chart 4. Representative spectra in 88% formic acid of the reaction products of native and denatured DNA, sRNA, and guanosine with N-benzoyloxy-N-methyl-4-aminoazobenzene (N-benzoyloxy-MAB). The spectra of N-benzoyloxy-MAB and MAB are given for comparison. Note that the scale for the absorbance of the nucleic acid products has been doubled from 300 to 540 mμ to show the detail.

Chart 2) are logical candidates for the ultimate carcinogenic forms (22) of MAB and AAF.

It appears probable at present that the metabolic activation of many and possibly of all carcinogenic aromatic amines and amides consists of the 2 steps of N-hydroxylation and N-hydroxy esterification. Similarly, the reaction of these ultimate carcinogenic metabolites with nucleophilic components of proteins and nucleic acids may account for the known binding of MAB and AAF to these biopolymers in vivo (see below). The subsequent mechanism of carcinogenesis initiated by these bindings could then consist of either genetic or epigenetic processes or combinations thereof (22, 31). These processes may conceivably include mutations, activation of viruses, quasipermanent repressions and derepressions of a normal genome, and alterations of immunologic responses. These are among the more prominent hypotheses of the mechanisms of loss of growth control in carcinogenic processes.

The reactions of guanosine, RNA, and DNA with N-benzoyloxy-MAB at neutrality in vitro to form covalently bound derivatives may be useful guides in the determination of the structures...
It is hoped that the reactions between \( N \)-benzoyloxy-MAB and methionine (Ref. 16, and this paper), cysteine, tryptophan, tyrosine, and protein in \( \textit{vivo} \) at neutrality will contribute to the solution of the structure of the protein-bound dyes formed in \( \textit{vivo} \) in the livers of rats fed aminoazo dyes (21, 29, 46). The observation of Andersen (1, 30) in our laboratory that the partially purified polar dyes from hepatic protein-bound dye contained 1–2 sulfur atoms/dye molecule was the first evidence for the involvement of a sulfur-containing amino acid. A definitive role of methionine was shown by the release of 3-methylmercapto-MAB from alkaline digests of liver protein from rats fed DAB or MAB (43). Subsequently, Higashimakagawa et al. (12) have shown that two of the polar dyes isolated from the liver proteins of rats fed \( 3' \)-methyl-DAB contain \( ^{35} \)S from \( ^{38} \)S-labeled methionine. The role of other amino acids, especially cysteine, tryptophan, and tyrosine, in the protein-binding of the dyes in \( \textit{vivo} \) has not been ascertained. The structures of the products of reaction of the latter 3 amino acids with \( N \)-benzoyloxy-MAB are under investigation.

The metabolism, reactivity, and carcinogenicity of DAB-\( N \)-oxide have received study (44, 45, 48) in the examination of this derivative as a possible proximate carcinogenic metabolite of DAB. However, even if the \( N \)-oxide should prove to be a metabolite of DAB, it is unlikely that the \( N \)-oxide could play a role in the carcinogenic activity of MAB. Thus, while MAB is a metabolite of DAB (33) and has carcinogenic activity equal to that of DAB (18), there is now no evidence that MAB is methylated to DAB in \( \textit{vivo} \). Recent data from our laboratory have shown that the “DAB” thought to be present in the livers of rats fed MAB (26, 33) is really 3-methylmercapto-MAB (43). These data are consistent with the other observations discussed above that point to an ester of \( N \)-hydroxy-MAB as the ultimate carcinogen.

The reason for the requirement of a \( N \)-methyl group for strong carcinogenicity with most aminoaazo dyes related to MAB (3, 6, 20) remains obscure. Even in those cases where a strong potentiating group elsewhere in the molecule (e.g., a \( 4' \)-ethyl group) has permitted carcinogenic activity with \( N \)-ethyl derivatives, the corresponding \( N \)-methyl dyes have proved to be considerably more active (3, 6). In a 2-step enzymatic activation mechanism of \( N \)-hydroxylation and \( N \)-hydroxy esterification, a \( N \)-methyl group may permit a better steric fit with the enzymes involved than do other \( N \)-alkyl groups. Tests of this and alternative possibilities in \( \textit{vivo} \) and in \( \textit{vivo} \) will be possible when syntheses of \( N \)-hydroxy-MAB, of various esters of this hydroxylamine, and of related compounds with other \( N \)-alkyl groups are developed.

**ACKNOWLEDGMENTS**

The authors are grateful to Dr. John Scribner of the McArdle Laboratory for interpretations of the infrared and nuclear magnetic resonance spectra. The latter spectrum was carried out by the Department of Chemistry, University of Wisconsin. The authors are appreciative of the expert technical assistance of Mrs. Barbara Butler, Mrs. Judith Sorenson, and Mrs. Cornelia Whelan.

**REFERENCES**

1. Andersen, R. A. Tumor Inhibition and Carcinogenesis by the Metabolite \( N \)-Hydroxy-4-acetylaminostilbene and Charac-

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Dye</th>
<th>Nucleoside</th>
<th>Radioactive product % (based on limiting reactant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( N )-Benzoyloxy-MAB (MAB)</td>
<td>Guanosine-8-(^{14} )C</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>( N )-Hydroxy-4-aminoazobenzene</td>
<td>Guanosine-8-(^{14} )C, Guanosine-8-(^{14} )C</td>
<td>0.0, 0.1</td>
</tr>
<tr>
<td>2</td>
<td>( N )-Benzoyloxy-MAB (MAB)</td>
<td>Guanosine-8-(^{14} )C</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenosine-8-(^{14} )C</td>
<td>0.0</td>
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<tr>
<td>3</td>
<td>( N )-Benzoyloxy-MAB (MAB)</td>
<td>Guanosine-8-(^{14} )C</td>
<td>0.0</td>
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<tr>
<td>4</td>
<td>( N )-Benzoyloxy-MAB (MAB)</td>
<td>Guanosine-8(^{14} )H</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guanosine-8(^{14} )C</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guanosine-8(^{14} )H</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* The ingredients listed below in 1.4 ml of 28% ethanol were incubated under nitrogen at 37°C for the times indicated: Experiment 1, 0.02 \( \mu \) mole of guanosine, 0.5 \( \mu \) mole of dye, and 2 \( \mu \) moles of sodium citrate buffer, pH 7, for 3.5 hours; Experiment 2, 0.08 \( \mu \) mole of nucleoside, 0.5 \( \mu \) mole of dye, and 2 \( \mu \) moles of sodium citrate buffer, pH 7, for 3 hours; Experiments 3 and 4, 1.7 \( \mu \) moles of dye.

The minimum reactions which could be detected were about 0.05 and 0.02% for Experiments 1 and 2, respectively.
Lionel A. Poirier, James A. Miller, Elizabeth C. Miller, and Kei Sato


Cancer Research

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