Chemically Induced Binding of the Hepatocarcinogen
$N$-Monomethyl-4-aminoazobenzene to Nucleic Acids in Vitro$^1$

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SUMMARY

Chemical reactions between the hepatocarcinogen $N$-methyl-4-aminoazobenzene (MAB) and nucleic acids were carried out in neutral aqueous solution and at room temperature ($25^\circ$) in the presence of potassium persulfate or iodine. The data presented are consistent with the formation of a covalent linkage between MAB and DNA or RNA under these mild conditions. The covalently linked MAB cannot be extracted by organic solvents that remove the physically bound MAB from the nucleic acid. A chemical linkage was further indicated by gel filtration and equilibrium dialysis of the nucleic acid:MAB complexes. Hydrolysis of the persulfate-induced DNA- or RNA-bound dye with deoxyribonuclease or alkaline phosphodiesterase, and alkaline phosphatase yields various nucleoside-bound dyes. The reaction of persulfate provides a new model system for a study of the activation mechanism of MAB in vitro.

INTRODUCTION

Most chemical carcinogens form macromolecule-bound derivatives in vivo that may play important roles in the process of carcinogenesis by these compounds (23, 24). The macromolecules involved in this binding are tissue nucleophiles such as DNA, RNA, or protein. The carcinogenic aromatic amines and amides require metabolic activation for this reactivity in vivo. A major activation step appears to be $N$-hydroxylation, and Miller and Miller (22, 25) suggest that the ultimate activation step is $N$-hydroxy esterification. Evidence that $N$-hydroxylation is the initial activation step for the carcinogen 2-acetylaminofluorene was first obtained in 1960 by Cramer et al. (6), and the idea has been supported by recent studies on a variety of carcinogenic aromatic amines and amides (25).

$N$-Benzoyloxy-MAB, but not MAB, reacts non-enzymatically in vitro at neutral pH with protein, RNA, and DNA (29). These observations led to the suggestion that, in rat liver, the metabolically active form of MAB is probably an ester of $N$-hydroxy-MAB. This possibility was indicated by studies of the characterization of the protein-bound [Lin et al. (16–18)] and nucleic acid-bound dyes [Miller et al. (24)] in the livers of rats fed MAB. However, other electrophilic metabolites of the azo dye carcinogens such as free radicals and aminonium ions (9, 24) might also be active forms in vivo.

Complexes of azo dye covalently bound with RNA, DNA, and protein were found in the livers of rats fed DAB (8, 30, 34) and MAB (24). DAB was demethylated to MAB and then to AB (27) in rat liver in vivo. DAB and MAB are equally active in inducing liver tumors in the rat, whereas AB is inactive (21). It is likely that MAB is the key compound for the metabolic activation of azo dye carcinogen. The reactive forms of MAB in the target tissue could be an ester of $N$-hydroxy-MAB, of the free radical, or of an aminonium ion of MAB. In the work described below, attempts were made to find chemical agents that would mimic the activation of MAB in vivo in the binding of this azo dye carcinogen to nucleic acid and protein. Among many chemical agents tested, only potassium persulfate and iodine induced covalent binding of MAB to DNA and RNA in vitro. The preparation, purification, and partial characterization of the covalently bound nucleic acid:MAB complexes induced by both iodine and persulfate are the basis of this report.

MATERIALS AND METHODS

Materials. Highly polymerized calf thymus DNA and salmon sperm DNA, yeast soluble RNA (Sigma, type III), calf liver-soluble RNA (Sigma, type IV), yeast RNA (Sigma, type XI), guanosine, and deoxyguanosine were purchased from Sigma Chemical Co., St. Louis, Mo. Aniline-U$^{14}$C hydrochloride was obtained from New England Nuclear, Boston, Mass., and alkaline phosphatase and DNase were the products of Nutritional Biochemicals Corp., Cleveland, Ohio.

MAB (m.p., 87–88°) was synthesized according to the procedure of Ishikawa et al. (12). $N$-Benzoyloxy-MAB (m.p., 89–91°) was synthesized according to the methods of Poirier et al. (29). MAB-U$^{14}$C (radioisotopic activity, 0.28 mCi/m mole) was prepared from aniline-U$^{14}$C-hydrochloride and $N$-methyl-4-nitrosoacetanilide as described by Lin and Miller (15).

Rat liver rRNA and DNA were isolated by the method of Irving and Veazey (10).

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$^2$The abbreviations used are: MAB, $N$-monomethyl-4-aminoazobenzene; DAB, $N,N$-dimethyl-4-aminoazobenzene; AB, 4-aminoazobenzene; TLC, thin-layer chromatography.

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$^3$In a $^{14}$C-labeled compound, U indicates that the compound is uniformly labeled.
General Procedure for Preparation of Nucleic Acid:MAB Complexes. The indicated amounts of MAB and nucleic acid (RNA or DNA) (Table 1) were added to a solution (5 to 30 ml) of 0.05 M sodium phosphate buffer (pH 7.0) and 33% ethanol. Then a calculated amount of iodine or potassium persulfate was introduced. The reaction mixture was incubated in the dark at room temperature (25°) for 12 hr or for the time indicated. The nucleic acid was precipitated with 2 volumes of 95% ethanol and sodium chloride at a final concentration of 0.05 M. The precipitate (70 to 80% recovery) was washed 4 times with ether or absolute ethanol and then redissolved in 0.05 M phosphate buffer (pH 7.0). The process was repeated 4 times, and the final nucleic acid precipitate was dissolved in 0.05 M sodium phosphate or 80% formic acid for spectral analysis. The absorbances at 250 and 360 nm in neutral solution were used to estimate the extent of binding of MAB to nucleic acids. The molecular extinction coefficient of nucleic acid bound-MAB at 350 nm in neutral medium is 1.2 X 10^4, while the absorbances of DNA and RNA at 260 nm are approximately E^0.1% (in phosphate buffer, pH 7.0) = 185, and E^0.1% (in H_2O) = 200, respectively (10). The bindings were expressed as μmoles of MAB per g of nucleic acid. Nucleic acid:MAB-14C complexes were obtained when MAB-U-14C was used as a reactant.

Formation of Guanosine:MAB Complexes Induced by Potassium Persulfate. MAB (38 mg, 0.18 mmole) was dissolved in 62 ml 95% ethanol and mixed with 250 ml 0.05 M sodium phosphate buffer, pH 7.0, containing 400 mg (0.35 mmole) of guanosine. The solution was mixed with 9 ml of 0.15 M potassium persulfate and incubated at room temperature (25°) for 12 hr. The major protein of nonpolar dye was removed by 1-butanol (150 ml each extraction) and dried in a vacuum. The residues were dissolved in 20 ml of acetone and passed through a column (2.1 x 25 cm) of silica gel (Wako-Gel B-5; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) previously equilibrated with acetone. The column was washed with acetone until the eluate contained no yellow color. The MAB:guanosine complex adsorbed at the top of the column was then eluted with 300 ml of methanol and concentrated in a vacuum in a rotatory evaporator at 60°. The products were subjected to TLC analyses.

Syntheses of Guanosinyl-MAB and Deoxyguanosinyl-MAB. Guanosinyl-MAB was prepared from N-benzoyloxy-MAB and guanosine by the method of Miller et al. (24). Ten mg of N-benzoyloxy-MAB were dissolved in 10 ml of methanol and mixed with a solution of 20 ml 0.05 M sodium phosphate buffer, pH 7.0, containing 10 mg of guanosine. The mixture was incubated at room temperature for 20 hr and, after removal of the methanol, the polar product was extracted into 1-butanol and purified by silica gel column chromatography as described above. The guanosinyl-MAB product was further purified with TLC on cellulose. The chromatogram was developed with the aqueous phase of 1-propanol:1-butanol:water (1:4:5, v/v/v). The R_f of the guanosinyl-MAB was about 0.37.

Deoxyguanosinyl-MAB (R_f = 0.44) was obtained similarly from deoxyguanosine.

Spectrophotometric Method. The electronic absorption spectra of samples were taken with a Beckman DK-2A or Beckman DU spectrophotometer in cells with a 1-cm optical path.

Gel Filtration Chromatography. The purified nucleic acid:MAB-14C complex was dissolved in 0.01 M phosphate buffer, pH 7.0, and 1 ml of the solution was applied to a Sephadex G-200 column (1.5 x 33 cm) that had been equilibrated previously with 0.01 M sodium phosphate buffer.

Table 1

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Concentration of nucleic acid (mg/ml)</th>
<th>Concentration of MAB (mM)</th>
<th>Inducer</th>
<th>Concentration of inducer (mM)</th>
<th>Bindinga (μmoles MAB/g nucleic acid)</th>
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</thead>
<tbody>
<tr>
<td>Calf liver tRNA</td>
<td>0.83</td>
<td>0.83</td>
<td>Iodine</td>
<td>4.2</td>
<td>0.8</td>
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<tr>
<td>Calf liver tRNA</td>
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<td>0.83</td>
<td>Persulfate</td>
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<td>5.9</td>
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<tr>
<td>Yeast tRNA</td>
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<td>0.83</td>
<td>Iodine</td>
<td>4.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Yeast RNA</td>
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<td>0.83</td>
<td>Persulfate</td>
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<tr>
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<td>0.83</td>
<td>Iodine</td>
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<td>23.4</td>
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<td>0.25</td>
<td>Persulfate</td>
<td>4.0</td>
<td>19.6</td>
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<tr>
<td>Salmon sperm DNA</td>
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<td>0.75</td>
<td>Iodine</td>
<td>6.0</td>
<td>4.5</td>
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<tr>
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<td>Persulfate</td>
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<td>19.6</td>
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<td>Rat liver tRNA</td>
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<td>0.83</td>
<td>Iodine</td>
<td>4.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Rat liver tRNA</td>
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<td>0.83</td>
<td>Persulfate</td>
<td>4.0</td>
<td>88.0</td>
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<tr>
<td>Rat liver DNA</td>
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<td>0.79</td>
<td>Iodine</td>
<td>3.8</td>
<td>13.1</td>
</tr>
<tr>
<td>Rat liver DNA</td>
<td>0.16</td>
<td>0.79</td>
<td>Persulfate</td>
<td>7.6</td>
<td>12.1</td>
</tr>
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</table>

The amount of MAB in rat liver tRNA and DNA preparations was estimated from the molecular extinction coefficient at 350 nm; we found this value to be 1.2 X 10^4 by estimating from the purified rat liver DNA-bound MAB-14C. The amount of nucleic acid was determined from the absorbance at 260 nm. The same constant was used in estimating the amount of MAB bound to other nucleic acid preparations.
pH 7.0. The column was eluted with the same buffer solution at a flow rate of 1 ml/5 min; 3-ml fractions were collected. The absorbances at 260 nm were determined, and the radioactivities were measured in a Packard liquid scintillation counter.

Equilibrium Dialysis. The nucleic acid:MAB complexes were dialyzed in a cellophane bag against 0.01 M sodium phosphate buffer (pH 7.0) containing 10% ethanol at 4° for 3 days. The solution outside the bag was replaced twice daily. The absorbances of aliquots of the solution inside the bag were determined daily at 260 and 350 nm. The quantities of nucleic acids and dye were calculated from these absorbances.

Enzymatic Hydrolysis. The yeast RNA:MAB complex (24 mg) was incubated in 15 ml of 0.03 N KOH at room temperature (25°) for 48 hr and then neutralized with 6 N HClO4. The KClO4 precipitate was removed by filtration and washed with 10 ml of water. The filtrates and washings were combined and buffered to pH 9.0 with 240 mg of Trizma base (Tris, from Sigma). The mixture was incubated at 37° for 72 hr with 6 mg of alkaline phosphatase. The nucleoside-dye derivatives were recovered by extraction with 1-butanol, washed with water, and dried in a vacuum at 60°. The residue was dissolved in a small amount of methanol and subjected to TLC analysis.

The DNA:MAB complex (15 mg) was dissolved in 20 ml of water, and the solution was made 0.01 M with respect to MgSO4. The mixture was incubated at 37° with 2 mg of DNase for 4 hr. The pH of the mixture was then adjusted to 8.7 by the addition of 60 mg of Trizma base; 3 mg of phosphodiesterase (Sigma) from Crotalus adamanteus, type II, and 1.0 mg of alkaline phosphatase were added. After incubation at 37° for 72 hr, the mixture was extracted with 1-butanol. The combined extracts were washed with water and dried in a vacuum at 40°, and the residue was dissolved in 0.5 ml of methanol for TLC.

TLC. Cellulose plates were prepared from Camag D-O cellulose powder, and silica gel plates were prepared on glass from Wako-Gel B-5. Silica gel plates on plastic film were purchased from Eastman Organic Chemicals, Rochester N. Y. (Chromagram Sheet 6061). The following solvent systems were used: Solvent A, aqueous phase of 1-propanol:1-butanol:H2O (1:4:5, v/v/v); Solvent B, methanol:benzene (3:7, v/v); Solvent C, Solvent A:glacial acetic acid (25:1, v/v); Solvent D, Solvent A:concentrated NH4OH (100:1, v/v); and Solvent E, acetone:1-butanol:H2O (1:5:10, v/v/v, aqueous phase).

RESULTS

Chemical Systems That Were Examined for Induction of Binding. In order to establish some model systems for chemical activation of MAB, we examined the following chemical systems: hydrogen peroxide:ferrous ion (Fenton's reagent) (19); iodine (13, 14); the ascorbic acid model hydroxylating system (32); hydrogen peroxide (57 mM); sodium sulfate (0.9 mM); m-chloroperbenzoic acid (4 mM); sodium perborate (13 mM); potassium persulfate; sodium periodate (10 mM); and sodium perchlorate (10 mM).

For a satisfactory model system, it is essential that the binding product be formed under mild conditions, i.e., at neutral pH, at room temperature, and at a low concentration of inducer. Only potassium persulfate and iodine induced a detectable amount of MAB bound to nucleic acids under these conditions.

Formation of Nucleic Acid:MAB Complexes. After yeast RNA was incubated with MAB in the presence of the inducer, iodine or potassium persulfate, the yellow nucleic acid was isolated and purified as described in "Materials and Methods." The purified product had a broad absorption band, between 300 and 400 nm, in neutral solution. This absorption band was diminished and a new band around 530 nm appeared when the product was dissolved in 80% formic acid (Chart 1). The spectrum of the yeast RNA:MAB complex (Chart 1, IV) produced by the reaction of nucleic acid with N-benzoyloxy-MAB (29) is shown for comparison. The spectra of the RNA-bound dyes produced with either MAB and iodine (Chart 1, I), MAB and potassium persulfate (Chart 1, II), or N-benzoyloxy-MAB are similar. A representative spectrum of the DNA:MAB complex induced by persulfate is also given in Chart 1, III. No nucleic acid-bound dye could be detected spectrophotometrically when the inducer, iodine or persulfate, was omitted from the reaction mixture. Similar spectra were observed for different DNA:MAB and RNA:MAB complexes. However, the extent of binding varied greatly among different nucleic acid preparations, as shown in Table 1. In the iodine system, yeast RNA was bound to MAB to the greatest extent, while yeast rRNA was bound less. In the persulfate system, rat liver rRNA, yeast RNA, rat liver DNA, calf thymus DNA, and salmon sperm DNA all were relatively susceptible to attack by MAB.

Chemical Nature of the Nucleic Acid:MAB Complex. In order to elucidate the nature of binding between the nucleic acids and MAB in the complexes, we subjected the purified products to gel filtration chromatography and equilibrium dialysis.

Gel Filtration Chromatography on Sephadex G-200. The purified rat liver DNA:MAB-14C complex and rat liver RNA:MAB-14C complex induced by iodine or potassium persulfate were analyzed on columns of Sephadex G-200 as described in "Materials and Methods." The results were presented in Chart 2. The radioactivities and nucleic acids were eluted together from the column with the void volume. Trace amounts of yellow nonpolar dye were retained on the top of the columns. When the radioactive nucleic acid:MAB complex was subjected to repeated gel filtration chromatography, the radioactivity remained intimately associated with the nucleic acid. This would have been unlikely had the radioactivity been associated with nucleic acid by simple adsorption or other weak physical binding. This also appeared to be impossible because the radioactivity was still associated with the nucleic acid after repeated benzene, ether, and alcohol extractions.

Equilibrium Dialysis. Table 2 gives the results of equilibrium dialyses of the nucleic acid:MAB complexes. The specific bindings are expressed by component ratios as μmoles of MAB per g nucleic acid. The specific binding of the yeast RNA:MAB complex induced by either persulfate or iodine was fairly constant during the course of dialysis (Experiment 1). When a
Without dialysis, the above-described mixture was incubated at room temperature (25°) for 12 hr and then was purified by DNA:MAB complex, the reason for which is not known. The decrease in specific binding during the 1st day of dialysis suggested either that the MAB moiety in the complex is stable on treatment with 80% formic acid, as shown in Experiment 4A. Afterward, there was a slight increase in specific binding of the complex. The RNA:MAB complexes was induced by alcohol precipitation and ether washing, as described in “Materials and Methods.” The product of a solution of MAB and yeast RNA with a component ratio of 31.4 was subjected to equilibrium dialysis, the free MAB was dialyzed out very rapidly. The mixture of a solution of MAB and yeast RNA with a component ratio of 31.4 was subjected to equilibrium dialysis, the free MAB was dialyzed out very rapidly. The ratio dropped to 0.5 at the end of 3 days of dialysis (Experiment 2). Without dialysis, the above-described mixture was incubated at room temperature (25°) for 12 hr and then was purified by repeated precipitations with alcohol and 3 washings with ether, as described in “Materials and Methods.” The product gave a component ratio of 0.5, which was not significantly altered by prolonged dialysis (Experiment 3). This indicated that the procedure of alcohol precipitation and ether washing very effectively removed the free MAB. The RNA:MAB complex is stable on treatment with 80% formic acid, as shown in Experiment 4B, whereas the DNA:MAB complex is partially decomposed by the same treatment Experiment 4A. The decrease in specific binding during the 1st day of dialysis may be due to the depurination of DNA in this acidic medium. Afterward, there was a slight increase in specific binding of the DNA:MAB complex, the reason for which is not known. The stability of the nucleic acid:MAB complexes in equilibrium dialysis suggested either that the MAB moiety in the complexes is nondialyzable or that it is not in a free state. It is probable that MAB is covalently bound to these nucleic acids.

### Kinetic Properties of Persulfate and Iodine Systems

The relationship between the concentration of inducer and the formation of calf thymus DNA:MAB complex is shown in Chart 3. There is a large increase in the amount of calf thymus DNA:MAB complex when the concentration of potassium persulfate is raised from 0 to 1.0 mM. This is followed by a small increase as the concentration of the inducer rises from 1.0 to 3.0 mM, after which the amount of the complex gradually reaches a plateau. It is obvious that the kinetics of iodine-induced calf thymus DNA:MAB complex is different from that of the potassium persulfate system. A slow increase in the formation of the complex occurred when the concentration of iodine was increased from 0 to 2.0 mM, after which there was a large increase when the iodine concentration in the reaction mixture was higher than 2.0 mM. Chart 4 shows the rate of formation of the yeast RNA:MAB complex in the presence of 3.3 mM potassium persulfate or 1.3 mM iodine at room temperature in 0.05 M sodium phosphate buffer (pH 7.0); ethanol (33%). Both inducers gave a similar reaction pattern, but with different magnitudes. In both systems, the formation of the complex rapidly increased from 0 to 2 hr, became slow afterward, and reached a plateau within 4 hr. The formation of calf thymus DNA:MAB complex was found to be highly dependent on the concentration of MAB (Chart 5). The denatured DNA preparations (Chart 5, C and D) were highly dependent on the concentration of MAB.

### Chart 1

Representative spectra of the nucleic acid:MAB complexes induced by iodine or potassium persulfate. I, spectrum of yeast RNA:MAB complex induced by persulfate; II, spectrum of yeast RNA:MAB complex induced by iodine; III, spectrum of rat liver DNA:MAB complex induced by persulfate and purified by gel filtration on Sephadex G-200. The spectrum of the yeast RNA:MAB complex produced by the reaction of N-benzoyloxy-MAB with yeast RNA is given for comparison (IV). Spectra taken in 0.05 M phosphate buffer (pH 7.0); —¿, spectra taken in 80% formic acid. In the buffer system, all complexes showed absorption at 260 nm while the UV absorption spectra were discontinued around 255 to 260 nm in the formic acid system because of the strong absorption of the acid at this region.

### Chart 2

Gel filtration patterns of rat liver DNA:MAB and rRNA:MAB complexes. The rat liver DNA:MAB complex was induced by persulfate (a) or iodine (b). The reactions were carried out in 7 ml 0.05 M sodium phosphate buffer (pH 7.0):33% ethanol solvent mixture. In the persulfate system (SYST.), the mixture contained rat liver DNA, 0.5 mg; MAB-14C, 2.3 Amoles; and potassium persulfate (K2S2O8), 0.12 mmole. In the iodine system, the reaction mixture contained rat liver DNA, 0.25 mg; MAB-14C, 1.2 Amoles; and iodine, 0.08 mmole. The rat liver rRNA:MAB complex was induced by persulfate (c) or iodine (d). The reaction mixture contained rat liver rRNA, 4.1 mg; MAB-14C, 7.3 Amoles; and potassium persulfate, 0.02 mole, in 7 ml of 0.05 M sodium phosphate buffer (pH 7.0):33% ethanol solvent mixture. The above reaction mixtures were incubated at 25° for 12 hr, the nucleic acid:MAB complexes were isolated, and an appropriate aliquot of the complexes was dissolved in 0.01 M phosphate buffer, pH 7.0, and subjected to Sephadex column chromatography as described in “Materials and Methods.” The radioactivity scale in c is 5 times larger than that in d.
Binding of MAB to Nucleic Acids

Table 2
Equilibrium dialysates of nucleic acid-MAB complexes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Conditions</th>
<th>Component ratio&lt;sup&gt;a&lt;/sup&gt; (µmoles MAB/g nucleic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% ethanol solution of yeast RNA: MAB complex induced by A. Persulfate</td>
<td>Day 0: 5.7, Day 1: 6.2, Day 2: 6.6, Day 3: 6.4</td>
</tr>
<tr>
<td></td>
<td>B. Iodine</td>
<td>Day 0: 3.0, Day 1: 3.0, Day 2: 3.1</td>
</tr>
<tr>
<td>2</td>
<td>10% ethanol solution of MAB and yeast RNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Day 0: 31.4, Day 1: 9.4, Day 2: 0.8, Day 3: 0.5</td>
</tr>
<tr>
<td>3</td>
<td>Three times repeated precipitation from Experiment 2</td>
<td>Day 0: 0.5, Day 1: 0.6, Day 2: 0.5, Day 3: 0.5</td>
</tr>
<tr>
<td>4</td>
<td>80% formic acid solution</td>
<td>A. Rat liver DNA: MAB complex induced by persulfate: Day 0: 22.4, Day 1: 16.5, Day 2: 17.1, Day 3: 18.1</td>
</tr>
<tr>
<td></td>
<td>B. Yeast RNA: MAB complex induced by iodine</td>
<td>Day 0: 7.8, Day 1: 7.8, Day 2: 8.2, Day 3: 8.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated from the absorbances at 350 nm and 260 nm, as described in Table 1.

<sup>b</sup> Prepared by mixing 4 mg yeast RNA in 4.5 ml of 0.05 M phosphate buffer, pH 7.0, and 0.126 µmoles (26.6 µg) MAB in 0.5 ml of absolute ethanol.

D) were more susceptible to induced binding than were the native preparations (Chart 5, A and B).

Partial Characterization of the Structure of the Yeast RNA:MAB Complex. The yeast RNA:MAB complex induced by potassium persulfate was hydrolyzed to mononucleotides with potassium hydroxide and subsequently to nucleosides with alkaline phosphatase, as described in "Materials and Methods." The nucleoside-bound dye was subjected to TLC analysis on the thin layers of cellulose with 4 different solvent systems (Solvents A, C, D, and E). One of the major components of the nucleoside dye was identical chromatographically to the authentic guanosinyl-MAB which was prepared from N-benzoyloxy-MAB and guanosine, as described in "Materials and Methods." The authentic nucleoside dye was shown by Miller et al. (24) to be the major product of rRNA-boundazo dye after the administration of MAB-3H to rats. Furthermore, the prepared nucleoside dye was hydrolyzed with 2 N HCl at 100° for 1 hr, and the hydrolysate

Chart 4. The reaction rate for the persulfate- or iodine-induced binding of MAB to yeast RNA. The reaction mixtures were composed of 9 ml 0.05 M sodium phosphate buffer (pH 7.0):33% ethanol solution; MAB, 6 mmoles; yeast RNA, 20 mg; and either potassium persulfate, 0.03 mmole, or iodine, 0.012 mmole. Reactions were run for the time indicated at 25°. The yeast RNA was precipitated with ethanol and purified as described in "Materials and Methods."

Chart 3. Effect of the concentration of inducer on the formation of calf thymus DNA:MAB complex. The reaction mixture contained 5 ml of 0.05 M sodium phosphate buffer (pH 7.0):33% ethanol solution, 0.5 mg of calf thymus DNA, 3.2 µmoles of MAB, and the indicated concentration of the inducer, iodine or persulfate. The reaction was run for 12 hr at 25°. The DNA was precipitated and purified and the binding was estimated as described in "Materials and Methods."

Chart 5. Effect of MAB concentration on the formation of calf thymus DNA:MAB complex induced by iodine or potassium persulfate. The reaction mixture was composed of 5 ml 0.05 M sodium phosphate buffer (pH 7.0):33% ethanol solution; MAB, 6 mmoles; yeast RNA, 20 mg; and either potassium persulfate, 0.03 mmole, or iodine, 0.012 mmole. Reactions were run for the time indicated at 25°. The extents of binding were estimated from the radioactivity and the absorbance at 260 nm. A, native calf thymus DNA:MAB complex induced by the iodine system; B, native calf thymus DNA:MAB complex induced by the persulfate system; C, heat-denatured calf thymus DNA:MAB complex induced by the persulfate system; D, heat-denatured calf thymus DNA:MAB complex induced by the iodine system.
was submitted to paper chromatography (28) and found to contain D-ribose. The yeast RNA:MAB complex induced by iodine gave some polar dyes but no detectable amount of guanosinyl-MAB under the same conditions.

Hydrolysis of calf thymus DNA:MAB complex induced by persulfate with DNase, phosphodiesterase, and alkaline phosphatase, as described in "Materials and Methods," gave a major polar deoxyribonucleoside dye (RF = 0.65 on a cellulose plate in Solvent A). This polar dye was different from the authentic deoxyguanosinyl-MAB (RF = 0.44 on a cellulose plate in Solvent A) that was prepared from N-benzoyloxy-MAB and deoxyguanosine (24). No polar dye was observed from the enzymatic hydrolysis of the DNA:MAB complex induced by the iodine system. Further studies on the structure of these ribonucleoside- and deoxyribonucleoside-bound dyes are in progress.

DISCUSSION

Physical Binding in the Nucleic Acid:MAB Complexes. Freshly prepared nucleic acid:MAB complexes are devoid of physically bound MAB, as revealed by gel filtration and equilibrium dialysis. The complexes slowly liberated nonpolar dye, i.e., MAB on prolonged storage, or upon acid or alkaline treatment. Synthetic guanosinyl-MAB also liberated a small amount of MAB under the same conditions. The molecular extinction coefficient of MAB at 510 nm in acidic medium is 5.3 X 10^4, while that of nucleic acid-bound MAB is about one-twentieth that of MAB (J.-K. Lin, J. A. Miller, and E. C. Miller, unpublished data). For this reason, an aged nucleic acid:MAB complex might show a higher absorbance at 500 to 550 nm in 80% formic acid.

Levels of Binding. The levels of binding of MAB-14 C to nucleic acids were 12.1 μmoles/g rat liver DNA and 88 μmoles/g rat liver rRNA in the potassium persulfate system, and 13.1 μmoles/g rat liver DNA and 8.6 μmoles/g rat liver rRNA in the iodine system. These values were much higher than the binding of the DAB metabolites in vivo (30, 34) or in vitro as determined from studies of binding of DAB with calf thymus DNA in the presence of microsomal fractions of rat liver (20). According to Roberts and Warwick (30), the maximum levels of binding in the liver of rats treated with DAB-3 H were 0.014 μmole/g DNA and 0.114 μmole/g rRNA. Very close values, 0.011 μmole/g DNA and 0.049 μmole/g rRNA, were obtained when the rats were treated with MAB-3 H (J.-K. Lin, J. A. Miller, and E. C. Miller, unpublished data). The binding ratio of RNA to DNA in rat liver was found to be 0.114:0.014 = 8.1, which is very close to that in the persulfate system (88:12.1 = 7.2) and is quite different from that in the iodine system (8.6:13.1 = 0.7). Furthermore, the level of binding induced by persulfate or iodine reached the same order of magnitude as that obtained from the reaction of N-benzoyloxy-MAB and calf thymus DNA (29). These data suggest that the reactive intermediate of the persulfate system might be similar in chemical type to that of DAB or MAB in rat liver. Persulfate induced binding of AB, MAB, and DAB to calf liver tRNA at levels of 0.5, 5.9, and 0.7 μmoles dye per g RNA, respectively.

Reaction in the Iodine System. 3-Iodo-MAB and AB were produced in the iodine system so that N-demethylation and iodination occurred in the reaction mixture. Since neither of these products could be bound to nucleic acid, it is suggested that some other reactive intermediate may be present. Oxidation of an aldehyde or ketone hydrazone with iodine in a neutral medium gives the corresponding azine; the reaction is interpreted as proceeding through a radical mechanism (1). Lesko et al. (14) proposed that the iodine-induced chemical linkage of 3,4-benzpyrene to DNA is mediated through a benzpyrene-free radical. Therefore one might speculate that a MAB-free radical may be the reactive intermediate in the formation of nucleic acid:MAB complexes; the reaction may also involve attack by nucleic acids on an aryl iodine complex of MAB.

Reaction of Persulfate System. The reaction of aromatic amines and persulfate has been studied by Boyland et al. (3) since 1953. The main product of this reaction was found to be the corresponding aromatic amine sulfamic acid (3–5, 31). Behrman (2) proposed a mechanism involving a nucleophilic displacement by the amine nitrogen on the peroxyxidase oxygen to yield the corresponding arylhydroxylamine-O-sulfate (or arylamine-N-sulfate). This intermediate is then assumed to rearrange to the o-aminophenyl sulfamic acid (or arylamine sulfate). This concept was further supported by the studies of Venkatasubramanian and Sabesan (33). MAB is an arylalkyl secondary amine. The nucleophilic displacement mechanism may be operative in the reaction between MAB and persulfate ion. The presence of MAB-3-sulfate in the reaction mixture (11) and the formation of guanosinyl-MAB or nucleic acid:MAB complex in the mixture of MAB, potassium persulfate, and guanosine or nucleic acid strongly suggests that the N-monomethylamino group of MAB might be attacked by persulfate ion, and that the reactive intermediate MAB-N-sulfate might be formed. This intermediate would be expected to be quite reactive and labile. Presumably, it would react with guanosine or nucleic acid to form bound dye or would rearrange to MAB-3-sulfate. However, further investigations are required to substantiate the presence of MAB-N-sulfate in this system. We hope that the chemical activation of MAB by persulfate will be a useful model system for further studies of the metabolic activation of MAB, since several studies (7, 23, 26) suggested that 2-acetylaminofluorene-N-sulfate is the reactive form of the carcinogen 2-acetylaminofluorene in vivo.

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