THE METABOLISM OF METHYLATED AMINOazo DYES

II. OXIDATIVE DEMETHYLATION BY RAT LIVER HOMOGENATES*

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At least one N-methyl group is required for the carcinogenic activity of 4-dimethylaminoazobenzene and related dyes in the liver of the rat (1). The N-dimethyl and N-monomethyl derivatives have equal carcinogenic potencies, while the corresponding primary aminoazo dyes exhibit low activities in proportion to the extent to which they become remethylated in vivo (2). Other studies have revealed that in vivo 4-dimethylaminoazobenzene is subject to a stepwise demethylation in which the first step appears to be readily reversible, while the second step, leading to the primary aminoazo dye, is reversible to only a small extent (2, 3).

This stepwise demethylation has been demonstrated also in fortified rat liver homogenates (4) and the present communication concerns the mechanism of the demethylation of the N-monomethyl dyes in vivo. This reaction has been found to be an oxidative process which requires at least two enzymes, triphosphopyridine nucleotide, diphosphopyridine nucleotide, and hexosediphosphate. The products of the reaction are formaldehyde and the corresponding primary aminoazo dye. These results are in agreement with the recent demonstration (5) that in vivo the N-methyl groups of the dyes are incorporated into the β-carbon of serine and the methyl groups of choline.

Methods

The young adult, male, albino rats used in these experiments were maintained on a grain diet (6). The rats were killed by decapitation and liver samples transferred immediately into ice-cold 1.15 per cent KCl. 10 per cent homogenates were prepared in this same medium with a Potter-Elvehjem homogenizer. The complete reaction mixture was prepared at 0° and contained the following ingredients, adjusted to pH 7.4, in a final

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Holtzman-Rolfsmeyer Company, Madison, Wisconsin.

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volume of 3.0 ml.: 0.3 to 0.5 ml. of homogenate, 0.4 ml. of 0.03 M hexosediphosphate, 0.1 ml. of diphosphopyridine nucleotide (DPN) solution (200 γ), 0.1 ml. of triphosphopyridine nucleotide (TPN) solution (200 γ), 0.2 ml. of 0.6 M nicotinamide, 0.2 ml. of 0.01 M adenosinetriphosphate (ATP), 0.1 ml. of 0.1 M MgCl₂, 0.1 ml. of 0.2 M KCl, and 0.5 ml. of 0.1 M KH₂PO₄-K₂HPO₄ buffer at pH 7.4. The monomethylaminoazobenzene dyes (70 to 150 γ in 0.1 ml. of 95 per cent ethanol) were added last to the iced reaction mixture from a micro blow pipette. The reactions were customarily carried out in open 25 ml. Erlenmeyer flasks with mechanical shaking in a water bath at 37°.

At the termination of the incubation period (0 to 40 minutes) the reaction was stopped by the addition of 3.0 ml. of acetone. Subsequently 4.0 ml. of benzene were added to the flasks and the free aminoazo dyes were extracted with brief shaking into the acetone-benzene phase. An aliquot of the clear acetone-benzene phase was taken to dryness in a vacuum desiccator and the residual dyes separated and recovered quantitatively by chromatography on activated alumina with mixtures of petroleum ether and benzene, as previously described (7). The quantity of the monomethylated dye remaining and the amount of 4-aminoazobenzene derivative formed in the demethylation reaction were determined from the optical densities of the orange to red solutions obtained on extraction of the eluted dyes into 7 N HCl (7). The optical density measurements were made with a Cenco-Sheard spectrophotometer at wave-lengths corresponding to the absorption maxima of the dyes in this medium.

All the ring methyl derivatives of 4-monomethylaminoazobenzene tested as possible substrates were prepared by the diazoamino rearrangement (7). The 3- and 3'-methyl derivatives have been described before (1); the 4'-, 2', and 2-methyl derivatives are new and their melting points were 101–102°, 73–74°, and 43–44°, respectively. All but one of the corresponding derivatives of 4-aminoazobenzene used as colorimetric standards were prepared by coupling the appropriately substituted nitrosobenzenes and p-aminoacetanilides (8), followed by hydrolysis in alcoholic alkali. The 2-methyl derivative was obtained through the coupling of the o-methyl sulphonate of m-toluidine with diazotized aniline, followed by hydrolysis in dilute alkali (9, 10). The 2'-methyl derivative has not been described before and its melting point was 45–46°; the 2-, 3-, and 4'-methyl derivatives had melting points as given in the literature (11, 12), while the 3'-methyl dye melted at 95–96° as compared with the reported value of 89–90° (10). All the dyes were purified by chromatography on alumina (7).

* We wish to thank Dr. G. A. LePage for the preparations of diphosphopyridine nucleotide and adenosinetriphosphate.

* Uncorrected.
Results

Choice of Dyes—In preliminary experiments with 4-dimethylaminoazobenzene and 4-monomethylaminoazobenzene it was observed that the loss of the first methyl group from the tertiary amine proceeded very slowly in the system described above; under the same conditions the removal of the methyl group from 4-monomethylaminoazobenzene occurred rapidly. However, in this system this compound is subject to reductive cleavage at the azo linkage and to hydroxylation at the 4' position (4); thus the concurrent operation of these reactions during the demethylation process resulted in a considerable deficit in recoverable dye. Accordingly a series of 4-monomethylaminoazobenzene dyes with ring methyl substitutions was compared. From the results in Table I it is apparent that both 3-methyl-4-monomethylaminoazobenzene and 2-methyl-4-monomethylaminoazobenzene were not cleaved or hydroxylated to any significant extent, since it was possible to account for essentially all of the added dye as residual monomethyl dye and the expected demethylated derivative. The 3-methyl dye was therefore used in the experiments reported here.

Cofactor Requirements—The dependence of the demethylation reaction on the addition of certain activating cofactors to the homogenate system is illustrated in Fig. 1. In the complete system 41 γ of 3-methyl-4-aminoazobenzene were formed during the incubation period. With the omission of hexosediphosphate the amount of demethylated product formed decreased to 25 γ. When either TPN or DPN was omitted from the reaction mixture, the demethylation activity was decreased, with the formation of only 8 and 15 γ of 3-methyl-4-aminoazobenzene, respectively, and essentially no demethylation took place in the absence of both cofactors. The activation curves for both TPN and DPN are presented in Fig. 2;

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dye metabolized</th>
<th>Demethylated dye recovered</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAB</td>
<td>52.7</td>
<td>28.8</td>
<td>59</td>
</tr>
<tr>
<td>4'-Methyl-MAB</td>
<td>29.6</td>
<td>16.2</td>
<td>59</td>
</tr>
<tr>
<td>3'-Methyl-MAB</td>
<td>41.0</td>
<td>24.4</td>
<td>64</td>
</tr>
<tr>
<td>2'-Methyl-MAB</td>
<td>34.8</td>
<td>24.0</td>
<td>76</td>
</tr>
<tr>
<td>2-Methyl-MAB</td>
<td>38.5</td>
<td>35.0</td>
<td>97</td>
</tr>
<tr>
<td>3-Methyl-MAB</td>
<td>23.0</td>
<td>22.3</td>
<td>96</td>
</tr>
</tbody>
</table>
each cofactor was tested in the presence of 200 γ of the other coenzyme. In this experiment the rat liver homogenate was prepared in distilled water and incubated at 37° for 5 minutes prior to addition to the cold reaction mixture; in other experiments it had been demonstrated that this preliminary incubation decreases the level of endogenous cofactors (13). Under these conditions it is apparent that the addition of 200 γ of each co-

![Chart](http://www.jbc.org/)

**Fig. 1.** The requirement of the demethylation system for cofactors. System as under "Methods" with 0.5 ml. of homogenate, 0.4 ml. of 0.03 M hexosediphosphate (HDP), 200 γ of triphosphopyridine nucleotide, and 200 γ of diphosphopyridine nucleotide as indicated. Each flask contained 150 γ of dye; incubation time, 30 minutes.

factor is more than adequate for the saturation of the enzyme system; however, the nature of this requirement for both coenzymes remains to be elucidated.

Adenosinetriphosphate produced no further activation of the enzyme system in the presence of hexosediphosphate, but in other trials in which this oxidizable substrate was omitted ATP gave a slight activation. It would appear that if ATP is involved in this system its participation is indirect, such as in the maintenance of some component of the enzyme system.

**Requirement for Aerobiosis**—The dependence of the demethylation reaction on the presence of oxygen was readily demonstrated. For example,
in a representative experiment 19.1 γ of 3-methyl-4-aminoazobenzene were formed under aerobic conditions, in contrast to 3.3 γ produced in the reaction carried out in an atmosphere of nitrogen.

Identification of Reaction Products—For the identification of the reaction products 50 mg. of rat liver (0.5 ml. of homogenate) were incubated for 30 minutes with 147 γ of 3-methyl-4-monomethylaminoazobenzene in the complete system containing 0.01 M semicarbazide as the trapping agent.

![Graph](attachment:image.png)

**Fig. 2.** The activation of the demethylation system by varied levels of diphosphopyridine and triphosphopyridine nucleotides. System as under "Methods" with 0.5 ml. of homogenate and 150 γ of added dye. Incubation time, 30 minutes.

Duplicate flasks were inactivated with acetone and the residual dyes measured in the manner described above. The reaction in another duplicate set of flasks was stopped by the addition of 2.0 ml. of 5 per cent perchloric acid. After centrifugation 4.0 ml. aliquots were diluted to 14.0 ml. with distilled water and then distilled. Exactly 10.0 ml. of distillate were collected and analyzed for formaldehyde by the highly specific chromotropic acid procedure of MacFadyen (14). All values were corrected for a distillation efficiency of 84 per cent under the conditions employed. The absorption maximum (570 με) of the colored test solution was identical with that of an authentic formaldehyde standard. The results of this experiment are presented in Table II. Of the 29.0 γ of 3-methyl-4-monomethyl-
aminoazobenzene metabolized 28.6 γ could be accounted for as 3-methyl-4-aminoazobenzene (27.2 γ). Similarly the quantity of formaldehyde recovered from the reaction mixture (3.94 γ) corresponded to 29.5 γ of the metabolized 3-methyl-4-monomethylaminoazobenzene. Zero time control flasks with added dye contained no measurable formaldehyde, and the incubated control flasks in which the dye was omitted (ethanol controls) yielded an amount of formaldehyde equivalent to only 0.2 γ of dye; this value was subtracted from the observed figures. Thus it was possible to account stoichiometrically for the metabolized azo dye as the demethylated derivative, 3-methyl-4-aminoazobenzene, and for the methyl group as formaldehyde.4

Effect of Glutathione—In an attempt to activate the demethylation enzyme system further 5.0 mg. of reduced glutathione were added to the complete system. Unexpectedly, it was observed that the aqueous phase of the reaction mixture remained highly colored after repeated extractions with the acetone-benzene mixture. The addition of 7.0 N HCl or 20 per cent trichloroacetic acid to the aqueous phase yielded the characteristic bright red color of secondary or tertiary aminoazo dyes of this series. This color, however, was unstable in acid solution and rapidly faded to an orange-colored solution. After neutralization to pH 7.0 it was possible to extract and identify 3-methyl-4-aminoazobenzene by mixed chromatograms and absorption curves. From this observation it was concluded that a product or reaction intermediate of the demethylation reaction was conjugated with the added glutathione to give a highly polar and acid-labile complex. It seems probable that glutathione effectively trapped a hydroxymethyl intermediate as a mercaptal derivative during the oxidation of the N-methyl group. A similar reaction was observed in the absence of enzyme

4 Formaldehyde has also been found as a metabolite of 4-dimethylaminoazobenzene. Personal communication from Dr. C. J. Kensler and Dr. C. G. Mackenzie.

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**Table II**

<table>
<thead>
<tr>
<th>Measurement made</th>
<th>Amount of dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate metabolized</td>
<td>29.0</td>
</tr>
<tr>
<td>Accounted for as demethylated dye</td>
<td>28.6</td>
</tr>
<tr>
<td>“ “ “ formaldehyde</td>
<td>29.5</td>
</tr>
</tbody>
</table>
when glutathione, 3-methyl-4-aminoazobenzene, and high concentrations of formaldehyde were combined in aqueous solution.

Distribution of Demethylation System in Rat Tissue—An assay of other rat tissues revealed that the liver was the only organ tested to possess an active demethylation system. Heart, spleen, kidney, brain, intestine, and primary hepatoma induced by azo dye did not contain measurable demethylation activity.

Miscellaneous Substances Tested—A wide selection of chemicals was tested for possible effects on the demethylation system. The following compounds were found to be without effect at the concentrations tested: 0.003 M glycine, 0.003 M methionine, 0.003 M Na formate, 0.003 M glycocyanine, 0.003 M urea, 100 γ of riboflavin-adenine dinucleotide, 500 γ of folic acid, 1000 γ of pyridoxal, 1000 γ of pyridoxamine, 7.5 γ of vitamin B12, 1000 γ of α-tocopherol, 500 γ of cocarboxylase, 500 γ of biotin, 1000 γ of γ-inositol, and 1000 γ of ascorbic acid per flask. Cytochrome c, 6.6 × 10⁻⁶ M, produced a 90 per cent inhibition of the demethylation activity.

DISCUSSION

The enzyme system mediating the demethylation of 3-methyl-4-monomethylaminoazobenzene and related dyes requires the presence of oxygen, diphosphopyridine nucleotide, triphosphopyridine nucleotide, and hexose-diphosphate for optimal activity. Since the products of this reaction are the primary amine and formaldehyde, it would appear that the demethylation reaction is an oxidative process rather than a transmethylation or hydrolysis. In Fig. 3 the apparent steps of the demethylation reaction are illustrated. The evidence for the formation of the hydroxymethyl intermediate resides in the production of formaldehyde in the reaction and in the ability of glutathione to combine with an intermediate in the reaction to yield the observed water-soluble azo dye complex which could be hydrolyzed subsequently to the free primary aminoazo dye. The great tendency of hydroxymethyl groups to dissociate to formaldehyde or to condense with compounds containing active hydrogen to form methylene derivatives is well known (15). Although the authors are unaware of any instances of the direct condensation of mercaptans with simple hydroxymethyl derivatives, the reaction postulated for glutathione in this case seems likely, since the literature contains many examples of the formation of mercaptals from mercaptans and various aldehydes, including formaldehyde, which presumably react in solution as substituted hydroxymethyl derivatives (15–18).

From the known high reactivity of hydroxymethyl amines in the field of polymer chemistry it is appropriate to consider the possibility that this is the form of the azo dye which has been found to combine with certain in-
tracellular proteins (19). If such a combination were to result in the inactivation of a specific protein concerned with the maintenance of differentiated cell growth, this reaction might then represent the initial step in the carcinogenic process induced by these chemicals. In this connection it is of interest that the liver, which is the site of tumor formation and protein-bound dye formation, is also the only tissue in the rat which was found to catalyze the demethylation reaction.

In recent studies from this laboratory (5) in which rats were fed aminoazo dyes labeled in the N-methyl groups with C^{14}, the isotope was found in the β-carbon of serine and the N-methyl groups of choline. The isolation of stoichiometric amounts of formaldehyde as a product of the oxidative demethylation in a rat liver homogenate system is in agreement with these findings and demonstrates at least one pathway by which the N-methyl group of the azo dye enters the 1-carbon metabolic pool.

Upon differential centrifugation of rat liver homogenates in isotonic sucrose solution it was found\(^6\) that the particles and the supernatant fluid were both unable to demethylate the dye. However, full activity was regained upon reconstitution of the homogenate. Since the activity of each fraction was destroyed by heating at 100° for 3 minutes, it is likely that at least two enzymes are involved in the over-all reaction. A similar situation was reported by Mackenzie \(et\ al\). (20) for the oxidative demethylation of dimethylaminoethanol by rat liver particles and supernatant fluid. These authors also noted that dimethylglycine and sarcosine were demethylated by the liver particles alone. Hence it is possible that dimethylaminoethanol is a natural substrate of the system described here.

SUMMARY

The enzymatic oxidative demethylation of N-monomethyl dyes related to the hepatic carcinogen 4-dimethylaminoazobenzene has been observed in properly fortified rat liver homogenates. Oxygen, triphosphopyridine nucleotide, diphosphopyridine nucleotide, and hexosephosphate are required for optimal activity. With one of these dyes, 3-methyl-4-monomethylaminoazobenzene, it has been possible to account stoichiometrically for the metabolized dye as the primary aminoazo dye and formaldehyde. However, if glutathione is added to these reaction mixtures, a highly polar dye is formed which liberates the primary aminoazo dye upon acid treatment. A similar product is observed non-enzymatically from the reaction of the primary aminoazo dye, formaldehyde, and glutathione. It thus appears that an N-hydroxymethyl derivative is a possible intermediate in the enzymatic demethylation of these dyes.

BIBLIOGRAPHY

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