The Metabolism of N,N-Dimethyl-p-Aminoazobenzene and Related Compounds


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Studies on the metabolism of azo compounds related to p-aminoazobenzene in the rat so far have indicated that: (a) Fission of the azo linkage occurs (4, 12); (b) Dealkylation of N-methyl derivatives (10) and the N-ethyl derivative (5) occurs, at least to some extent, prior to reduction fission; (c) Partial reduction to hydrazo compounds which undergo a benzidine rearrangement in acid solution may also occur (2, 3).

The results obtained in this laboratory bearing on this problem are reported below.

METHODS

Three per cent solutions of the compounds in cottonseed oil were mixed with the diet of brown rice and carrot. This diet has been shown to favor the production of liver cancer (14) in the rat.

N,N-dimethyl-p-aminoazobenzene (DMB), N-methyl-p-aminoazobenzene (MMB), and p-aminoazobenzene (AB) were determined by chromatographic analysis and absorption characteristics in acid solution essentially as outlined by the Wisconsin group (9). N,N-diethyl-p-aminoazobenzene (DEB) was absorbed less firmly on aluminum oxide than was DMB. N,N-diethanol-p-aminoazobenzene (DE-ol-B) was absorbed much more strongly and did not elute with benzene but was eluted with a 2:1 benzene-methanol mixture.

The absorption maxima of these azo compounds in 7N HCl were determined in a Beckman spectrophotometer and are as follows:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption Maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>500 µM</td>
</tr>
<tr>
<td>MMB</td>
<td>508 µM</td>
</tr>
<tr>
<td>DMB</td>
<td>518 µM</td>
</tr>
<tr>
<td>DEB</td>
<td>520 µM</td>
</tr>
<tr>
<td>De-ol-B</td>
<td>528 µM</td>
</tr>
</tbody>
</table>

The absorption spectra were measured between 240 and 560 nm. Other absorption maxima were found at 320 and 270 nm, but the absorption coefficients were much lower than at the 500 nm bands.

In the tissue slice experiments 200 mgm. (wet weight) of tissue were used. The medium consisted of 3 ml. of Krebs-Ringer-phosphate, 0.4 ml. of 2 per cent glucose, and 0.1 ml. of 95 per cent ethanol containing 50 µgm. of the azo compound. The tissues were incubated for 90 minutes at 37.5° C. in test tubes and shaken in a Warburg bath. At the end of the incubation period the contents of the tubes were ground in a glass homogenizer. The extraction procedure used was the same as for tissue samples (9).

In the brown rice diet experiments a 3 per cent solution of the compound was added to the diet to make the concentration of the dye 0.06 per cent, the diet mixed, an aliquot weighed out, and the material ground and extracted in the same manner as the tissues.

RESULTS

Ten rats were fed the brown rice and carrot diet containing 0.06 per cent N,N-dimethyl-p-aminoazobenzene (DMB). The rats were killed and the tissues were analyzed for DMB, MMB, and AB. The results obtained are in agreement with those of the Wisconsin group with the exception of the distribution of the 3 dyes in the stomach contents. The results of typical experiments are shown in Table I.

The finding that a considerable amount of the DMB had been demethylated to MMB and AB in the stomach contents led us to investigate the stability of DMB in the brown rice diet. The results are shown in Table II. It can be seen that demethylation of DMB in the diet fed was responsible for the unexpected finding of large amounts of MMB and AB in the stomach contents. Only a very small amount of DMB was found to be demethylated in 4 weeks in cottonseed oil, whereas demethylation occurred immediately on mixing with the brown rice diet. As is shown in Table II if the brown rice diet was heated at 90° C. for 5 days before DMB was mixed with it, the demethylation did not occur. During the heating period the weight of the brown rice decreased between 10 to 14 per cent. The mechanism of removal of the methyl groups is not known.
When DEB or DE-ol-B in cottonseed oil was mixed with the diet no detectable de-ethylation or de-ethanolation occurred. As shown in our previous report (5), the stomach contents of rats fed DEB contained only that dye in detectable amounts.

The observation that DEB, a non-carcinogen (at least when fed to rats on a brown rice diet), is deethylated (5) and yields the same concentration of AB in tissue and blood cells as do the N-methyl derivatives made it of interest to study N,N-diethanol-p-aminazobenzene (DE-ol-B), also noncarcinogenic under the same conditions.

The results are summarized in Table III. The earlier results obtained with DEB are included for comparison.

**TABLE III: THE ACCUMULATION OF AB IN THE BLOOD CELLS OF RATS FED DEB AND DE-ol-B**

<table>
<thead>
<tr>
<th>Number of days on diet</th>
<th>AB from DEB γ/ml cells</th>
<th>AB from DE-ol-B γ/ml cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

Only small amounts of AB were found in the blood cells of the rats fed DE-ol-B in contrast to the results obtained with DMB and DEB. Apparently, the rat does not split the N-C₂H₄OH linkage as readily as the N-CH₃ or N-C₅H₇. This suggests that the mechanism of removal of -C₅H₇ groups does not involve an oxidation to -C₅H₈OH.

The destruction of the large amounts of DMB and AB (10) in the rat and the accumulation of AB in the blood cells of rats fed DMB suggested that it might be possible to measure the rate of destruction of DMB and AB by liver slices in vitro. It was thought possible to demonstrate a conversion of DMB to AB in these in vitro experiments. However, the results shown below (Table IV) indicate that under the conditions of these experiments AB is destroyed (metabolized) in vitro at least as rapidly, if not more so, as is DMB. When DMB was destroyed by the liver slices in vitro, no AB could be detected. No attempt has been made to determine the nature of the in vitro metabolism, but as only basic compounds are extracted in the procedure used it would indicate either that the azo linkage was reduced, or that the molecule was oxidized to a phenolic compound which would not have been extracted.

**TABLE IV: AMOUNTS OF AZO COMPOUNDS DESTROYED ON INCUBATION OF 50 γ WITH 200 MGM. OF TISSUE SLICES AT 37.5 °C FOR 90 MINUTES**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue</th>
<th>No. of mgm.</th>
<th>Average destroyed in γ</th>
<th>Range in γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMB</td>
<td>Normal liver</td>
<td>5</td>
<td>34.6</td>
<td>29.0-40.8</td>
</tr>
<tr>
<td>DMB</td>
<td>Liver tumor*</td>
<td>5</td>
<td>11.6</td>
<td>5.6-16.7</td>
</tr>
<tr>
<td>AB</td>
<td>Normal liver</td>
<td>5</td>
<td>38.4</td>
<td>31.3-44.8</td>
</tr>
</tbody>
</table>

* Classified as cholangiomas on histologic examination by Dr. S. Spitz.

In view of the fact that normal liver tissue destroyed DMB readily, it was of interest to determine whether liver tumors produced by this agent possessed this capacity and if so to what extent. The results included in Table IV indicate that the liver tumors studied were able to destroy DMB, but, on an equivalent wet weight basis and under the same conditions, did so to a significantly less extent. The average destruction of DMB and AB by 200 mgm. of normal liver slices was 34.6γ.
and 38.4%, respectively. The tumor slices (200 mgm.) destroyed 11.6y of DMB under the same conditions.

In several experiments in which 50y of DMB was incubated with blood cells only a small amount (5 per cent) was destroyed and small amounts (approximately 2y) of MMB and (ca. 1y) AB were recovered.

**DISCUSSION**

While the data presented above are of interest in connection with the metabolism of closely related azo dyes, which vary tremendously in their capacity to produce a neoplasm in rat liver under standard conditions, little light is thrown on the mode of action of the carcinogenic ones. The finding that the N,N-diethyl derivative, non-carcinogenic under the conditions of our test, leads to the same accumulation of AB in the tissues as the N,N-dimethyl, N-monomethyl and AB suggests that this dealkylation pathway may not be directly related to the problem of carcinogenesis. The observations that the administration of a methyl acceptor compound, guanido-acetic acid, is without effect on the production of liver tumors by MMB (8), and that o-aminoazotoluene, in which the amino group is unsubstituted, is carcinogenic, are also consistent with this view.

The recent observations of Kirby (7) that p-aminoazobenzene may under certain dietary conditions produce liver tumors in the rat, and our observation (13) of one tumor (427 days) in a rat fed 4'-methyl-p-aminoazobenzene are two examples of other p-aminoazobenzene compounds with unsubstituted amino groups that have produced liver cancer.

While the in vitro experiments dealing with the destruction of DMB are by no means adequate to evaluate the rate of destruction, a calculation based on the data available, assuming the total liver weight to be 4 gms., reveals that the liver would destroy approximately 11,000y of DMB per 24 hour period. Actually, on tumor producing diets, the rats eat about 6,000y per day. The failure to find any AB when DMB was incubated with the liver slices raises the problem as to whether these results can be extrapolated to the intact animal, or whether possibly the small amounts of AB found in vitro in the rat, chiefly in the blood cells, may be due to extrahepatic demethylation. The results obtained with the liver tumors (cholangiomas) should not be interpreted as evidence of a loss of hepatic cell ability to destroy DMB. The cholangiomas are presumably derived from bile duct epithelial cells and hence any comparison would have to be between these two types of tissue. The tissue slice technic is obviously not suitable for determining the ability of normal bile duct epithelial cells to destroy DMB.

The finding that brown rice ‘catalyses’ the demethylation of DMB necessitates further quantitative study of the influence of the addition of protective supplements on the stability of DMB in this diet. Preliminary experiments have indicated that the inclusion of 15 per cent yeast does not influence this phenomenon. The observation that heating the rice renders the DMB much more stable and the observations of the Wisconsin group (9) indicating stability of DMB in their basal diets without rice, suggests that the presence of a ‘catalyst’ in the rice is responsible for this effect.

This further complication in the use of a brown rice diet, in addition to rice variation and the presence of unknown factors, makes it desirable to discard the use of the brown rice for further nutritional experiments with the azo dyes. Further use is not warranted although its use permitted: (a) The discovery of the carcinogenic action of the azo dyes (15); (b) The demonstration that the incidence of tumors in rats can be greatly decreased by the addition of crude dietary supplements such as yeast (1), and liver (11); (c) The first demonstration of the protective effect of a pure compound, riboflavin (6).

**SUMMARY**

1. In agreement with earlier observations it has been found that when N,N-dimethyl-p-aminoazobenzene is administered orally to rats the bulk of the azo compound found in the blood and tissues is p-aminoazobenzene. However, this represents only a very small portion of the N,N-dimethyl-p-aminoazobenzene fed.

2. The recovery of relatively large amounts of N-methyl-p-aminoazobenzene and p-aminoazobenzene from the stomach contents led to the finding that when N,N-dimethyl-p-aminoazobenzene in cottonseed oil is mixed with ground brown rice some demethylation occurs. Heating the brown rice for 5 days prior to mixing inhibited the demethylation.

3. Feeding N,N-diethanol-p-aminoazobenzene, in contrast to N,N-diethyl-p-aminoazobenzene, led to the accumulation of only small amounts of p-aminoazobenzene in the blood cells.

4. Liver slices under the conditions of our experiments have been found to destroy both N,N-dimethyl-p-aminoazobenzene and p-aminoazobenzene. No conversion of N,N-dimethyl-p-aminoazobenzene to p-aminoazobenzene by liver slices was observed in vitro.

5. Under the conditions of our experiments liver tumor slices destroyed only one-third as much N,N-dimethyl-p-aminoazobenzene as did the normal liver tissue.

**ACKNOWLEDGEMENT**

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