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4-Dimethylaminoazobenzene and related compounds exert a powerful and specific carcinogenic action on the livers of rats (1). This effect depends to a considerable extent upon the presence of at least one methyl substituent on the amino nitrogen. Metabolic studies (2, 3) have shown, however, that dealkylation is an important step in the biochemical transformations of this class of compounds. On the other hand, 2-dimethylaminofluorene (2-Me2AF) as well as the dealkylated products 2-methylaminofluorene (2-MeAF) and 2-aminofluorene (2-AF) all exhibit carcinogenic activity (4, 5). It was, therefore, of some interest to investigate the metabolism of these compounds with special reference to the fate of the methyl group and the possible reversible conversion of the tertiary and secondary alkyl amine to the primary amine. The study was carried out in normal rats by using 2-methyl-C14-aminofluorene (2-Me-C14-AF) to trace the methyl carbon atom. Parallel experiments were performed in riboflavin- and pantothenic acid-deficient rats to determine the effect of lower cofactor levels on the metabolic conversion of the methyl group, and thereby gain information on the mechanism of the reaction.

Incidental to these biochemical studies, the carcinogenic effects of 2-MeAF and of 2-Me2AF were reexamined in Buffalo strain female rats. Contrary to the results reported by previous investigators (4, 5), an appreciable yield of hepatomas was obtained.

Materials and Methods

The metabolic studies were undertaken with 2 to 3 month-old female Buffalo rats. Some animals were placed on a complete diet, and some on vitamin-deficient semisynthetic diets, as follows: vitamin-free casein

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(Scott) 360 gm., cerelose 390 gm., modified McCollum No. 185 salt mixture 45 gm., lard (fortified with vitamins A and E) 10 gm., lard 190 gm., and choline 5 gm. A vitamin mixture, consisting of folic acid 10 mg., biotin 1 mg., pyridoxine hydrochloride 20 mg., thiamine 40 mg., riboflavin 10 mg., niacin 200 mg., and calcium pantothenate 140 mg., was added to a portion of the cerelose prior to incorporation in the diet mixture. Omission of the riboflavin or calcium pantothenate from the mixture gave the corresponding deficient diets. Signs of severe riboflavin or pantothenic acid deficiencies were present in 7 to 9 and 12 to 15 weeks, respectively.

2-Me-C\textsuperscript{14}-AF was obtained from Dr. F. E. Ray (6). The radioactive compound was fed by stomach tube in a 0.4 N hydrochloric acid solution containing a few drops of ethanol at a level of 10 mg. per 100 gm. of body weight. The animals were kept in glass metabolism cages designed to allow separate collection of respiratory CO\textsubscript{2}, urine, and feces. Food and water were available ad libitum. At the end of 72 hours, the animals were sacrificed under ether anesthesia by withdrawing blood from the abdominal aorta into a heparinized syringe. The organs were removed and dried in vacuo prior to the determination of radioactivity by combustion.

The carcass and an aliquot of the fresh liver were homogenized with ethanol in a Waring blender. Choline was determined in the ethanol extracts of the minced carcass or liver. By the procedure of du Vigneaud \textit{et al.} (7), choline was isolated as the reineckate. The choline reineckate was decomposed and the choline precipitated as the phosphotungstate, which was recrystallized from 50 per cent ethanol to constant specific activity. The crude protein residue from the ethanol extractions was treated as described by Miller \textit{et al.} (2) for serine determination. Aniline was used to remove excess HCl from the protein hydrolysate (8) and the serine crystallized to constant specific activity from water-ethanol. The purity was checked by chromatographing a sample on paper with the solvent system tert-butanol-formic acid-water, 70:15:15 volume per volume. The serine and choline were degraded to formaldehyde or trimethylamine, respectively, by the standard procedures (7, 9) and the radioactivity of these fragments was determined. Creatinine was isolated as the picrate from the urine and crystallized to constant specific activity as potassium creatinine picrate.

Urines of all the rats were chromatographed on paper in the three different solvent systems described earlier (10), and autoradiographs were made of the developed chromatograms. The excretion of unchanged 2-Me-C\textsuperscript{14}-AF in the urine was determined by carrier isotope dilution methods. In a typical experiment, 0.5 to 1.0 ml. of urine containing 300,000 to 500,000 c.p.m. was added to 200 to 230 mg. of unlabeled MeAF dissolved in 5 ml. of 0.4 N HCl. After mixing, the
free amine was precipitated with ammonium hydroxide. The amine was chromatographed in benzene solution on an alumina column, then recrystallized five times as the hydrochloride from ethanol-ether. After conversion to the free amine, the compound was allowed to react with 3,5-dinitrobenzoyl chloride in benzene solution to yield the corresponding crude amide, m.p. 163-164°. This derivative was chromatographed in benzene solution on an alumina column and recrystallized from ethanol three times to give yellow needles, m.p. 166-168°.

C_{31}H_{30}O_{2}N_{4}. Calculated, C 64.78, H 3.88; found, C 64.96, H 3.86

The specific radioactivity per millimole of the material was determined at each step by plating an aliquot on glass disks until constancy was achieved in three successive operations. The amount of labeled compound present in the original urine sample was then calculated from this specific activity and the amount of unlabeled carrier used.

The possible presence in the urine of 2-Me_{2}AF produced by methylation in vivo of 2-Me-C^{14}-AF was similarly investigated by carrier isotope dilution. After chromatography on alumina and nine recrystallizations from dilute ethanol, the specific activity of the compound decreased at each step and finally reached zero.

The excretion of the demethylation product of 2-MeAF, 2-AF, in the urine was determined by the use of colorimetric methods (11).

**Determinations of Radioactivity**—Tissues, feces, and other dry samples were converted to CO_{2} by wet combustion (12) and counted as duplicate BaCO_{3} plates. Liquid samples or dilute solutions were plated directly on glass disks; suitable correction factors to convert counts of direct plates to those obtained by combustion were determined on representative samples and applied to all direct plates. Counting was carried out in a windowless gas flow counter with a 48 per cent efficiency for C^{14}. Samples with a very low radioactivity were counted by a Robinson type windowless counter (13) which had a background counting rate of 3.8 to 4.0 c.p.m.

The carcinogenic studies were carried out in three experiments, each containing eighteen female Buffalo strain rats 2$^{1/2}$ to 3$^{1/2}$ months of age at the start of the experiment. The animals were housed individually in raised screen bottom cages and had access to a mixed diet of natural foodstuffs$^{1}$ and tap water at all times. The chemicals 2-MeAF, 2-Me_{2}AF, and 2-acetylaminofluorene (2-AAF) were incorporated into the diet at a level of 250 mg. per kilo of diet, or at 1.23, 1.20, and 1.12 mmoles per kilo of diet.

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$^{1}$ The diet was composed of the following constituents in per cent by weight: commercial casein 3.00, skim milk powder 22.75, whole wheat (hard spring) meal 60.52, yeast 1.00, whole liver powder (Wilson) 1.00, NaCl 1.40, iron citrate 0.13, cod liver oil 2.00, corn oil 5.20, propylene glycol 3.00.
respectively. The 2-AAF-containing diet was intended to serve as a positive control. The rats were maintained on their respective diets until they developed visible tumors or until death appeared imminent, at which time a careful autopsy was performed. The tissues were fixed in Zenker's formol solution and subsequently stained by hematoxylin and eosin.

EXPERIMENTAL

Studies in Metabolism; Distribution of Radioactivity in Normal Animals—Rats on the control diet excreted 54 per cent of the carbon 14 from the administered 2-Me-C¹⁴-AF in the respiratory carbon dioxide in the 72 hour experimental period (Table I). Of this, 46, 6, and 2 per cent were excreted on the 1st, 2nd, and 3rd day, respectively. The specific radioactivity of the barium carbonate derived from the expired carbon dioxide was highest in the 6 to 8 hour period and dropped off markedly after 24 hours. The urinary activity followed a similar course, a total of 22 per cent of the dose being eliminated in that manner. Radioactivity was found in all the organs analyzed.

Radioactivity was present in the choline and serine isolated from the carcass and the liver (Table II) and in the urinary creatinine. Degradation of the choline samples showed that 60 and 26 per cent of the C¹⁴ was located in the trimethylamine portion of carcass and liver choline, respectively. Degradation of the pure serine indicated that 98 to 103 per cent of the C¹⁴ was incorporated in the β position of this amino acid.

Solvent system A yielded the best separation of the complex mixture of urinary metabolites on the paper chromatograms. The autoradiographs showed intense spots with \( R_f \) values of 0.04, 0.12, 0.21, 0.25, and 0.49; other spots appeared at 0.30, 0.44, 0.64, 0.70, 0.82, 0.91, and 0.97. The last spot corresponded to that obtained with unchanged 2-Me-C¹⁴-AF. Solvent system B or C gave a more reliable indication of the relative amounts of 2-Me-C¹⁴-AF in comparative experiments since this compound alone moved near the solvent front; other metabolites had low \( R_f \) values or did not move at all. Thus, the unchanged compound appeared on the chromatograms of a urine sample collected in the first 24 hour period. However, only a trace of 2-Me-C¹⁴-AF was present in urine obtained during the 2nd day.

Carrier experiments showed that only a minor amount, approximately 3 per cent of the urinary radioactivity collected on the 1st day, was due to 2-Me-C¹⁴-AF. Similar carrier experiments with 2-dimethylaminofluorene gave a product counting within background rates which indicated that, if 2-MeAF is further methylated in vivo, the product is not excreted in the urine.

An aliquot of the urine collected on the 1st day contained 455,000 c.p.m. per ml., equivalent to 737 \( \gamma \) of 2-Me-C¹⁴-AF. Colorimetric analysis for
diazotizable material gave the equivalent of 30 γ per ml. of free 2-AF, which thus accounted for 4.1 per cent of the urinary metabolites.2

**Table I**

*Distribution of Radioactivity 72 Hours after Single Oral Dose of 2-Methyl-C14-aminofluorene in Normal, Riboflavin-Deficient, and Pantothenic Acid-Deficient Rats*

<table>
<thead>
<tr>
<th>Per cent of dose administered</th>
<th>Normal*</th>
<th>Riboflavin-deficient†</th>
<th>Pantothenic acid-deficient†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Average</td>
<td></td>
</tr>
<tr>
<td>Respiratory CO₂</td>
<td>55</td>
<td>53</td>
<td>54</td>
</tr>
<tr>
<td>Urines</td>
<td>19</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Feces</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Carcass</td>
<td>4.6</td>
<td>5.4</td>
<td>5.0</td>
</tr>
<tr>
<td>Gastrointestinal tract + contents§</td>
<td>2.4</td>
<td>4.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Other organs</td>
<td></td>
<td>5.7</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* The results with two animals administered 3.27 and 1.41 × 10⁷ c.p.m., respectively.
† The results with two animals administered 3.03 and 1.21 × 10⁷ c.p.m., respectively.
‡ Radioactivity of one deficient rat administered 2.54 × 10⁷ c.p.m.
§ Stomach, small intestine, large intestine, cecum.
|| Bladder, blood, brain, heart, kidney, liver, lungs, spleen.

**Table II**

*Incorporation of Radioactivity from 2-Me-C14-AF into Choline and Serine*

<table>
<thead>
<tr>
<th></th>
<th>Normal rat</th>
<th>Riboflavin-deficient rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent of dose</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Liver choline</td>
<td>0.13</td>
<td>580 (92)*</td>
</tr>
<tr>
<td>Carcass choline</td>
<td>0.11</td>
<td>820 (61)*</td>
</tr>
<tr>
<td>Liver serine†</td>
<td>0.53</td>
<td>200</td>
</tr>
<tr>
<td>Carcass serine†</td>
<td>0.83</td>
<td>20</td>
</tr>
</tbody>
</table>

Specific activity is stated in counts per minute per mg. of compound.
* Carrier choline hydrochloride was added after the isolation of choline reineckate prior to the recrystallization steps. The amount added is indicated in parentheses.
† 225 mg. of D,L-serine carrier were added to 300 mg. of crude protein prior to hydrolysis.

**Distribution of Radioactivity in Pantothenic Acid-Deficient Animals**—The distribution and excretion data are given for only one pantothenic acid-

* Detailed colorimetric studies on the metabolism of 2-MeAF and 2-Me₂AF have recently been reported by Dyer (14).
deficient rat because it was difficult to secure survival of a rat for the required time of 72 hours after a dose of 2-MeAF in a severely deficient animal. The signs of the pantothenic acid deficiency appeared suddenly and the resistance of the treated animals decreased rapidly, so that death often occurred before the entire experiment could be concluded. However, the initial rate of radioactivity excreted in the urine and exhaled as CO₂, as well as the chromatograms of the urines collected in two partial runs, supports the results described for the single rat. 52 per cent of the radioactivity was exhaled in 72 hours, with 43, 6, and 3 per cent being found after 1, 2, and 3 days, respectively. The specific activity of the CO₂ was highest between 6 and 9 hours. The urine accounted for 21 per cent of the dose, and contained 14.6, 4.1, and 2.7 per cent, respectively, in the 3 successive days. The chromatograms of the urine showed the same number of spots with identical Rₛ values as those of the normal animals. Hence, this deficiency did not affect the metabolism of 2-MeAF.

Distribution of Radioactivity in Riboflavin-Deficient Rats—In this case also some difficulty was experienced in keeping the animals on the deficient diet alive for the 3 day experimental period after administration of the carcinogen. The results listed in Table I are averages obtained with two animals. Further data derived from three incomplete runs with other rats on a deficient diet support these results. In 1, 2, and 3 days, 23.5, 5, and 2.5 per cent respectively of the dose, or a total of 31 per cent, was expired as carbon dioxide, while the urines accounted for 37, 6, and 2 per cent, or a total of 45 per cent. A large portion of this radioactivity was due to excretion of unchanged 2-Me-C¹⁴-AF, as indicated by the intense spot with an Rₛ value corresponding to this compound on the autoradiographs of the chromatograms in three solvent systems and by carrier isotope dilution experiments. In the first 24 hour period, 28 per cent of the urinary activity was associated with 2-MeAF, as compared to 3 per cent in normal rats.

Thus, the increased excretion of unchanged 2-MeAF, the lowered exhalation of radioactive carbon dioxide, the decreased activity (Table I) in the organs and carcass, and the appreciable decline in the specific and total activity of the serine and choline (Table II) indicated that riboflavin deficiency diminished the ability of the rat to demethylate the carcinogen.

Studies on Carcinogenicity—2-MeAF was fed for the entire experimental period averaging 11.7 months. 2-MeAF was administered for an average of 11.6 months, but the animals were continued on the control diet 3.5 months longer. The carcinogen intake (Table III), calculated from individual food consumption, varied because of differences in food intake and survival time. Survival was longer and there was a greater increase in weight for both groups receiving the methyl derivatives than for the animals fed 2-AAF.
Under our conditions, 2-MeAF and 2-Me₂AF produced multiple tumors at points distant from the portal of entry (Table IV). With 2-MeAF, 2-Me₂AF, and 2-AAF, fourteen of sixteen, fourteen of seventeen, and six-

TABLE III
Average Body Weight and Carcinogen Intake

<table>
<thead>
<tr>
<th>Period of experiment in wks.</th>
<th>No. of rats surviving</th>
<th>Average body weight, gm.</th>
<th>Average daily intake, mg. per kilo body weight</th>
<th>Average total intake per rat cumulative period, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-MeAF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-40</td>
<td>13</td>
<td>195</td>
<td>12.2*</td>
<td>666</td>
</tr>
<tr>
<td>41-50</td>
<td>9</td>
<td>223</td>
<td>12.4</td>
<td>860</td>
</tr>
<tr>
<td>51-60</td>
<td>6</td>
<td>234</td>
<td>10.8</td>
<td>1038</td>
</tr>
<tr>
<td>61-75</td>
<td>1</td>
<td>249</td>
<td>9.6</td>
<td>1298</td>
</tr>
</tbody>
</table>

| 2 Me₂AF                     |                       |                          |                                               |                                                  |
| 1-42                        | 15                    | 214                      | 11.5*                                         | 748                                             |
| 43-54                       | 13                    | 236                      | 10.3†                                         | 850                                             |
| 55-76                       | 5                     | 246                      | 10.0†                                         | 1060                                            |

| 2-AAF                       |                       |                          |                                               |                                                  |
| 1-33                        | 17                    | 187                      | 12.1*                                         | 522                                             |
| 34-40                       | 8                     | 216                      | 10.7                                          | 634                                             |
| 41-50                       | 3                     | 212                      | 10.3                                          | 787                                             |

* Continuous ingestion.
† Alternate ingestion of 2-Me₂AF at weekly intervals. Intake values are based on the weeks in which 2-MeAF was ingested.

TABLE IV
Distribution and Frequency of Tumors Induced in Female Rats by Ingestion of 2-MeAF and 2-Me₂AF

<table>
<thead>
<tr>
<th>Compound</th>
<th>Site and frequency of tumor formation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breast</td>
</tr>
<tr>
<td>2-MeAF</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td>2-Me₂AF</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td>2-AAF</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

* All the tumors except one mammary tumor were verified by microscopic examination of the tissues.
teen of sixteen rats were tumor-bearing. Bielschowsky and Bielschowsky (4) found no liver tumors in female rats in the experiments with the methyl derivatives, while only a low incidence of liver tumors was obtained in the male pie-bald rats of the Sheffield strain. In addition, Miller et al. (5) found no liver tumors in either male or female rats fed 2-Me$_2$AF for 8 months and kept on a control diet 2 months longer. The Buffalo strain of rat used in our laboratory is somewhat resistant to the development of liver tumors by 2-IAF (15). Therefore, the induction of liver tumors by 2-MeAF and 2-Me$_2$AF in approximately 50 per cent of our female rats seems all the more significant.

DISCUSSION

The similarity in the rate of excretion and the total amount of radioactivity in the respiratory CO$_2$ of normal rats treated with the liver carcinogen 4-dimethyl-4'-aminoazobenzene (3) and with 2-Me-C$^{14}$-AF are noteworthy, although of course this relationship might be coincidental. These two compounds are similar in structure in so far as the alkyl group is attached to an aromatic amino nitrogen. Furthermore, both compounds are carcinogenic to the liver, although 2-MeAF is not exclusively so. On the other hand, of the dealkylated products, 4-aminoazobenzene is carcinogenic only to the extent that methylation occurs (16), whereas 2-aminofluorene is a potent carcinogen which affects a number of tissues.

Our experiments show that further methylation of 2-MeAF appears somewhat unlikely as 2-Me$_2$AF was not excreted in the urine. This is in contrast to the easily reversible methylation of 4-methylaminoazobenzene to form 4-dimethylaminoazobenzene (17). Correspondingly, 2-MeAF is not present in the urine of rats fed 2-acetylaminofluorene-9-C$^{14}$, although 2-AF is obtained (18).

The occurrence of radioactivity in the $\beta$-carbon of liver and carcass serine, in the methyl and ethanolamine parts of choline, and in urinary creatinine suggests that the pathway of the methyl carbon of 2-MeAF leads to formaldehyde or related 1 carbon compounds (19) similar to that of the methyl group of 4-methylaminoazobenzene. However, there is a striking difference between the effect of a low riboflavin level on the demethylation of an azo dye, as described by Miller et al. (2), and our findings on the demethylation of 2-Me-C$^{14}$-AF. In our experiments the respiratory radioactivity and the activity incorporated in the serine and choline in deficient rats were considerably lower than in normal animals. In contrast, Miller et al. (2) found an increase in the radioactivity excreted in the CO$_2$ and incorporated into the serine with riboflavin-deficient rats fed 3'-methyl-4-dimethyl-4'-aminoazobenzene.

Our results clearly indicate that the vitamin deficiency actually inhibited
the initial reaction in the sequence of steps from 2-MeAF to serine, choline, and carbon dioxide, since the chromatograms as well as the isotope dilution analysis demonstrated a considerable excretion of unaltered 2-MeAF in the urine of the animals on a deficient diet as compared to those on a normal diet. Thus, the enzyme system carrying out demethylation may be a flavoprotein, or the reaction could be indirectly coupled to a flavoprotein as suggested by La Du et al. (20). An earlier case of a demethylase containing flavin-adenine dinucleotide as a prosthetic group has been recorded by Moritani et al. (21) and Hirohata (22).

SUMMARY

1. The respiratory carbon dioxide, urine, and feces, collected over a 72 hour period, accounted for 54, 22, and 12 per cent, respectively, of the radioactivity from 2-methyl-C14-aminofluorene administered to normal rats. Riboflavin deficiency altered this distribution to 31, 45, and 4 per cent, respectively, while pantothenic acid deficiency had no appreciable effect.

2. Liver and carcass serine and choline and urinary creatinine were radioactive. The specific radioactivity of serine and choline was lower in riboflavin-deficient than in normal animals.

3. Unchanged 2-Me-C14-AF accounted for 3 and 28 per cent of the urinary radioactivity in normal and riboflavin-deficient rats, respectively.

4. Evaluation of the effect of dietary conditions on the demethylation of 2-Me-C14-AF leads to the tentative conclusion that a flavin cofactor plays a rôle in this reaction.

5. 2-Dimethylaminofluorene, a potential metabolite of 2-Me-C14-AF, was not present in the urine. A diazotizable amine amounted to about 4 per cent of the urinary metabolites excreted in 24 hours.

6. Multiple tumors, including hepatomas, were induced in Buffalo strain female rats by feeding 2-methylaminofluorene, 2-dimethylaminofluorene, and 2-acetylaminofluorene at a level of 0.250 gm. per kilo of diet over 7.5 to 11.5 months.

BIBLIOGRAPHY

THE FATE OF THE METHYL GROUP IN 2-METHYL-C$^{14}$-AMINOFUORENE, INCLUDING STUDIES OF THE CARCINOGENICITY OF 2-METHYL- AND 2-DIMETHYLAMINOFUORENE

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