The Effects of a Marginally Lipotrope-deficient Diet on the Hepatic Levels of S-Adenosylmethionine and on the Urinary Metabolites of 2-Acetylaminofluorene in Rats

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SUMMARY

Hepatic levels of S-adenosylmethionine (AdoMet), of glutathione, and of the microsomal enzymes p-nitroanisole demethylase and benzo(a)pyrene hydroxylase were measured in male and female rats fed a diet marginally deficient in choline and methionine and void of folic acid (lipotrope deficient) or an adequate diet for 0 to 14 weeks with and without added 2-acetylaminofluorene (AAF). The urinary metabolites of AAF were determined throughout the experimental period. After 2 to 4 weeks of dietary administration, the hepatic AdoMet levels were 43% lower in male rats fed the lipotrope-deficient diet than in male rats fed the lipotrope-adequate diet; no differences were found in hepatic AdoMet of females fed the lipotrope-deficient or lipotrope-adequate diets for 2 to 14 weeks. Administration of AAF to lipotrope-deficient female rats for 2 weeks led to a transient decrease in hepatic levels of AdoMet. The administration of AAF for 2 to 14 weeks did not significantly affect hepatic AdoMet in female rats fed the lipotrope-adequate diet or in male rats fed either diet. Female rats fed the lipotrope-deficient diet and treated with AAF excreted decreased proportions of N-hydroxy-2-acetylaminofluorene and increased proportions of 5-hydroxy-2-acetylaminofluorene in their urine. However, the urine of lipotrope-deficient male rats treated with AAF contained increased proportions of N-hydroxy-2-acetylaminofluorene and decreased levels of 5-hydroxy-2-acetylaminofluorene. The urinary excretion of 7-hydroxy-2-acetylaminofluorene by male and female lipotrope-deficient rats treated with AAF was generally similar to that in lipotrope-adequate rats. The lipotrope-deficient diet did not appear to alter the hepatic levels of glutathione, p-nitroanisole demethylase, or benzo(a)pyrene hydroxylase in male or female rats. Benzo(a)pyrene hydroxylase activity was lower in the livers of lipotrope-deficient male rats treated with AAF for 8 to 14 weeks than in the livers of lipotrope-deficient rats not receiving the carcinogen. The altered metabolism of AAF correlated well with the previously reported effects of a marginal lipotrope deficiency on AAF carcinogenesis.

INTRODUCTION

The dietary content of the lipotropes methionine, choline, folic acid, and vitamin B₁₂ influences induction of hepatic tumors by several different chemicals. While chemical carcinogens often alter lipotrope metabolism. Induction of hepatic carcinoma by aflatoxin B₁₂, dibutylnitrosamine, or AAF was increased in rats fed a diet marginally deficient in lipotropes and high in fat (19–22). However, diets that contained increased amounts of vitamin B₁₂ enhanced induction of hepatic carcinoma by N,N-dimethyl-4-aminooazobenzene (3, 11), AAF (13), and diethylaminoethylamine (17). Marginal deficiency of lipotropes depressed hepatic microsomal oxidase activity and decreased the clearance of diethylaminoethylamine from blood and liver of rats (21, 23). Diethylaminoethylamine treatment decreased hepatic AdoMet content, induced folate deficiency as measured by folate stores in the liver and by formiminoglutamatic acid excretion in the urine, and precipitated an acute lipotrope deficiency in animals fed the marginally deficient diet (2, 18, 19).

AAF is an excellent compound with which to study the metabolic interactions of diet and carcinogens. AAF metabolism has been extensively studied; the pathways for its activation and inactivation are known more fully than those for many other hepatocarcinogens and can be directly related to susceptibility to AAF carcinogenesis of different organs, species, and sexes (5, 6, 8, 9, 12, 24). Male Sprague-Dawley and female Fischer rats fed AAF in a diet marginally deficient in lipotropes had a higher incidence of hepatocarcinomas and a shorter latent period before death with tumor than did rats fed AAF in an adequate control diet (20). Female Sprague-Dawley rats developed no liver tumors in response to AAF, but mammary tumors were induced in high incidence; rats fed the adequate diet had a higher incidence of mammary tumors than lipotrope-deficient rats in 1 experiment and an approximately equal incidence in a 2nd experiment. Further, methionine, a known nucleophilic target of AAF in vivo (12), has been shown to inhibit the carcinogenic activity of low doses of AAF (10).

To study the role of lipotropes in AAF carcinogenesis, we have measured the urinary metabolites of AAF and the hepatic levels of AdoMet in male and female rats fed either the marginally lipotrope-deficient or the lipotrope-adequate diet. To obtain further information relating carcinogen metabolism to diet, the hepatic levels of glutathione, a potent...
tial nucleophilic target of AAF \textit{in vivo}, and of 2 microsomal oxidases were also determined.

**MATERIALS AND METHODS**

**Animals.** Male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were fed a diet adequate in all known respects for rats (Diet 1) or a diet marginally deficient in lipotropes and high in fat (Diet 2) (Table 1) for 3 weeks. AAF was then incorporated into the experimental diets in the amounts and for the periods used in the carcinogenesis experiments cited (20).³ Male rats were fed AAF: 0.02%, 2 weeks; 0%, 1 week; 0.0125%, 5 weeks; 0.02%, 1 week; 0%, 1 week; and 0.0125%, 6 weeks. Female rats were fed AAF: 0.02%, 2 weeks; 0%, 1 week; and 0.0125%, 11 weeks. There were 27 male or female rats per diet group fed AAF and 18 fed Diet 1 or Diet 2 alone. AAF intake was determined by measurement of food intake 1 week in 4. Results were averaged and projected through the intervening periods of urine collection. After 3 weeks of ingestion of the diets alone and after 2, 8, or 14 weeks of AAF feeding, groups of 6 AAF-treated and 4 diet-control male or female rats from each of the 2 diet groups were killed by decapitation. Their livers were taken for assay of GSH; of 2 microsomal oxidases, BPOH and PNA;³ and of AdoMet. Histological sections were prepared from all livers by routine methods.

For determination of the urinary metabolites of AAF at specified time periods, 3 male or female AAF-treated and 2 male or female diet-control rats were placed in metabolism cages, and the urine was collected in the presence of 0.1 ml toluene for 24 hr. The urine was frozen and stored for subsequent analysis of the hydroxylated metabolites of AAF. Urine samples were obtained from female rats after 2, 8, and 14 weeks of AAF feeding; with male rats the urine collections were made after 3, 9, and 16 weeks of AAF administration. Urine volumes were recorded but were not found to vary substantially between groups. Food, with and without carcinogen, and water were available throughout the period of urine collection.

The same rats were used at each of the urine collection periods. The urinary metabolites of AAF were determined spectrophotometrically following their isolation by the paper chromatographic techniques customarily used in this laboratory (5, 25).

The microsomal oxidases were measured using standard methods (21). GSH was determined spectrophotometrically (7). Hepatic AdoMet was assayed by a new method using isotope dilution, reineckate precipitation, and thin-layer chromatography developed in this laboratory (15). The urine samples containing AAF metabolites and the liver extracts for AdoMet determinations were prepared at Massachusetts Institute of Technology and sent frozen to the National Cancer Institute for subsequent analyses; all samples were coded, and were thus run blind at the National Cancer Institute.

**RESULTS**

**Growth.** Rats fed Diet 1, which is adequate in all respects known, grew normally; rats fed Diet 2 grew at a slightly decreased rate, although the differences were not great. AAF depressed weight gain in both diet groups (Chart 1).

**Histology.** In the livers of male rats examined 8 weeks after the beginning of AAF treatment there was moderate to marked proliferation of bile ducts and formation of clusters of eosinophilic or vacuolated cells; at 14 weeks the changes were much more extensive. There were a great number of eosinophilic nodules than at 8 weeks and small foci of markedly basophilic hepatocytes; both changes are associ

\begin{table}[h]
\centering
\caption{Composition of Diets 1 and 2}
\begin{tabular}{l|cc}
\hline
Component & Diet 1 & Diet 2 \\
\hline
Casein & 22 & 3 \\
\hline
Methodol-extracted peanut meal & 12 & 12 \\
Gelatin & 6 & 6 \\
Fibrin & 1 & 1 \\
Sucrose, dextrose, and dextrin & 55.7 & 36.3 \\
Cellulose flour & 2 & 2 \\
Mazola oil & 2 & 2 \\
Beef fat & 30 & 30 \\
Wesson oil & 15 & 15 \\
Vitamin mix & 2³ & 2³ \\
Salt, Rogers-Harper's & 5 & 5 \\
L-Cystine & 0.5 & 0.5 \\
Choline chloride & 0.3 & 0.2 \\
\hline
\multicolumn{3}{l}{³ Vitamins, mg/kg diet: vitamin A, 10; vitamin D₂ (IU), 3000; vitamin E, 169; menadione, 1; niacin, 50; calcium pantothenate, 20; riboflavin, 4; thiamine-HCl, 8; pyridoxine-HCl, 8; folic acid, 10; inositol, 250; vitamin B₁₂, 0.05.}
\end{tabular}
\end{table}

\[\text{Chart 1. Body weights of rats fed experimental diet. AAF was added to diets when rats were 6 weeks of age. There were 18 control and 27 AAF-fed rats per group initially.}\]
ated with hyperplasia in carcinogen-treated livers (21). The changes were the same in the 2 dietary groups. There was a small amount of fat in hepatocytes of both dietary groups treated with AAF and control animals fed Diet 2.

In female rats the changes induced by AAF in the liver were much less marked. After 8 weeks there was minimal bile duct proliferation in some but not all livers; after 14 weeks there was still minimal bile duct proliferation with occasional small foci of vacuolated or hyperbasophilic hepatocytes. In a few rats there were small cystic bile ducts. As in male rats there were scattered fatty cysts in Diet 2 control animals.

Liver, AdoMet, GSH, and Oxidases. Feeding the marginally lipotrope-deficient diet (Diet 2) to male rats resulted in a 43% decrease in hepatic AdoMet compared to the levels seen in male rats fed Diet 1 (Table 2). AdoMet levels in livers of female rats were not altered by Diet 2. However, administration of AAF for 2 weeks to female rats fed Diet 2 did lead to a significant decrease in hepatic content of AdoMet (Table 2). The administration of AAF to male rats fed Diet 1 or 2 and to female rats fed Diet 1 had no significant effect on the AdoMet levels in liver; the 24% decrease in hepatic AdoMet seen in male rats fed Diet 1 plus AAF for 8 weeks and the 25% increase in AdoMet observed in female rats fed Diet 1 plus AAF for 2 weeks were not statistically significant. GSH concentration of the liver was not affected by diet. In male rats it tended to rise slightly during AAF treatment, but in female rats there was no consistent effect.

Both microsomal oxidases were lower in control male and female rats fed Diet 2 than in rats fed Diet 1; there was wide variation in enzyme level between animals, and differences between diet groups were not statistically significant (Table 2). In male rats fed Diet 1, PNA was increased after 8 weeks of AAF feeding and was significantly greater than it was in rats fed AAF in Diet 2 at that time. There was no evidence of enzyme induction in rats fed Diet 2, but there was a signifi-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver wt (g)</th>
<th>AdoMet (μg/g liver)</th>
<th>GSH (μg/g liver)</th>
<th>PNA (μg p-nitrophenol liberated/g liver/hr)</th>
<th>BPOH (quinine units/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet 1</td>
<td>11.3 ± 2.1a</td>
<td>62.6 ± 5.2</td>
<td>2.8 ± 0.3</td>
<td>199 ± 24</td>
<td>19.5 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>(5.7-16.0)c</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Diet 1 + AAF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk</td>
<td>8.3 ± 0.3</td>
<td>64.2 ± 13.1</td>
<td>3.2 ± 0.1</td>
<td>192 ± 14</td>
<td>22.8 ± 0.7</td>
</tr>
<tr>
<td>8 wk</td>
<td>14.2 ± 0.6</td>
<td>47.3 ± 2.9</td>
<td>3.5 ± 0.1</td>
<td>258 ± 14</td>
<td>14.6 ± 1.6</td>
</tr>
<tr>
<td>14 wk</td>
<td>16.6 ± 2.2</td>
<td>62.2 ± 9.4</td>
<td>3.7 ± 0.1</td>
<td>178 ± 8</td>
<td>17.8 ± 1.8</td>
</tr>
<tr>
<td>Diet 2</td>
<td>12.7 ± 1.9</td>
<td>35.6 ± 5.9</td>
<td>3.0 ± 0.1</td>
<td>148 ± 19</td>
<td>16.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>(7.4-15.5)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Diet 2 + AAF</td>
<td></td>
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</tr>
<tr>
<td>2 wk</td>
<td>7.0 ± 0.8</td>
<td>36.2 ± 7.2</td>
<td>3.0 ± 0.3</td>
<td>149 ± 8</td>
<td>14.7 ± 7a</td>
</tr>
<tr>
<td>8 wk</td>
<td>13.4 ± 1.2</td>
<td>31.4 ± 4.2</td>
<td>3.3 ± 0.1</td>
<td>160 ± 27a</td>
<td>11.2 ± 2.0a</td>
</tr>
<tr>
<td>14 wk</td>
<td>16.3 ± 1.6</td>
<td>44.6 ± 4.9</td>
<td>3.6 ± 0.3</td>
<td>145 ± 12</td>
<td>10.5 ± 2.1a</td>
</tr>
<tr>
<td>Diet 1</td>
<td>7.7 ± 0.4</td>
<td>58.5 ± 4.9</td>
<td>2.5 ± 0.2</td>
<td>177 ± 43</td>
<td>13.8 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>(6.5-8.4)</td>
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<td></td>
</tr>
<tr>
<td>Diet 1 + AAF</td>
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<tr>
<td>2 wk</td>
<td>7.0 ± 0.7</td>
<td>73.0 ± 4.2</td>
<td>2.8 ± 0.3</td>
<td>186 ± 14</td>
<td>18.2 ± 3.2a</td>
</tr>
<tr>
<td>8 wk</td>
<td>8.0 ± 0.5</td>
<td>54.7 ± 0.9</td>
<td>2.5 ± 0.3</td>
<td>129 ± 31</td>
<td>9.1 ± 1.8</td>
</tr>
<tr>
<td>14 wk</td>
<td>9.7 ± 0.5</td>
<td>51.3 ± 10.1</td>
<td>2.7 ± 0.1</td>
<td>262 ± 120</td>
<td>10.1 ± 1.3</td>
</tr>
<tr>
<td>Diet 2</td>
<td>8.4 ± 0.9</td>
<td>58.4 ± 7.2</td>
<td>2.8 ± 0.2</td>
<td>109 ± 7</td>
<td>9.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>(6.5-10.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet 2 + AAF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk</td>
<td>8.2 ± 0.5</td>
<td>28.5 ± 3.2a</td>
<td>3.2 ± 0.2</td>
<td>147 ± 20</td>
<td>11.5a</td>
</tr>
<tr>
<td>8 wk</td>
<td>8.4 ± 0.3</td>
<td>62.7 ± 3.3</td>
<td>2.5 ± 0.1</td>
<td>104 ± 6</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>14 wk</td>
<td>8.0±</td>
<td>44.6 ± 4.8</td>
<td>2.8 ± 0.1</td>
<td>187 ± 23a</td>
<td>12.8 ± 2.0</td>
</tr>
</tbody>
</table>

* There were 8 diet controls and 3 AAF-treated rats per group, except where noted otherwise. The 8 diet controls were obtained by sacrificing 2 rats at 0, 2, 8, and 14 weeks; times are given from the beginning of AAF ingestion.

* Mean ± SE.

* Numbers in parentheses, range of control liver weights.

* Significantly different from rats fed Diet 1 and AAF at same time period.

* Significantly different from diet control (p < 0.05).

* Mean of 2 animals.

* Single value.
significant depression of BPOH in them after 8 and 14 weeks of AAF treatment. No consistent effect of AAF treatment on BPOH in rats fed Diet 1 was found. In female rats fed either diet, PNA was increased over control levels after 14 weeks of AAF feeding but not earlier. BPOH was not significantly affected by AAF treatment.

The total hepatic contents of AdoMet, GSH, PNA, and BPOH in each of the groups were less in female rats than in male rats, largely because the females have smaller livers (Table 2). No other consistent findings could be observed following determination of the total liver contents of AdoMet, GSH, PNA, and BPOH.

**AAF Metabolism.** The metabolism of AAF was markedly altered by diet in both male and female rats (Table 3). The relative amounts of N-hydroxy-AAF and 5-hydroxy-AAF were most affected. In general, feeding Diet 2 to male rats tended to increase the relative amount of N-hydroxy-AAF excreted and to decrease 5-hydroxy-AAF. For example, the ratio of the amounts of N-hydroxy-AAF to the amounts of 5-hydroxy-AAF excreted by male rats fed Diet 1 and AAF for 9 weeks was 0.11; the corresponding ratio for male rats fed Diet 2 was 1.0. With female rats the situation was reversed. Feeding Diet 2 to female rats decreased the relative amounts of N-hydroxy-AAF excreted into the urine while increasing 5-hydroxy-AAF excretion (Table 3). Thus, no N-hydroxy-AAF could be detected in the urine of lipotrope-deficient female rats treated with AAF for 8 and 14 weeks, whereas N-hydroxy-AAF accounted for approximately 20% of the acetylated hydroxy metabolites of AAF excreted by female rats fed Diet 1 (Table 3). 5-Hydroxy-AAF accounted for approximately 56% of the hydroxy-2-acetylaminofluorene metabolites detected in the urines of female rats fed Diet 1 plus AAF (Table 3); this value was increased to 76% in female rats fed Diet 2. 7-Hydroxy-2-acetylaminofluorene excretion was relatively unaffected by diet, although a slight increase in the proportion of 7-hydroxy-2-acetylaminofluorene in male rats and a significant decrease in that in female rats was noted in animals fed Diet 2 (Table 3). The levels of 1- and 3-hydroxy-2-acetylaminofluorene seen in the urines of animals treated with AAF were extremely low; random 24-hr urine samples obtained from rats treated with AAF contained only ~5 µg/rat/day for each metabolite. The 1- and 3-hydroxy metabolites were not quantitated further.

**DISCUSSION**

The results indicate that feeding AAF-treated male and female rats a lipotrope-deficient diet alters the urinary excretion of N-hydroxy-AAF in a manner consistent with the effects of the diet on carcino genesis by AAF (20). Male Sprague-Dawley AAF-treated rats fed Diet 2 excreted a higher proportion of N-hydroxy-AAF and developed more hepatomas than did AAF-treated rats fed Diet 1 (20). On the other hand, female Sprague-Dawley rats fed the deficient diet excreted less N-hydroxy-AAF and, in 1 experiment, developed fewer mammary tumors than did corresponding animals fed the adequate diet. The correlation between the proportion of N-hydroxy-AAF in the urine and carcinogenic activity of AAF has been well documented (5, 6, 12, 16, 24–26).

The relationship between AAF and hepatic AdoMet levels is more difficult to discern. Previous studies have shown that chronic diethylnitrosamine administration decreases the AdoMet levels in rat liver (1). Our present studies on AdoMet confirm and extend previous observations that the...
hepatic levels of AdoMet are greatly altered by the exogenous supply of methyl donors and that female rats are more resistant than males to dietary deficiencies of methyl donors (1, 2, 14). The chronic administration of AAF did lead to a significant drop in the levels of AdoMet seen in the livers of female rats fed the adequate diet for 2 weeks; these hepatic AdoMet levels returned to normal after 8 and 14 weeks of AAF feeding, when no N-hydroxy-AAF could be detected in the urine. The 25% decrease in the hepatic AdoMet of male rats fed AAF in Diet 1 for 8 weeks and the increase in hepatic AdoMet in female rats fed the same diet and AAF for 2 weeks did not prove to be statistically significant (p < 0.12 in each case). It is possible that increasing the number of animals used in these groups would have demonstrated a significant effect of AAF feeding on hepatic AdoMet. The high variabilities in hepatic AdoMet levels in rats (1, 2) would seem to require relatively high numbers of animals to demonstrate that a 20 to 30% change was statistically significant. The present experiments were designed to determine whether the effects of the lipotrope-deficient diet on AAF carcinogenesis occurred through an alteration of AAF metabolism or by the induction of a methyl deficiency which may be an essential step in the induction of cancer by AAF. The diet-induced alterations of AAF metabolism were closely correlated with its carcinogenicity.

AAF administration of male rats increased the hepatic GSH levels, confirming the findings of Fiala et al. (4). PNA plus BPOH were consistently but not significantly lower in rats fed Diet 2 than in rats fed Diet 1 and were variably affected by AAF; they did not correlate with dietary effects of AAF carcinogenesis. The amounts of urinary hydroxy-2-acetylaminofluorene metabolites relative to the AAF ingested were lower in the present investigation than in previous studies, possibly because of the low dietary levels of AAF used in this investigation (24, 26).

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