Effects on Hepatic Microsomal N- and C-oxygenation of Aromatic Amines by in Vivo Corticosteroid or Aminofluorene Treatment, Diet, or Stress

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SUMMARY

Various environmental, endocrine, and inherent conditions, which enhance the tumor-inducing capacity of carcinogenic aromatic amines, are associated with an increased N-oxygenation of these compounds in vivo. The impairment of protein-synthesizing functions of liver ribosomes by reactive metabolites of the carcinogenic amines is, to a certain extent, correlated with these functions.

Here the effect of similar conditions on microsomal N-oxygenation and C-oxygenation in vitro was studied, using N-oxide formation and N-demethylation of N,N-dimethylaniline as test reactions. Increased microsomal N-oxygenation, and to a considerably smaller extent N-demethylation was obtained by in vivo treatment with corticosteroid or 2-aminofluorene. Vitamin E depletion, protein deficiency, or stress caused an increase in N-oxygenation accompanied by decreased N-demethylation. These conditions thus gave rise to an increase in the N-oxygenation/C-oxygenation ratio.

Guinea pig liver microsomes showed a higher N-demethylating capacity and a lower N-oxygenation than did rat liver microsomes.

The N-oxygenation of N,N-dimethylaniline by liver microsomes thus seems to be well correlated with the tumor-inducing properties and ribosome-impairing capacity of carcinogenic aromatic amines under various in vivo conditions.

INTRODUCTION

Carcinogenic aromatic amines, e.g., AF₂, AAF, and N-methylated aminoazo dyes, generally exert their tumor-inducing action at sites distant from the area of application (51, 65). This suggests that a metabolic process is required for the manifestation of their carcinogenic properties. As the liver is the main target organ for these substances (51, 65), considerable interest has been focused on the metabolism of aromatic amines in liver cells. It has been shown that microsomal enzymes catalyze the stepwise transformation of aromatic amines into water-soluble conjugates which can be eliminated from the body (67). During this process, however, reactive intermediates can be formed which manifest themselves by their ability to bind to proteins and nucleic acids in the cells (12, 47).

This binding occurs primarily to the microsomal membranes themselves (22-24). The ribosomes of liver cells, most of which are attached to these structures, are liable to functional disturbances by the action of the reactive metabolites. When animals are treated with carcinogenic amines, or when liver slices are incubated in vitro in the presence of these substances, an impairment of the functions of ribosomes in protein synthesis in vitro has been observed (5, 6, 25-27). The manifestation of the ribosomal inhibition (6, 7, 25, 26), as well as the carcinogenicity of the amines (16, 51, 65, 66), depends, however, on a variety of genetic, environmental, and hormonal factors. There is a certain, although not complete, correlation between the manifestation of the carcinogenic properties and the effects on protein synthesis in vitro (6, 7, 25).

In the last few years substantial evidence has been produced in favor of metabolites of aromatic amines oxygenated on the nitrogen atom being proximal carcinogenic metabolites, in contrast to the less carcinogenic metabolites oxygenated on various carbon atoms (48-50). N-oxygenation of various kinds of amines has been demonstrated in vivo as well as in isolated microsomal systems (11, 28, 59-61, 69, 70). Furthermore, N-oxygenated derivatives of aromatic amines are metabolized in vivo and in vitro to the same ring-hydroxylated or N-dealkylated products as the parent compounds themselves (19, 20, 28, 39, 46, 54, 60-62). It has therefore been postulated by some authors that the N-oxygenated metabolites are intermediates in the microsomal NADPH₂-dependent C-oxygenation in ortho, para or α-position, i.e., ring-hydroxylation and N-dealkylation of aromatic amines (39, 44, 60, 69). However, this view has not been accepted unanimously (29, 44).

With monocyclic aromatic amines, like DMA, no N-oxygenated products can be demonstrated in vivo (19, 20). Nevertheless, in experiments with isolated microsomes, significant amounts of DMA-N-oxide are formed. The accumulation of the

1 This work was supported by research grants from the Swedish Cancer Society.

2 The abbreviations used are: AAF, 2-acetylaminofluorene; AF, 2-aminofluorenone; AN, 2-aminoanithalene; DAB, 4-dimethylaminoazobenzene; DMA, N,N-dimethylaniline; DMA-N-oxide, N,N-dimethylaniline-N-oxide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH₂, reduced nicotinamide adenine dinucleotide phosphate; N-OH-AAF, N-hydroxyacetylaminofluorene.

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N-oxygenated product is provoked by ultrasonication of the microsomes or exposing them to cholate treatment (3, 25, 69, 70).

The aim of the present investigation was to study whether the accumulation of N-oxygenated metabolites of aromatic amines in microsomal systems is influenced by in vivo conditions related to tumor induction. The microsomal N-oxygenation and C-oxygenation of DMA to DMA-N-oxide and N-monomethylaniline, respectively, was used as a test system for the intactness of the microsomal detoxication pathway for aromatic amines. It was shown that various environmental, hormonal, and genetic factors increase the relative amount of N-oxygenated products formed in microsomal systems. The increased N-oxygenation in vitro is correlated with the effect of the same factors on tumor induction by aromatic amines, and also with their influence on the effect of these carcinogens on microsomal protein-synthesizing functions. Some data giving evidence of such correlations have been published previously (4, 25).

MATERIALS AND METHODS

Animals. Male rats (150-250 gm) of a Sprague-Dawley strain purchased from Anticimex AB, Rotebro, Sweden, and male guinea pigs (300-550 gm) of a local breed were used in the experiments. The animals were starved for 16-20 hours before being killed.

Diets. The standard diet used for rats was Anticimex rat pellets No. 210 (21.9% digestible protein, vitamin E content 30 ~g/gm). The guinea pigs were fed Fors rabbit pellets, fresh swedes, and hay. The vitamin E-deficient diet employed was a slight modification of diet III of Hultin and Arrhenius (26) (Torula yeast 30%, potato starch 29%, sucrose 33%, stripped lard 5%, salts, and vitamins). The protein-free diet was obtained by substitution of potato starch for Torula yeast in the diet. AAF and ~-tocopherol acetate dissolved in acetone were added to a small portion of the diet. The acetone was allowed to evaporate before this mixture was added to the whole diet batch. As in earlier experiments (26), the animals were fed the standard diet until the age of 6 weeks in order to prevent manifest symptoms of vitamin E deficiency.

The stripped lard (freed from vitamin E on molecular distillation) was obtained from Distillation Products Industries, Rochester, N.Y., and dried Torula yeast from Waldhof Zellstoff Fabrik, Mannheim, W. Germany.

Chemicals. AF and AAF were prepared as described before (7); ~-hydrocortisone (prednisolone) was purchased from Merek, Sharpe and Dohme, Haarlem, Netherlands (Codeleor); Celite (diatomaceous earth) from Johns Manville International Corp., New York, N.Y.; ~-tocopherol acetate from Itoffman La Roche, Basel, Switzerland; ATP, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase from Sigma Chemical Co., St. Louis, Mo.; pyruvate kinase from Boehringer GmbH, Mannheim, W. Germany. Phosphoenolpyruvate was synthesized as described before (7). The L-leucine-14C was supplied by New England Nuclear Corporation, Boston, Mass. The labeled compound was diluted with unlabeled L-leucine to a specific activity of 2.5 mc/mmole L-leucine.

Pretreatment of Animals. For short-term experiments AF or AAF was suspended in an agate mortar with 2 ml of 10% gum acacia; 1 ml was given by stomach tube and 1 ml was injected intraperitoneally. Prednisolone was obtained as a suspension (10 mg/ml) and injected i.p. Celite was suspended in 1 ml saline and injected i.p.

In Vitro Systems

Preparation of Subcellular Fractions. The animals were killed by decapitation, and the livers were removed rapidly and minced in the appropriate ice-cold medium. After repeated washings the mince was homogenized in 2.5 volumes of the same medium using a glass homogenizer with Teflon pestle. The homogenate was centrifuged at 14,000 X g for 6 min in a refrigerated International PR-1 centrifuge. The supernatants were centrifuged in a Spinco L centrifuge at 105,000 X g for 55 min. The microsomal pellets were either suspended in medium with a small manual homogenizer to a concentration of 10-15 mg microsomal protein/ml and used immediately in the experiments, or washed by resuspension in the original volume of medium and a second 105,000 X g centrifugation. The pellet was resuspended as above and used in the experiments.

Oxidative N-demethylation and N-oxide Formation. The medium used for the preparation of microsomes was 0.2 M potassium phosphate buffer, pH 7.5, or in some cases 0.175 M KCl in 0.035 M Tris buffer, pH 7.5 (35°C). Unless otherwise mentioned, the microsomes were washed by recentrifugation in the same medium.

The standard incubation system contained, in final volumes of 2.0 ml, 0.2 ml microsomal suspension (2-3 mg microsomal protein), 0.1 ~moles NADP, 10 ~moles glucose-6-phosphate, 120 ~moles nicotineamide, 0.25 Kornberg units glucose-6-phosphate dehydrogenase, 10 ~moles DMA and 200 ~moles/ml potassium phosphate buffer pH 7.5. The tubes were incubated aerobically with gentle shaking at 35°C. The reaction was terminated by the addition of 1 ml 0.9 M perchloric acid, except in some early demethylation experiments in which 1 ml of 20% trichloroacetic acid was used. This difference did not affect the final analytical result.

Enzymatic NADPH2-dependent Lipid Peroxidation. The incubation conditions were the same as for N-demethylation and N-oxygenation, except that DMA was omitted. The incubation was interrupted by the addition of 0.5 ml of 40% trichloroacetic acid and 0.25 ml 5 M HCl (26).

Amino Acid Incorporation. The homogenization medium contained 0.25 M sucrose, 0.075 M KCl, 0.01 M MgCl2 and 0.035 M Tris buffer, pH 7.8. The mitochondrial supernatants were incubated at 35°C with L-leucine-14C and a nucleoside triphosphate-generating system consisting of ATP, phosphoenolpyruvate and pyruvate kinase as described earlier (7). Aliquots (0.1 ml) of the incubation mixture were transferred to filter paper disks, which were immersed in cold 10% trichloroacetic acid (40).
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ANALYTICAL METHODS

Formaldehyde produced in the oxidative N-demethylation of DMA was determined by the method of Nash (53) slightly modified. Proteins were removed from the acidified incubation mixtures by centrifugation. Two ml of the supernatants were mixed with 0.6 ml of Nash reagent, and the color was allowed to develop for 30 minutes at 35°C. At this low temperature maximum color intensity was reached within this period. At the higher temperature originally recommended (60°C) the color had a tendency to fade after 5 min in trichloroacetic acid or perchloric acid. The optical density at 415 nm was determined in a Beckman DB spectrophotometer, and the amount of formaldehyde was calculated from standards run in parallel.

DMA-N-oxide was determined by the method of Ziegler and Pettit (69). Malondialdehyde formed in lipid peroxidation was determined as described earlier (26). All determinations of N-demethylation, N-oxygenation and lipid peroxidation were corrected for the small increase in O.D. obtained in the absence of NADPH₂. The protein content of the microsomal suspensions was determined by the method of Lowry et al. (38).

Labeled proteins were processed by the method of Mans and Novelli (40). The radioactivity was determined using a Packard TriCarb liquid scintillation counter with a counting efficiency of 45% for ¹⁴C. The values obtained were recalculated to cpm/mg microsomal protein.

The enzyme activities within groups of control animals from the same experiment showed rather small variation. In agreement with others (32, 33), however, certain variations were observed between different batches of animals.

The experimental values are expressed as percent of control animals within the same experiment, with standard errors and probability for identity with control calculated from all experiments. Primary values for control animals from all experiments with standard errors are given in each table.

RESULTS

Effects of Vitamin E Deprivation and AF-treatment in Vivo on Microsomal N- and C-oxygenation of DMA. It was shown previously that vitamin E protects liver ribosomes against the harmful effects of large doses of AF or AN, administered in vivo (25, 26). The experimental data suggested that this effect of vitamin E might be related to a higher efficiency of the liver microsomes in transforming the aromatic amines to nontoxic metabolites. The experiments described in this section illustrate this effect of vitamin E.

Rats, which had been maintained on standard food until the age of 6 weeks, were fed a vitamin E-depleted diet 30-40 days. As is shown in Table 1, microsomal preparations from depleted rats had markedly decreased capacities to N-demethylate DMA, whereas the accumulation of DMA-N-oxide was much higher than with corresponding preparations from normal animals. The total amount of DMA oxygenated (expressed as the sum of DMA-N-oxide and formaldehyde produced) was increased somewhat. Addition of 2 mg α-tocopherol acetate per gm diet for 3-4 days strongly reduced the accumulation of N-oxide and increased the N-demethylation to values well above normal. The sum of N-oxide and formaldehyde was decreased under the influence of the vitamin. The quotient N-oxide/formaldehyde was increased by more than 100 percent by the vitamin depletion and decreased to values below the controls after vitamin E administration.

Table 2 illustrates how a 4-hour AF treatment in vivo affects the oxidative N-demethylation of DMA by microsomes isolated from normal and vitamin E-depleted rats. The treatment with

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of experiments</th>
<th>1 N-demethylation (HCHO formed)</th>
<th>2 N-oxygenation (N-oxide formed)</th>
<th>1 + 2 Total DMAoxidation (N-oxide+HCHO)</th>
<th>2/1 N-demethylation (N-oxide/HCHO)</th>
<th>Lipid peroxidation (ETBA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-depleted</td>
<td>8</td>
<td>66 ± 2</td>
<td>190 ± 11</td>
<td>114 ± 3</td>
<td>295 ± 13</td>
<td>261 ± 47</td>
</tr>
<tr>
<td>Probability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-depleted vs. control</td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>E-supplemented</td>
<td>6</td>
<td>123 ± 9</td>
<td>8.1 ± 22</td>
<td>83 ± 1</td>
<td>6.8 ± 7.9</td>
<td>0</td>
</tr>
<tr>
<td>Probability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-supplemented vs. control</td>
<td></td>
<td>P = 0.05</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Effect of vitamin E depletion of rats on microsomal N- and C-oxygenation and NADPH₂-dependent lipid peroxidation by liver microsomal systems.

| Control values (6 animals): oxidative N-demethylation, 2.51 ± 0.30 mmoles HCHO/mg microsomal protein/min; N-oxygenation, 1.23 ± 0.31 mmoles DMA-N-oxide/mg microsomal protein/min; lipid peroxidation 0.029 ± 0.003 ETBA/mg microsomal protein/min. All values calculated over incubation interval of 0-20 min at 35°C. |
| Abbreviations: DMA, N, N-dimethylaniline; DMA-N-oxide, N, N-dimethylaniline-N-oxide; NADPH₂, reduced nicotinamide adenine dinucleotide phosphate. |
| Animals fed E-deficient diet for 30-36 days, followed by 3-4 days on the same diet supplemented with 2 mg α-tocopherol acetate/gm diet. |
| The presence of 5 mmoles DMA/ml in the incubations reduced lipid peroxidation to 2% of control values. |
Microsomal Oxygenation of Aromatic Amines

Table 2

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of experiments</th>
<th>1 N-demethylation (HCHO formed)</th>
<th>2 N-oxygenation (N-oxide formed)</th>
<th>1/2 Total DMA* oxygenated (HCHO+N-oxide)</th>
<th>2/1 N-oxygenation (HCHO/N-oxide)</th>
<th>Lipid peroxidation (ETBA)</th>
<th>Amine acid incorporation (rpm/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF-treated* (4 hr*)</td>
<td>4</td>
<td>117 ± 5</td>
<td>339 ± 28</td>
<td>184 ± 2</td>
<td>271 ± 26</td>
<td>8.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Probability AF-treated vs. control</td>
<td></td>
<td>P &lt; 0.1</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>AF-treated* (18 hr*)</td>
<td></td>
<td>117</td>
<td>244</td>
<td>162</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E-depleted</td>
<td></td>
<td>135 ± 18</td>
<td>207 ± 19</td>
<td>172 ± 17</td>
<td>183 ± 5</td>
<td>2.2 ± 0.6</td>
<td>62*</td>
</tr>
<tr>
<td>Probability E-depleted AF-treated* (4 hr*)</td>
<td>4</td>
<td>P &lt; 0.2</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.025</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>E-depleted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Effect of short-term AF-treatment in vivo on microsomal N- and C-oxygenation and NADPH₂-dependent lipid peroxidation by liver microsomal systems.

Abbreviations: AF, 2-aminofluorene; DMA, N,N-dimethylaniline; DMA-N-oxide, N,N-dimethylaniline-N-oxide; NADPH₂, reduced nicotinamide adenine dinucleotide phosphate.

Effect of dietary AAF. Dietary AAF, at a level which causes a high incidence of liver tumors in rats within 7–9 months, has been shown to increase the output of conjugated N-hydroxylated metabolites in the urine of the animals within a few weeks (50). In order to examine whether there was a change in the metabolism of aromatic amines in vivo under these conditions, rats were fed a standard diet containing 0.06% AAF for 20–25 days. As is seen in Table 3, this treatment greatly increased the accumulation of N-oxide by the liver microsomal systems. The N-demethylation was also significantly increased, although to a much smaller extent. As a result, the N-oxide/formaldehyde quotient was doubled. No significant change was observed in the NADPH₂-dependent lipid peroxidation in vitro.

Effects of Protein-free Diet. The carcinogenic potency of the aromatic amine DAB is increased when the protein content of the diet is reduced (51). Mori noted that a polished rice diet containing 0.03% AAF gave a liver tumor incidence of 44%, as compared with 20% when protein was added to the diet in the form of dried beef liver powder (65).

Rats were fed a protein-free diet for 12–15 days (Table 3). This treatment caused a pronounced reduction in the N-demethylating activity of the liver microsomes (cf. 43). At the
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Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of experiments</th>
<th>1 Oxidative demethylation (HCHO formed)</th>
<th>2 N-oxygenation (DMA-N-oxide formed)</th>
<th>2/1 N-demethylation (HCHO/N-oxide)</th>
<th>Lipid peroxidation (ETBA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF dietc</td>
<td>7</td>
<td>127 ± 5</td>
<td>222 ± 21</td>
<td>174 ± 13</td>
<td>93.6 ± 0.1</td>
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<tr>
<td>Probability AAF vs. controls</td>
<td></td>
<td>P &lt; 0.001</td>
<td>P = 0.001</td>
<td>P &lt; 0.002</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Protein-free dietd</td>
<td>5</td>
<td>52 ± 8</td>
<td>131 ± 11</td>
<td>292 ± 40</td>
<td></td>
</tr>
<tr>
<td>Probability protein-free vs. controls</td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.002</td>
<td>P &lt; 0.20</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Effects of dietary AAF or protein-free diet on the oxidative N-demethylation, N-oxygenation and NADPH$_2$-dependent lipid peroxidation by rat liver microsomal preparations.

a Control values (12 animals): oxidative N-demethylation, 2.35 ± 0.09 mmoles HCHO/mg microsomal protein/min; N-oxygenation, 2.06 ± 0.09 mmoles DMA-N-oxide/mg microsomal protein/min; lipid peroxidation, 0.0590 ± 0.0017 ETBA/mg microsomal protein/min. All values calculated over incubation interval of 0-20 min at 35°C.

b Abbreviations: AAF, 2-acetylaminofluorene; DMA, N,N-dimethylaniline; DMA-N-oxide, N,N-dimethylaniline-N-oxide; NADPH$_2$, reduced nicotinamide adenine dinucleotide phosphate.

c 0.06% AAF added to standard rat diet.

d For composition see Methods.

Table 4

<table>
<thead>
<tr>
<th></th>
<th>No. of experiments</th>
<th>1 Oxidative demethylation (HCHO formed)</th>
<th>2 N-oxygenation (DMA-N-oxide + formaldehyde formed)</th>
<th>1 + 2 Total oxygenated DMA (HCHO+N-oxide)</th>
<th>2/1 N-oxygenation (N-demethylation HCHO/O-N-oxide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>18</td>
<td>2.14 ± 0.12</td>
<td>1.94 ± 0.14</td>
<td>4.08 ± 0.23</td>
<td>0.92 ± 0.06</td>
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<tr>
<td>Guinea pig</td>
<td>18</td>
<td>2.90 ± 0.16</td>
<td>1.20 ± 0.26</td>
<td>4.10 ± 0.30</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>Guinea pig/rat x 100</td>
<td>18</td>
<td>141 ± 9</td>
<td>60 ± 9</td>
<td>102 ± 7</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>Probability guinea pigs vs. rats</td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.9</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Comparison of oxidative N-demethylation and N-oxygenation by microsomal preparations from guinea pigs and rats.

a Abbreviations: DMA, N,N-dimethylaniline; DMA-N-oxide, N,N-dimethylaniline-N-oxide.

b Mean values ± standard error for quotients obtained within individual experiments.

c Incubation, 40 min at 35°C.

same time, there was a small but significant rise in the formation of N-oxide and the N-oxide/formaldehyde quotient was more than doubled. As the increase in N-oxide formation was smaller than the decrease in N-demethylation, the total amount of DMA oxygenated was somewhat decreased, but this effect was not statistically significant in the present experiments.

Species Variations. Guinea pigs are known to be refractory to the carcinogenic action of aromatic amines (51, 65). Table 4 shows a comparison of the N-oxide formation and the N-demethylation of DMA in preparations of liver microsomes from guinea pigs and rats. The total amount of DMA oxygenated (DMA-N-oxide + formaldehyde produced) was, with high significance, the same in preparations from rats and guinea pigs. Guinea pig microsomes, however, performed the metabolism to the N-demethylated product (measured as formaldehyde) more efficiently than did the rat preparations and showed a markedly lower accumulation of N-oxygenated metabolites as indicated by the low N-oxide/formaldehyde quotient.

Effects of Prednisolone or Celite Treatment in Vivo. The carcinogenic potency of aromatic amines is dependent on a variety of exogenous and endogenous hormonal factors including adrenal corticosteroids. Adrenalectomy or hypophysectomy abolishes the carcinogenic action of AF; administration of corticosteroids, on the other hand, shortens the latent period and increases the frequency of tumors in rats given carcinogenic aromatic amines in the diet (16, 66). It was shown in previous reports that short-term treatment with AF or AAF in vivo gives rise to a corticoid-dependent stimulation of the protein-synthesizing activity of the liver ribosomes (7) that fairly soon overcompensates the early inhibitory action of the carcinogens. It was therefore of interest to study the effect of corticosteroids and adrenal-dependent stress in vivo on the metabolism of aromatic amines in microsomal systems.

Animals were given prednisolone (50-100 mg/kg body weight) intraperitoneally 16-20 hours before decapitation; N-oxide and formaldehyde production from DMA were measured in preparations of liver microsomes from these and control ani-
Microsomal Oxygenation of Aromatic Amines

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of experiments</th>
<th>N-demethylation (HCHO formed)</th>
<th>N-oxygenation (DMA-N-oxide formed)</th>
<th>Lipid peroxidation (E_TBA)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>1+2</td>
</tr>
<tr>
<td>Prednisolone (50-100 mg/kg</td>
<td>38</td>
<td>111 ± 5</td>
<td>208 ± 18</td>
<td>144 ± 6</td>
</tr>
<tr>
<td>body wt., 16-20 hours)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Probability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prednisolone vs. control</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Celite (200-225 mg/kg</td>
<td>28</td>
<td>86 ± 4</td>
<td>178 ± 10</td>
<td>118 ± 4</td>
</tr>
<tr>
<td>body wt., 16-20 hours)</td>
<td></td>
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<tr>
<td>Probability</td>
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<tr>
<td>Celite vs. control</td>
<td>P &lt; 0.002</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
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<tr>
<td>Probability Celite vs.</td>
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<td></td>
</tr>
<tr>
<td>prednisolone</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.2</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.2</td>
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</table>

Effect of treatment with prednisolone or celite in vivo on oxidative N-demethylation, N-oxygenation, and NADPH$_2$-dependent lipid peroxidation by rat liver microsomes.

*a Control values (40 experiments): N-demethylation, 2.14 ± 0.11 mmoles HCHO/mg microsomal protein/min; N-oxygenation, 1.30 ± 0.00 mmoles DMA-N-oxide/mg microsomal protein/min. All values calculated over incubation period of 0-20 min at 35°C.

*b Abbreviations: DMA, N,N-dimethylaniline; DMA-N-oxide, N,N-dimethylaniline-N-oxide; NADPH$_2$, reduced nicotinamide adenine dinucleotide phosphate.

The formation of metabolites which interfere with macromolecules of the cells is considered to be a basic characteristic of chemical carcinogenesis. When carcinogenic amines are metabolized in the endoplasmic reticulum of liver cells, reactive metabolites are formed which chemically attack adjacent cell components (22-24) and soon manifest their noxious character by a tendency to disorganize the membranes of the endoplasmic reticulum (14, 18, 52, 55), and interfere with the anabolic functions of the ribosomes (5-7, 25-27, 52). The chemical nature of the reactive derivatives has been discussed extensively (12, 47). During the last few years special attention has been focused on the role of N-oxygenated metabolites in this connection. These compounds have a high carcinogenic potency (48, 49) and a greater ability than the parent amines or ring-hydroxylated derivatives to react with nucleic acids and proteins in vivo (42, 68). Furthermore, N-oxygenated metabolites tend to accumulate during continued feeding of carcinogenic aromatic amines (50).

A number of genetic, hormonal, or dietary factors have been described which influence the carcinogenic potency of aromatic amines (16, 51, 57, 65, 66) and the degree of microsomal and ribosomal damage produced by short-term treatment with these chemicals (6, 7, 25, 26). It seemed of interest to examine whether the potentiating or protective effects of these factors were correlated with specific alterations in the pattern of oxygenation of aromatic amines by isolated liver microsomes. The monocyclic, noncarcinogenic amine DMA was chosen as a test substance. This substrate, in contrast to AF or AAF, does not interfere with microsomal detoxication mechanisms under the incubation conditions used (3) (3); thus the effects of in vivo treatment studied here will not be obscured by in vitro damage.

DISCUSSION

The formation of metabolites which interfere with macromolecules of the cells is considered to be a basic characteristic of chemical carcinogenesis. When carcinogenic amines are metabolized in the endoplasmic reticulum of liver cells, reactive metabolites are formed which chemically attack adjacent cell components (22-24) and soon manifest their noxious character by a tendency to disorganize the membranes of the endoplasmic reticulum (14, 18, 52, 55), and interfere with the


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(a) The toxic effects of heated fat and its non-urea adduct fraction have been interpreted by Kummerow as indirectly due to vitamin E deficiency (35). When heated fat (and particularly its non-urea adduct fraction) is included in the diet of rats, the carcinogenic effect of simultaneously administered AAF is potentiated (57) and a higher proportion of N-OH-AAF is excreted in the urine (41).

It has previously been shown that the inhibitory effects of brief AF or AN treatments in vivo on the ability of isolated rat liver microsomes to incorporate amino acids into protein is markedly enhanced after feeding a vitamin E-depleted diet (25, 26). It was therefore of interest to find that these potentiating effects of vitamin E depletion on the action of aromatic amines were clearly correlated with an increased accumulation of N-oxygenated products in the DMA test system (Table 1).

The mode of action of vitamin E has been discussed extensively but is by no means clarified (17, cf. 31). It is a potent antioxidant acting by the transfer of one electron to electrophilic radicals, followed by a proton migration (15, 63). Propagating radicals in lipid peroxidation are thereby effectively inactivated (10, 15). It seems unlikely, however, that the altered pattern of DMA oxygenation in vitro in vitamin E deficiency is caused by peroxidation of lipoproteins in the endoplasmic reticulum, since the lipid peroxidation is strongly inhibited by the added substrate (Table 1, Footnote d). Furthermore, all attempts have failed to demonstrate an increased level of peroxides or peroxide-dependent functional disturbances in vitamin E-deficient animals in vivo (13, 17). An electrophilic radical has, however, been proposed to be an intermediate in N-oxygenation of aromatic amines, including AF3,4 (3); furthermore, N-acetoxy-2-acetylaminofluorene, which acquires a similar electrophile with electron singlet characteristics (21), binds in model systems to nucleophilic groups of proteins and nucleic acids, e.g., methionine sulfur and guanine bases (37, 45). It has also been shown that electrophilic reagents can selectively inhibit the N-demethylation of aromatic amines, while N-oxygenation is still functional2 (2, 3). By the transfer of electrons to the above-mentioned radicals, vitamin E would prevent N-oxygenation and also their deleterious effects on microsomal functions, e.g., N-demethylation and protein synthesis.

Evidence has been produced indicating that, in vivo, a metabolite of α-tocopherol represents the active form of the vitamin (17, 63). The inability of α-tocopherol to counteract the effects of vitamin E deficiency on oxygenation of aromatic amines in vitro favors this view.

(b) When rats are fed an AAF-containing tumor-promoting diet for some weeks, their normally low urinary output of N-oxygenated AAF metabolites is increased severalfold (50). The increased accumulation of N-oxygenated DMA in liver microsomal systems, when feeding the animals a similar AAF diet (Table 3), affords a good correlation between the changed oxygenation pattern in this in vitro system and N-hydroxylation of AAF in tumor induction experiments.

Similarly, more marked effects on DMA oxygenation in vitro are obtained by short-term treatment in vivo with relatively large doses of AF or AAF (Table 2). As will be shown in a subsequent paper,4 reactive intermediates formed in the oxygenation of AF in vitro selectively inhibit microsomal N-demethylation, and thereby give rise to an increased proportion of N-oxygenated products. A similar effect of metabolic intermediates of AF in vivo would explain the changed pattern of DMA-oxygenation in the present experiments. The slight increase in the rate of N-demethylation observed is most readily explained on the basis of a simultaneous (7) corticoid stimulation, vide infra.

(c) Induction of liver tumors in rats by AF, acetylated AF, and aminoazo dyes is strongly inhibited by hypophysectomy and complete adrenalectomy (16, 66). To be effective against aminoazo dye carcinogenesis the adrenalectomy must be performed before or during the first weeks of carcinogen treatment (58). Administration of corticoids simultaneously with the carcinogenic amines increases the tumor incidence and drastically shortens the latent period; such effects are not observed when corticoids are administered after the carcinogen feeding has ceased (18). Thus, that function in the tumor-inducing process which is affected by endocrine factors is associated with the early initiation phase.

A corticoid-dependent stimulation of protein synthesis by liver ribosomes is observed in rats treated with AF or AAF for 4–20 hours (7). The experiments in Table 3 show that a single dose of prednisolone causes a slight increase in N-demethylation and a more marked rise in the N-oxygenation of DMA, resulting in a doubling of the N-oxygenation/N-demethylation quotient. These facts suggest that some microsomal detoxication enzymes are included among those proteins that are synthesized at an increased rate. It may be speculated that the integrity of the detoxication chain is less strict under such conditions, or that some enzyme components, e.g., cytochrome P450, which takes part in C-oxygenation but not N-oxygenation (30, 70), become rate-limiting (3, 56). In addition, corticosteroids may act directly on the endoplasmic reticulum, causing a slight “dissociation” of the flavin and cytochrome enzymes similar to that obtained with cholate treatment or ultrasonication in vitro3 (3, 25, 69, 70).

The changes in the proportion of AAF excreted as N-OH-AAF in vivo under various endocrine conditions described by Lotlikar et al. (36), taken together with the effect of corticoids on N-oxygenation and N-demethylation in vitro described above, point to an increased production of reactive intermediates in the detoxication of carcinogenic amines as one explanation for the effect of corticoids on tumor induction with aromatic amines. This interpretation is also consistent with the extreme histologic damage caused by simultaneous administration of carcinogenic aromatic amines and corticoids (C. M. Goodall, personal communication).

Nonspecific stress induced in rats by the intraperitoneal injection of Celite has been shown to mimic the corticoid-dependent stimulatory effect of a single dose of AF or AAF on the protein-synthesizing capacity of liver microsomes (7). The effects obtained by Celite-induced stress on microsomal N-oxygenation were not significantly different from the corticoid effects at the dose levels used; there was, however, some difference in the effect on N-demethylation. A stress reaction, however, involves a complex pattern of hormonal and neural mechanisms (8, 64). The adrenocorticotropic hormone which
is included in this mechanism not only increases the output of corticoids from the adrenals, but also directly affects the reduction and conjugation of steroids in the liver cells, and can therefore induce qualitative and quantitative changes of the biologic activities of these hormones in the liver (9).

(d) The effect of varied levels of dietary protein on the carcinogenicity of AAF and ammnoazo dyes is a matter of some controversy at present (51, 65). Mori has shown that addition of protein in the form of dried beef liver powder to a polished rice diet containing AAF decreased the liver tumor incidence from 44% to 20% (65). MeLean et al. (43) demonstrated that the N-demethylation of pyramion in vivo was affected by the dietary protein level; a partial inhibition was obtained when the protein content of the diet was reduced below 6%. The present results clearly indicate that this inhibition selectively affects C-oxygenation and thus gives rise to a relative increase in N-oxygenation.

(e) Guinea pigs are known to be refractory towards the carcinogenic action of AAF, AAF, and ammnoazo dyes (51, 65). In vivo guinea pigs do N-oxygenate AF or AAF only to a very limited extent (34, 50). Consistent with these facts was the ability of their liver microsomes to oxygenate DMA as efficiently as rat liver microsomes, but essentially by way of an N-demethylation. The N-oxide/HCHO quotient was much smaller than in the rat.

In conclusion, these experiments indicate that several conditions which favor tumor induction by aromatic amines, and which potentiate the effect of these agents on ribosomal functions, are associated with a changed pattern of microsomal oxygenation of DMA, favoring the accumulation of N-oxide in the system. Although N-oxygenation of DMA has not been demonstrated in vivo (19, 20), but only under the less physiology in vitro conditions, the increased tendency toward N-oxygenation demonstrated in these experiments indicates that constitutional changes in the membranous structures have taken place. These changes favor a type of metabolism which, with potent carcinogens, leads to the production of harmful intermediates, even in vivo, interfering with the function of cellular macromolecules.

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