Enzymic Lesions of Nicotinamide Adenine Dinucleotide Biosynthesis in Hepatomas and in Azo Dye Carcinogenesis

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SUMMARY

Several enzymes involved in nicotinamide adenine dinucleotide (NAD) biosynthesis were assayed in transplanted and azo-dye-induced primary hepatomas in order to determine the enzymic lesion responsible for the low level of NAD present in these tumors. The same enzymes were also investigated in precancerous livers of 3'-methyl-4-dimethylaminobenzene (3'-Me-DAB) fed rats and in rats receiving 3'-Me-DAB and 4'-Me-DAB i.p., as well as rats fed α-naphthyl isothiocyanate. It was concluded that the low level of NAD in the tumors examined was probably due to the deletion of enzymes in the tryptophan-to-NAD pathway and to decrease in activity of nicotinic acid mononucleotide pyrophosphorylase which catalyzes the rate-limiting step in the nicotinamide-to-NAD pathway. Changes in these enzymes in precancerous liver did not satisfy Reid's criteria for key steps in the carcinogenic process.

INTRODUCTION

The concentration of NAD and other pyridine nucleotides in malignant tissues has been the subject of much recent investigation. The levels of the pyridine nucleotides in tumors are generally much lower than in the corresponding normal tissues (9, 19, 22, 31, 39). A hypothesis linking this low level of NAD to the rapid cellular division characteristic of neoplastic tissue has been a number of these enzymes in normal rat liver, in precancerous liver, and in transplanted hepatomas. The nicotinamide deaminase activity of mouse mammary carcinoma is reduced to 20% of the normal level in azo-dye-induced primary hepatomas. The nicotinamide deaminase activity of a number of tumors has been reported recently (32), but no direct comparison was made with the corresponding normal tissues. Several enzymes related to NAD biosynthesis have been discovered since the investigations of Branster and Morton (2). We have compared the activity of a number of these enzymes in normal rat liver, in precancerous liver of azo-dye-fed rats and in primary and transplanted hepatomas in order to determine whether NMN-adenyl transferase is actually the rate-limiting step in the deficient NAD biosynthesis of tumor tissue.

MATERIALS AND METHODS

Animals. Male rats were obtained from Holtzman Rat Co., Madison, Wisconsin. These animals weighed 100–150 gm at the beginning of each experimental series.

Diets. The basal or control diet was the vitamin B complex test diet obtained from Nutritional Biochemicals, Inc., supplemented with vitamins to give a riboflavin-deficient diet (0.5 mg/kg of riboflavin) as described by Medes et al. (34). Pair-fed controls were used with the azo-dye- and α-naphthyl isothiocyanate-fed animals. 3'-Me-DAB and 4'-Me-DAB were synthesized from appropriate precursors by the method of Giese et al. (8). α-Naphthyl isothiocyanate was purchased from K & K Laboratories, Inc. The azo dyes were added to the basal diet to give a concentration of 0.06% and α-naphthyl isothiocyanate was fed at a level of 0.1%. Food intake of the control animals was restricted to equal that of the azo dye or α-naphthyl isothiocyanate-fed animals. In experiments in which azo dyes were injected, 3 ml of corn oil in which 40 mg of azo dye were dissolved by heating to 60–65°C were injected i.p. Control animals were injected with 3 ml of the warm corn oil.

Transplantable Tumors. The Novikoff hepatoma and another transplantable hepatoma induced at the Samuel Roberts Noble Foundation by feeding 3'-Me-DAB (hereafter referred to as DAB hepatoma) were obtained from Dr. D. E. Kizer of the Samuel Roberts Noble Foundation, Ardmore, Oklahoma. These tumors were maintained by intramuscular transplantation.

Enzyme Assays. Rats were killed by cervical dislocation and the livers or tumors removed, perfused with ice-cold 0.9% saline,
and homogenized in 4 volumes of water at 0°C in a Potter homogenizer. NMN-adenyl transferase activity was assayed on aliquots of the homogenate before centrifugation by the method of Branster and Morton (2). The homogenate was then centrifuged at 20,000 × g for 30 min and aliquots of the supernatant were assayed for 3-OHAA oxidase (5), α-aminomuconic semialdehyde dehydrogenase (6), quinolinate phosphoribosyl transferase; h, nicotinic acid mononucleotide pyrophosphorylase (EC 2.4.2.11); i, NMN-adenyltransferase (EC 2.7.7.1); j, nicotinamide deamidase.

RESULTS
Enzyme Levels in Hepatomas

In most animals, including rats, two pathways are available for the biosynthesis of NAD, the tryptophan-to-NAD pathway, and the more direct route from dietary niacin or niacinamide (21). Previous work (26, 41) demonstrated that the early enzymes of the tryptophan pathway (see Scheme I) tryptophan pyrrolase (a), kynurenine hydroxylase (b), and kynureninase (c) were absent from azo-dye-induced and Novikoff hepatomas. However, no studies have been reported on the later enzymes of the tryptophan-NAD pathway, and it is possible that intermediates employed in these studies. However, it has been recently reported (17, 36) that the “block” in the conversion of tryptophan to niacin observed in diabetes is actually due to a sharp increase in picolinic carboxylase activity which depletes 2-amino-3-carboxymuconic semialdehyde, the common intermediate of the glutarate and NAD pathways of tryptophan metabolism. No such increase in picolinic carboxylase occurs in hepatomas, ruling out this mechanism for decreased NAD formation. The activity of α-aminomuconic semialdehyde dehydrogenase (f), another enzyme of the glutarate pathway, is also greatly reduced in primary hepatoma and lost in the Novikoff tumor.

TABLE 1
Levels of 3-Hydroxyanthranilic Acid Oxidase and α-Aminomuconic Semialdehyde Dehydrogenase in Normal Rat Liver and Hepatomas∗

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>μoles product/min/mg protein (X10^4) at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-Hydroxyanthranilic acid oxidase</td>
</tr>
<tr>
<td>Normal liver</td>
<td>132.5 ± 12.4</td>
</tr>
<tr>
<td>Primary hepatoma†</td>
<td>5.5 ± 1.7</td>
</tr>
<tr>
<td>Tissue adjacent to hepatoma</td>
<td>102.5 ± 6.5</td>
</tr>
<tr>
<td>Novikoff hepatoma‡</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Livers of Novikoff hepatoma rats</td>
<td>145.0 ± 2.5</td>
</tr>
</tbody>
</table>

* Experimental conditions are described in the text. Values are the means from enzyme assays on tissues of 4 rats ± S.E.
† Induced by feeding 3'-methyl-4-dimethylaminoazobenzene.
‡ Obtained from Dr. D. E. Kizer, Samuel Roberts Noble Foundation, Ardmore, Oklahoma, and maintained by i.m. transplantation.

TABLE 2
Levels of Three Enzymes of NAD Biosynthesis in Normal Rat Liver and Transplanted Hepatomas∗

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>μoles product formed/min/mg protein (X10^4) at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quinolinate phosphoribosyltransferase</td>
</tr>
<tr>
<td>Normal liver [7]</td>
<td>89.3 ± 5.2</td>
</tr>
<tr>
<td>Novikoff hepatoma [6]</td>
<td>0</td>
</tr>
<tr>
<td>&quot;DAB&quot; hepatoma [6]</td>
<td>0</td>
</tr>
<tr>
<td>Liver of Novikoff tumor rats [6]</td>
<td>91.7 ± 8.8</td>
</tr>
<tr>
<td>Livers of DAB tumor rats [6]</td>
<td>66.7 ± 5.8</td>
</tr>
</tbody>
</table>

* Experimental conditions are described in the text. Numbers in brackets refer to number of animals assayed in each group. Values are average ± S.E.
† NMN, nicotinamide mononucleotide; DAB, dimethylaminooazobenzene.
‡ Two animals.
Liver Activity of NAD-synthesizing Enzymes in Precancerous
dye carcinogenesis have been studied by a number of investiga-
tors in the hope of identifying critical enzymic lesions in the de-
velopment of neoplasia (3, 23-25, 28, 43, 48). The data presented
in Table 4 show the changes in the activity of several enzymes re-
lated to NAD biosynthesis during 12 weeks of feeding with 3'-Me-
DAB and the basal diet. Pair feeding with the control diet for
8-12 weeks caused significant reductions in the activity of α-
aminomuconic semialdehyde dehydrogenase and NAMN pyro-
phosphorylase, suggesting that the level of these enzymes is de-
pressed by restricted food intake. During the early weeks of
feeding the 3'-Me-DAB diet, 3-OHAA oxidase and NAMN pyro-
phosphorylase activities were greatly reduced from control levels
as shown in Table 4. The activities of the other enzymes were not
appreciably affected during 12 weeks. It is known that feeding of
3'-Me-DAB results in extensive and progressive bile duct prolifer-
eration, e.g., Ref. 48, and it was possible that reduction of
certain enzyme activities in azo-dye-fed liver represented a rela-
tive increase in the population in bile duct cells, which cells may
cannot contain the enzyme under consideration. It has been demon-
strated that feeding α-naphthyl isothiocyanate results in massive
bile duct hyperplasia (29, 33, 47). However, this compound is not a
hepatocarcinogen (33). Therefore, experiments were carried out
to determine if feeding α-naphthyl isothiocyanate had any effect
on the activity of 3-OHAA oxidase and NAMN pyrophospho-
rylase. The results of these experiments are shown in Charts
1 and 2. The activity of these two enzymes was decreased by
feeding α-naphthyl isothiocyanate to about the same extent as
produced by feeding 3'-Me-DAB. The level of NAMN pyrophos-
phorylase in the α-naphthyl isothiocyanate controls did not de-
cline as in the 3'-Me-DAB controls. This is probably due to a
significantly larger food intake in the α-naphthyl isothiocyanate
control group.

Recently, it has been discovered that a single intraabdominal
injection of 3'-Me-DAB will produce changes in the liver in a few
days similar to those produced by weeks of feeding the dye (10,
20, 27, 40). These alterations were not observed when the very
weakly carcinogenic 4'-Me-DAB was injected under the same
conditions (27, 40). The effects of i.p. injection of 3'-Me-DAB
and 4'-Me-DAB on the activity of some of the hepatic enzymes
concerned with NAD biosynthesis are shown in Table 5. The
activities of α-aminomuconic semialdehyde dehydrogenase,
quinolinate phosphoribosyl transferase, and NAMN pyrophos-
phorylase were greatly reduced in 3'-Me-DAB-injected rats as
compared to 4'-Me-DAB-injected animals. The oil injection it-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>μmoles/min/mg protein (×10⁶) at 27°C</th>
<th>μmoles/min/mg protein (×10⁶) at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-OHAA oxidase</td>
<td>α-AMS dehydrogenase</td>
</tr>
<tr>
<td>Normal liver</td>
<td>145.0 ± 7.5</td>
<td>8.7 ± 2.2</td>
</tr>
<tr>
<td>Primary hepatoma</td>
<td>5.9 ± 1.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Liver adjacent to hepatoma</td>
<td>85.0 ± 4.5</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>QA phosphoribosyl transferase</td>
<td>NAMN pyrophosphorylase</td>
</tr>
<tr>
<td></td>
<td>68.3 ± 3.3</td>
<td>35.0 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>65.0 ± 5.0</td>
<td>21.7 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>81.3 ± 5.0</td>
<td>36.7 ± 10.0</td>
</tr>
<tr>
<td></td>
<td>NMM-adenyl transferase</td>
<td>541.7 ± 16.7</td>
</tr>
<tr>
<td></td>
<td>375.0 ± 41.7</td>
<td>375.0 ± 41.7</td>
</tr>
</tbody>
</table>

* 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; 3-OHAA, 3-hydroxyanthranilic acid; AMS, α-aminomuconic semialdehyde; QA, quinolate; NAMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide.

† Experimental conditions are described in the text. The average weight of rats in all groups was approximately 450 grams. Values are the means for enzyme assays on tissues of 5 rats ± S.E.
**TABLE 4**

The Activities of Enzymes Related to NAD Biosynthesis in Livers of Rats Fed 3'-Me-DAB a, b

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>No. of rats</th>
<th>Body weight average (g)</th>
<th>3-OHAA oxidase</th>
<th>a-AMS dehydrogenase</th>
<th>QA phosphoribosyl transferase</th>
<th>NAMN pyrophosphorylase</th>
<th>NMN-adenyl transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>127</td>
<td>197.0 ± 3.6</td>
<td>10.30 ± 0.66</td>
<td>128.3 ± 3.0</td>
<td>55.0 ± 3.0</td>
<td>750.0 ± 66.7</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>138</td>
<td>195.0 ± 6.1</td>
<td>9.50 ± 0.35</td>
<td>104.2 ± 4.5</td>
<td>29.2 ± 3.7</td>
<td>583.3 ± 58.3</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>125</td>
<td>173.0 ± 5.0</td>
<td>8.50 ± 0.49</td>
<td>108.3 ± 6.7</td>
<td>15.0 ± 3.5</td>
<td>580.0 ± 55.0</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>116</td>
<td>137.5 ± 7.2</td>
<td>10.00 ± 0.83</td>
<td>91.7 ± 2.2</td>
<td>33.3 ± 4.3</td>
<td>750.0 ± 58.3</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>132</td>
<td>125.0 ± 18.4</td>
<td>5.75 ± 1.68</td>
<td>91.7 ± 13.2</td>
<td>12.5 ± 3.7</td>
<td>666.7 ± 58.3</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>180</td>
<td>185.0 ± 7.9</td>
<td>10.00 ± 0.75</td>
<td>104.2 ± 7.0</td>
<td>33.3 ± 4.3</td>
<td>666.7 ± 41.7</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>130</td>
<td>114.7 ± 10.6</td>
<td>6.00 ± 0.43</td>
<td>95.8 ± 3.7</td>
<td>20.8 ± 4.3</td>
<td>750.0 ± 58.3</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>200</td>
<td>202.5 ± 2.2</td>
<td>7.00 ± 0.18</td>
<td>62.5 ± 4.2</td>
<td>12.5 ± 3.7</td>
<td>805.0 ± 41.7</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>156</td>
<td>102.5 ± 15.2</td>
<td>6.00 ± 0.83</td>
<td>79.2 ± 7.2</td>
<td>8.3 ± 3.7</td>
<td>570.0 ± 40.0</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>156</td>
<td>122.5 ± 6.5</td>
<td>6.75 ± 0.22</td>
<td>79.2 ± 5.5</td>
<td>20.8 ± 3.7</td>
<td>756.7 ± 23.0</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>169</td>
<td>178.3 ± 2.7</td>
<td>4.09 ± 0.29</td>
<td>62.5 ± 3.7</td>
<td>13.8 ± 4.3</td>
<td>616.7 ± 30.0</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>5</td>
<td>148</td>
<td>115.0 ± 8.9</td>
<td>4.00 ± 0.25</td>
<td>55.0 ± 6.0</td>
<td>18.3 ± 3.7</td>
<td>616.7 ± 30.0</td>
</tr>
</tbody>
</table>

a The experimental details are described in the text. Values are averages of assay for livers of number of rats indicated ± S.E.

Abbreviations: see Footnote a, Table 3.

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**CHART 1.** The hepatic 3-hydroxyanthranilic acid (3-OHAA) oxidase activity of rats fed α-naphthyl isothiocyanate for 18 weeks. α-Naphthyl isothiocyanate was fed at 0.1%. Each point represents averages of assays of five individual rat livers. Vertical lines on either side of each point designate standard error.

---

**CHART 2.** The hepatic nicotinic acid mononucleotide (NAMN) pyrophosphorylase activity of rats fed α-naphthyl isothiocyanate for 18 weeks. α-Naphthyl isothiocyanate was fed at 0.1%. Each point represents averages of assays on five individual rat livers. Vertical lines on either side of each point designate standard error.

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**DISCUSSION**

The main purposes of this investigation were to pinpoint the enzymic lesion responsible for the comparatively low level of NAD which is found in a variety of tumors, and to determine if any of the enzymic changes observed might be a key change in the carcinogenic process.

The data presented in Tables 1–3 do not support the earlier suggestion of Branstan and Morton (2) that decreased activity of 3-OHAA oxidase, quinolinate phosphoribosyl transferase, and NAMN pyrophosphorylase, while NMN-adenyl transferase activity was not greatly affected by oil or either azo dye.

self appeared to lower significantly the activity of 3-OHAA oxidase, quinolinate phosphoribosyl transferase, and NAMN pyrophosphorylase, while NMN-adenyl transferase activity was not greatly affected by oil or either azo dye.

NMN-adenyl transferase is the reason for the low NAD concentration of tumors. These data rather support the conclusion that, in the hepatomas examined, a decreased rate of NAD synthesis is due to deletion of enzymes on the tryptophan to NAD pathway and reduction of the activity of NAMN pyrophosphorylase, an enzyme of the nicotinamide-to-NAD pathway. Reduction of enzyme activity will only decrease product formation if that enzyme is already the rate-limiting step in a biosynthetic sequence (or becomes the rate-limiting step after decrease in activity). It has been proposed on the basis of the low activity of this enzyme in in vitro assays that nicotinamide deaminase (3) (Scheme I) is the rate-limiting step in NAD biosynthesis from nicotinamide in liver (42) and Ehrlich ascites tumor cells (32). However, other...
lines of evidence indicate that, at least in normal liver, NAMN pyrophosphorylase is the rate-limiting enzyme in this pathway. It has been reported that free nicotinic acid accumulates in the liver of nicotinamide-injected rats (44); this would not occur if nicotinamide deamidase were the rate-limiting step. It has also been reported that the level of hepatic NAD increases much more rapidly after injection of NAMN than of either nicotinic acid or nicotinamide (37), again suggesting that NAMN pyrophosphorylase is the rate-limiting enzyme in NAD biosynthesis from nicotinamide. It therefore appears plausible that the observed decrease in the activity of this enzyme in hepatomas coupled with the deletion of the tryptophan-NAD pathway could account for the reduced level of NAD observed in hepatomas.

Reid (43) has suggested three criteria to determine which biochemical effects of hepatocarcinogens are significant steps toward neoplasia. The first two of these are: (a) key changes will lie at steps which are rate limiting in normal liver; (b) any effect produced by diverse carcinogens but not by noncarcinogenic analogs is likely to be a key change. As has been discussed above, NAMN pyrophosphorylase meets the first criterion since it catalyzes what is probably the rate-limiting step in the formation of NAD from nicotinamide. The activity of this enzyme is also greatly decreased by feeding the noncarcinogenic a-naphthyl isothiocyanate (Chart 2), suggesting that this decrease may be due to bile duct hyperplasia rather than being a step in hepatocarcinogenesis.

It may be concluded from the results of this study that the deletion of the tryptophan-NAD pathway together with a decreased activity of NAMN pyrophosphorylase is a possible explanation for the reduced level of NAD observed in hepatomas. However, the decrease in NAMN pyrophosphorylase activity does not appear to be a significant change in carcinogenesis as defined by Reid's criteria (43).

TABLE 5

Response of Hepatic Enzymes Related to NAD Biosynthesis to Intraperitoneal Injection of 3'-Me-DAB and 4'-Me-DAB.

<table>
<thead>
<tr>
<th>Days after injection</th>
<th>Experimental conditions</th>
<th>μmoles/min/mg protein (X10⁶) at 27°C</th>
<th>μmoles/min/mg protein (X10⁶) at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-OHAA oxidase</td>
<td>α-AMS dehydrogenase</td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>197.0 ± 3.4</td>
<td>10.36 ± 0.66</td>
</tr>
<tr>
<td>2</td>
<td>3'-Me-DAB</td>
<td>127.0 ± 9.9</td>
<td>11.90 ± 0.45</td>
</tr>
<tr>
<td>2</td>
<td>4'-Me-DAB</td>
<td>115.0 ± 8.0</td>
<td>6.10 ± 0.22</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>125.0 ± 2.8</td>
<td>10.10 ± 0.61</td>
</tr>
<tr>
<td>4</td>
<td>3'-Me-DAB</td>
<td>115.0 ± 5.7</td>
<td>11.30 ± 0.66</td>
</tr>
<tr>
<td>4</td>
<td>4'-Me-DAB</td>
<td>95.0 ± 6.3</td>
<td>2.70 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>115.0 ± 6.3</td>
<td>9.50 ± 0.72</td>
</tr>
</tbody>
</table>

* Experimental details described in the text. Values are averages of assays for livers of 5 rats ± S.E.

ACKNOWLEDGMENTS

We are most grateful to Dr. Donald E. Kizer for providing the Novikoff and DAB hepatomas and for his frequent advice and encouragement during the course of these investigations. The contributions of Mr. S. J. Lan to the early phases of this work and the competent technical assistance of Miss K. Usuki are also gratefully acknowledged.

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