The Effect of Hepatocarcinogenesis upon 5-Hydroxytryptophan Decarboxylase and Serotonin Deaminase

DONALD E. KIZER AND SHUNG-KAI CHAN*

(Biomedical Division of The Samuel Roberts Noble Foundation, Inc., Ardmore, Okla.)

SUMMARY

Enzymes associated with the catabolism of 5-hydroxytryptophan (5-HT) were assayed in transplanted hepatomas, in primary lesions induced with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), and in 3'-Me-DAB precancerous rat liver. Among six transplanted rat hepatomas, 5-HT decarboxylase activity was essentially absent in five, whereas the Morris hepatoma 5123 manifested activity equivalent to that observed in rat liver. Mouse Hepatoma 134 retained slight but measurable activity, but no appreciable activity was observed in Hepatoma 129. When animals were fed diets containing 3'-Me-DAB for 12 weeks, the 5-HT decarboxylase activity of the liver was significantly depressed as early as 2 weeks. At 4 and 8 weeks the activity was about half the value observed in control animals, but at 12 weeks the activity was essentially the same as that of controls. When primary lesions developed, no 5-HT decarboxylase activity was observed, but the activity in the adjacent liver tissue was essentially normal. For the first 4 weeks the lowered decarboxylase activity in the liver of animals fed 3'-Me-DAB was not due to dietary restriction, nor was it reversed by pyridoxine supplementation of the diet. Removal of 3'-Me-DAB from the diets restored activity to normal levels in 7 days.

Serotonin deaminase activity was essentially absent in four transplanted rat hepatomas but was readily demonstrated in the Morris 5123. Activity was observed in mouse Hepatoma 134, but activity was absent in Hepatoma 129. No alteration of serotonin deaminase activity was observed during the 12 weeks on diets containing 3'-Me-DAB; however, activity was absent in primary lesions and was significantly lowered in liver tissue adjacent to primary lesions.

The primary pathway of 5-hydroxytryptophan (5-HT) catabolism appears to be mediated by two enzymes: 5-HT decarboxylase, which yields serotonin (5, 33, 39), and serotonin deaminase, which catalyzes a two-step reaction yielding 5-hydroxyindoleacetic acid (5-HIAA) (35, 40). The formation of 5-hydroxyindole compounds usually represents a minor pathway of tryptophan metabolism in humans (32); however, patients with argentaffinoma (malignant carcinoid) displayed markedly elevated levels of both serotonin (5-hydroxytryptamine) and 5-HIAA in the blood and urine (6, 27, 29, 30, 34, 38). It has been estimated that possibly 60 per cent of the dietary tryptophan of these patients is metabolized by the 5-hydroxyindole pathway (32). Udenfriend, Weissbach, and Bogdanski (36) postulated that the increased 5-HT metabolism in humans with argentaffinoma might be associated with the metabolic activity of the malignant lesions, and several studies (9, 14) indicated that these tumors could catabolize 5-HT. Furthermore, Benditt et al. (1) demonstrated the presence of serotonin in mast cells, and Sjördsm, Waalkes, and Weissbach (38) subsequently showed that the growth of a transplanted mast cell tumor (7) was accompanied by increased urinary excretion of 5-HIAA. Thus, the available evidence strongly indicated that both malignant carcinoids and mast-cell tumors contained enzymes for the catabolism of 5-HT.

* Present address: University of Wisconsin, Department of Physiological Chemistry, Madison 6, Wisconsin.

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Since it was demonstrated that the liver of rats and mice possessed both 5-HT decarboxylase activity (30) and serotonin deaminase activity (11, 12, 26, 40), it was of interest to determine whether these enzymes were present in a spectrum of transplanted lesions derived from hepatic tissue. It was of further interest to determine whether any alteration of 5-HT decarboxylase activity or serotonin deaminase activity occurred during hepatocarcinogenesis with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB).

MATERIALS AND METHODS

Three transplanted hepatomas—Novikoff solid, Novikoff ascites, and 3'-Me-DAB—were carried intraperitoneally in Holtzman rats, whereas Hepatomas 129 and 134 were transplanted intraperitoneally in C57 mice. The Morris hepatomas 5123 (20), 3683, and 3924-A (24) were supplied by Dr. Harold P. Morris of the National Cancer Institute, Bethesda, Maryland. Tumor-bearing animals were transported by air express shortly after transplantation, and the enzyme assays were performed at periods ranging from several days to a week or more after arrival.

Primary hepatomas were induced in female Holtzman rats by feeding 0.06 per cent 3'-Me-DAB in the semi-synthetic diet described by Medes, Friedmann, and Weinhouse (18) for 12 weeks. After the 12th week, animals were maintained on Rockland Chow diet until the lesions could be palpated. For the experiments in which the 5-HT decarboxylase activity and serotonin deaminase activity of precancerous livers were determined, control animals were maintained on the semi-synthetic diet without carcinogen. The 3'-Me-DAB used throughout these experiments was synthesized according to the method of Giese, Miller, and Baumann (10).

The formation of serotonin was used as the criterion for determining 5-HT decarboxylase activity. The extraction procedure and ultraviolet absorption method described by Udenfriend, Weissbach, and Clark (37) were used to determine the serotonin concentration. Serotonin creatinine·H₂SO₄ complex was used as a standard, and all absorption measurements were made in a Beckman Model DU Spectrophotometer at 275 μm. Protein concentration was determined by the method of Lowry et al. (15).

Serotonin deaminase activity of tissue homogenates was assayed by determining the ammonia production (2) in aliquots of the incubation mixture which were removed at 0, 20, 40, and 60 minutes. The tissue nitrogen content was determined by micro-Kjeldahl (16). In some experiments, the serotonin deaminase activity of mitochondria and supernatant fractions was determined. The fractions were separated by a Model L Spinco Ultracentrifuge, according to the procedure published by Schneider and Hogeboom (25).

Animals were sacrificed by cervical fracture and the tissues rapidly excised. Usually tissues from three animals were pooled; however, with Hepatomas 5123, 3683, and 3924-A assays were performed on tumors from individual animals. Livers were perfused with cold isotonic saline, and all tissues were homogenized in 0.1 M phosphate buffer.

The reaction mixture for the assay of 5-HT decarboxylase activity contained 0.002 M 5-HT, 0.0004 M pyridoxal phosphate, and homogenate in a total volume of 15 ml. Both the substrate and co-factor were dissolved in 0.1 M phosphate buffer. The mixture was incubated in Warburg reaction vessels at 38 °C. under a 95 per cent nitrogen-5 per cent CO₂ gas mixture.

Preliminary experiments indicated that serotonin synthesis was linear throughout a 60-minute incubation period and throughout a protein concentration of 14 mg/ml. Thus, all subsequent assays were incubated 30 minutes, and the tissue concentration in the homogenates was adjusted to yield a final protein content of 7–10 mg/ml. Experiments in which both the buffer composition and buffer pH were altered yielded information indicating maximum serotonin synthesis at a range of pH 7.6–8.0. The unit of enzyme activity was defined as μmoles of serotonin creatinine·H₂SO₄ equivalent/gm protein/30-minute incubation.

For the assay of serotonin deaminase activity, tissues were homogenized in sufficient 0.1 M phosphate buffer to effect a ratio of 1 gm. of tissue/10 ml. of buffer. The reaction mixture which contained 5 ml. of tissue homogenate and 5 ml. of

1. The authors wish to express appreciation to Drs. E. C. Moore and R. B. Hurlbert for supplying a transplant of the Novikoff ascites tumor.

2. The 3'-Me-DAB hepatoma was induced by feeding 3'-methyl-4-dimethylaminoazobenzene and has been carried through more than 50 transplant generations.

3. Transplants of the two mouse ascites hepatomas, 129 and 134, were originally made available through the generosity of Dr. Morris Belkin of the National Cancer Institute. The tumors have been subsequently maintained in C57 mice from our breeding stock.

4. The authors are indebted to California Corporation for Biochemical Research and to Vismara Terapeutici for the generous supply of serotonin.

5. 5-Hydroxytryptophan was purchased from Nutritional Biochemicals Company and pyridoxal phosphate from Sigma Chemical Company.
serotonin creatinine · H2SO4 was incubated 60 minutes at 38° C. in air. Preliminary experiments indicated that a serotonin concentration of 2.5 × 10−3 M was sufficient to yield a linear reaction velocity throughout a 60-minute incubation period, and the pH optimum appeared to lie between pH 7.5 and 8.0. Accordingly, 0.1 M phosphate buffer at pH 7.8 was used in all subsequent assays. The unit of serotonin deaminase activity was defined as μmoles of ammonia formed/mg of homogenate nitrogen/60-minute incubation.

RESULTS

A comparison of the 5-HT decarboxylase activity of normal liver homogenates with that of transplanted hepatomas is shown in Table 1. Although rat and mouse liver homogenates showed appreciable activity, five of the transplanted rat hepatomas and one transplanted mouse hepatoma were essentially devoid of 5-HT decarboxylase activity. Homogenates of the Morris hepatoma 5123 had activity equivalent to that observed in normal rat liver homogenates, whereas mouse Hepatoma 134 showed slight but measurable activity. Since it was possible that the absence of activity in some of the transplanted hepatomas was associated with factors other than deletion of the apoenzyme, mixing experiments were conducted in which varying amounts of tumor homogenate were mixed with liver homogenate. The addition of Novikoff hepatoma homogenates in excess of 50 per cent (on a protein basis) did not alter the specific activity of the liver enzyme. Furthermore, the addition of the supernatant of boiled liver homogenate failed to activate the 5-HT decarboxylase activity of the Novikoff hepatoma.

Since 5-HT decarboxylase activity was essentially absent in all the transplanted rat hepatomas except the Morris hepatoma 5123, it was of interest to determine the status of this enzyme during hepatocarcinogenesis with 3'-Me-DAB. The data from these experiments are shown in Table 2. Throughout a 12-week period, the livers of animals receiving the basal diet had a decarboxylase activity of 5.2–6.2 μmoles serotonin/gm protein. At the 4th and 8th weeks, the decarboxylase activity of animals ingesting 3'-Me-DAB was half that of the control animals. During the 12th week, decarboxylase activity of dye-fed animals was essentially the same as that of control animals. When primary hepatomas developed sufficiently to permit assay, the decarboxylase activity of liver tissue adjacent to the primary lesions also showed activity within the range routinely observed in normal liver. No decarboxylase activity was detected in primary lesions.

It was important at this juncture to determine whether 5-HT decarboxylase activity was altered to TABLE 2

5-HYDROXYTRYPTOPHAN DECARBOXYLASE ACTIVITY OF PRECANCEROUS LIVERS AND PRIMARY HEPATOMAS

<table>
<thead>
<tr>
<th>Time (weeks)*</th>
<th>Enzyme activity (μmoles serotonin/gm protein/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td>4</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td>8</td>
<td>5.3±0.1</td>
</tr>
<tr>
<td>12</td>
<td>5.8±1.1</td>
</tr>
</tbody>
</table>

* All animals were preconditioned 7 days on basal diet.
† Number of experiments: See Table 1.
‡ 3'-Me-DAB fed at a level of 0.06 per cent.

Conditions: See Table 1.

TABLE 1

5-HYDROXYTRYPTOPHAN DECARBOXYLASE ACTIVITY IN HOMOGENATES OF LIVER AND TRANSPLANTED HEPATOMAS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. experiments*</th>
<th>Enzyme activity (μmoles serotonin/gm protein/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morris hepatoma 5123</td>
<td>3</td>
<td>4.7±0.4</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>3</td>
<td>6.6±0.9</td>
</tr>
<tr>
<td>3'-Me-DAB hepatoma</td>
<td>3</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>Morris hepatoma 3683</td>
<td>1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Novikoff hepatoma 3924-A</td>
<td>2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Mouse liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatoma 134</td>
<td>3</td>
<td>5.4±0.1</td>
</tr>
<tr>
<td>Hepatoma 129</td>
<td>3</td>
<td>0.4±0.1</td>
</tr>
</tbody>
</table>
earlier than 4 weeks. Accordingly, two groups of animals were fed either the basal diet or basal +0.06 per cent 3'-Me-DAB, and the decarboxylase activity of each group was determined at 7-day intervals. As shown in Chart 1, when compared with animals fed the basal diet, the activity of the carcinogen-fed animals at 1 and 2 weeks was 62 and 54 per cent, respectively. At the end of the 2d week the carcinogen was removed from the diet, and the decarboxylase activity was determined at the 3d and 4th week. As shown in Chart 1, within 1 week the activity approached that of the control animals. Thus, the inclusion or deletion of 3'-Me-DAB brought about significant changes in the 5-HT decarboxylase activity of the liver within 7 days.

Since the ad libitum food intake of animals fed 3'-Me-DAB was usually less than that of animals fed the basal diet, it was possible that the lowered decarboxylase activity was associated with restricted dietary intake. Therefore, two groups of animals were paired by weight; one animal of each pair was fed the basal +3'-Me-DAB diet ad libitum, and the daily food consumption was determined. The second animal of each pair was fed the basal diet in an amount corresponding to the consumption of the carcinogen-fed animals. The results are shown in Chart 2. Although dietary restriction caused some lowering of the decarboxylase activity, animals ingesting 3'-Me-DAB manifested a lower decarboxylase activity. Thus, the low activity of the precancerous animals did not appear to be associated with a restricted dietary intake. In an additional experiment the pyridoxine level of the basal +3'-Me-DAB diet (4 mg/kg of diet) (18) was increased two- and threefold. These increases failed to exert any effect upon the lowered 5-HT decarboxylase activity of the carcinogen-fed animals.

The data derived from the experiments with the precancerous livers indicated a direct effect of the dye upon the decarboxylase activity. In view of this, it was important to determine whether injection of 3'-Me-DAB also might alter the decarboxylase activity. Therefore, animals were conditioned 7 days on basal diet and were given injections of 2 ml. of olive oil containing 5.0 mg. of 3'-Me-DAB. Two control groups were either sham-injected or given injections of the olive oil vehicle. The livers of three animals from each group were pooled at 6, 18, 24, 48, and 72 hours following injections, and the 5-HT decarboxylase activity was determined. The results from two such experiments indicated no appreciable alteration of the decarboxylase activity at any of the time periods.

The serotonin deaminase activity of normal liver and hepatoma homogenates is shown in Table 3. The distribution pattern was essentially the same as that observed with 5-HT decarboxylase activity. Activity was essentially absent in four transplanted rat hepatomas and one transplanted mouse hepatoma. On the other hand, the Morris
hepatoma 5123 retained activity approximating that of normal rat liver, and Hepatoma 134 also retained activity.

Mixing experiments failed to inhibit the deaminase activity of liver homogenates or to activate measurable deaminase activity in homogenates of the Novikoff hepatoma. Since it was demonstrated that deamination of serotonin was a diphosphopyridine nucleotide (DPN)-linked reaction (40), it was possible that the deaminase activity of transplanted hepatomas might be adversely affected by limiting DPN concentrations. In view of the stoichiometric relationship between deamination of serotonin and DPN reduction (40), DPN was added to homogenates of the Novikoff hepatoma in amounts equivalent to the deaminase activity observed in normal liver and at concentrations 2 and 3 times greater. These additions caused no appreciable increase in the serotonin deaminase activity of the Novikoff hepatoma. Thus, it appeared that activity losses were not associated with the presence of inhibitors or loss of essential co-factors.

**TABLE 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. experiments*</th>
<th>Enzyme activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morris 5123</td>
<td>4</td>
<td>0.41 ± 0.04†</td>
</tr>
<tr>
<td>Novikoff</td>
<td>3</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>4</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Novikoff ascites</td>
<td>3</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Morris 5924-A</td>
<td>2</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Mouse liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatoma 134</td>
<td>4</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Hepatoma 129</td>
<td>4</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

Conditions: 2.5×10^{-3} M serotonin; 0.1 M phosphate buffer; total volume 10 ml; incubation 60 minutes at 38° C. in air.

* Number of experiments: See Table 1.
† The unit of enzyme activity was μmoles ammonia/mg homogenate nitrogen/60 minutes.
‡ Average ± standard deviation: See Table 1.

As shown in Table 4, maintaining animals on diets containing 3'-Me-DAB for 12 weeks exerted no appreciable alteration in the serotonin deaminase activity of the liver. When primary lesions developed, however, no appreciable serotonin deaminase activity was detected. Furthermore, the activity of the liver tissue adjacent to the primary lesions was significantly reduced. Since the 5-HT decarboxylase activity of adjacent liver tissue approximated that of normal tissue, it would not appear that the reduced activity in this instance could be explained by dilution of liver tissue with malignant tissue.

Striebich, Shelton, and Schneider (31) reported that 3'-Me-DAB feeding resulted in significant reductions in the number of mitochondria in precancerous livers. Weissbach, Redfield, and Udenfriend (40) reported that, although some serotonin deaminase activity was demonstrable in liver homogenate supernatant fractions, most of the activity was manifested by mitochondrial fractions. Since no appreciable alteration of serotonin deaminase was observed throughout 12 weeks on 3'-Me-DAB diets, it was possible that the specific deaminase activity of precancerous liver mitochondria had increased or that the activity in supernatant fractions had increased. When both fractions were separated (25) from livers of animals fed 3'-Me-DAB for 4 weeks and compared with the fractions obtained from animals fed basal diets, there was no evidence indicating either increases in the specific activity of the mitochondrial serotonin deaminase or translocation of activity to supernatants.

**DISCUSSION**

The majority of the transplanted lesions had no appreciable 5-HT decarboxylase activity or serotonin deaminase activity, and the data indicated that the absence of activity was due to deletion rather than the influence of inhibitors or a paucity of co-factors. Thus, despite the fact that these enzymes were present in liver (11, 19, 26, 36, 40), the lesions which developed during hepatocar-
Carcinogenesis were largely devoid of demonstrable activity. There were, however, two notable exceptions. Both 5-HT decarboxylase activity and serotonin deaminase activity were observed in homogenates of the Morris 5123 and Hepatoma 184. This result would necessitate concluding that deletion of 5-HT decarboxylase activity and serotonin deaminase activity was not an essential step for malignant transformations per se in the liver.

Both decarboxylase and deaminase activities were essentially absent in the Novikoff hepatomas, which were induced with DAB (21); in the 3'-Me-DAB hepatoma, which was induced with 3'-Me-DAB (17); and in primary lesions which developed following 12 weeks on diets containing 3'-Me-DAB. Thus, without exception, carcinogenesis with either DAB or 3'-Me-DAB yielded lesions in which these enzyme activities were apparently deleted. Potter et al. (24) considered that the enzyme patterns in transplanted hepatomas were influenced by (a) malignant transformations occurring in both the parenchymal and biliary cells of the liver and (b) convergence toward a common enzyme pattern imposed by transplantation in noninbred animal strains. Either or both of these considerations could explain the absence of decarboxylase activity and deaminase activity in the 3'-Me-DAB hepatoma and Novikoff hepatomas, but the latter factor does not apply when considering the absence of these activities in 3'-Me-DAB-induced primary lesions. Miller and Miller (19) concluded from their studies with azo dyes that both parenchymal and bile-duct cells might undergo malignant transformation, and morphologic studies have supported such a contention (8, 22, 23). In addition, the apparent retention of tryptophan pyrrolase activity by primary lesions induced with DAB (13) and evidence indicating retention of tryptophan pyrrolase adaptation in both DAB- (13) and 3'-Me-DAB-induced (4) primary lesions also suggested parenchymal cell participation in malignant transformations induced by azo dyes. Thus, the absence of 5-HT decarboxylase activity and serotonin deaminase activity in 3'-Me-DAB-induced primary lesions may represent, in some cases, malignant transformations in cells which had no enzymatic capability for the catabolism of 5-HT. In other cases, however, the malignant transformations must have occurred in cells which had these enzymes. Here, the absence of the two enzyme activities must be related to the process of multiple deletions which was imposed by 3'-Me-DAB carcinogenesis.

Cantero (3) concluded that a decrease in enzyme systems during azo-dye carcinogenesis was indicative of the precancerous stage which was followed by further deletions of key enzymes in the liver-tumor stage. In the present study, early losses of 5-HT decarboxylase activity were observed, and these losses appeared to be associated with the ingestion of 3'-Me-DAB. The early activity losses were followed by the development of primary lesions in which 5-HT decarboxylase activity could not be demonstrated. Whether the early changes in decarboxylase activity were associated with azo-dye carcinogenesis must be determined by further experimentation.

ACKNOWLEDGMENTS
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REFERENCES
15. LOWRY, O. H.; ROSEBROUGH, N. J.; FARE, A. L.; and


The Effect of Hepatocarcinogenesis upon 5-Hydroxytryptophan Decarboxylase and Serotonin Deaminase

Donald E. Kizer and Shung-Kai Chan