Changes in the Adenylic Acid Deaminase Activity of Rat Liver during Carcinogenesis

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SUMMARY

Changes in the adenylic acid (AMP) deaminase activity of rat liver during azo dye hepatocarcinogenesis were studied to determine whether they were related to malignant transformation. The AMP deaminase activity of rats fed 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) for 4 weeks was increased 1.5- to twofold compared with that of control animals fed basal diets. The elevated activity persisted throughout 12 weeks. If 3'-Me-DAB was withdrawn from diets after 4, 8, or 12 weeks, AMP deaminase activity returned to control levels within 7-10 days. No appreciable increases in enzyme activity were observed during regeneration following partial hepatectomy. Animals fed 4'-fluoro-DAB showed increases in hepatic AMP deaminase activity; however, animals fed 4'-Me-DAB or o-aminoazotoluene failed to show increases throughout 12 weeks. Hypophysectomized rats fed 3'-Me-DAB for 6 weeks showed no increases in AMP deaminase activity. Similarly, the simultaneous feeding of p-hydroxypropiophenone and 3'-Me-DAB greatly delayed the usual increases in AMP deaminase activity. Feeding o-napthyl isothiocyanate caused early increases in deaminase activity; however, activity subsequently returned to the normal range. Animals that were fasted and then fed a low protein diet also showed elevated AMP deaminase activity. It was concluded that the elevation of AMP deaminase activity in precancerous rat liver (a) was related to azo dye carcinogenesis, (b) was not associated intrinsically with regeneration or with biliary proliferation, and (c) was not a phenomenon exclusively characteristic of precancerous liver.

The adenylic acid (AMP) deaminase activity of rat or mouse liver (18) was lower than the activity determined for several transplanted hepatomas (18) and for primary hepatomas induced with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) (5). In a subsequent study (6), it was observed that the AMP deaminase activity in the liver of rats fed 3'-Me-DAB was significantly elevated compared with the activity of rats fed a basal diet.

The relationship between changes in the activity of hepatic enzymes during azo dye carcinogenesis and ultimate neoplasia was discussed earlier by Cantero (4) and, more recently, by Weber (35). Cantero (4) emphasized the sequential nature of the changes in precancerous liver and believed that these changes were manifested by the tissue as well as at the cellular level. Weber (35) hoped that systematic analysis of the hepatocarcinogenic sequence might identify crucial lesions in the development of neoplasia. Reid (29) was also concerned with the recognition of key changes from among those probably irrelevant, and he defined criteria for the recognition of those key changes in precancerous liver. Some of these criteria (29) were used to evaluate precancerous changes in several enzymes associated with tryptophan metabolism (14, 15, 19), and it was concluded that these changes were probably irrelevant to either 3'-Me-DAB or ethionine carcinogenesis (16).

The purpose in the present experiments was to study further the precancerous changes in AMP deaminase activity and to determine the relevancy of those changes to 3'-Me-DAB carcinogenesis. On the basis of Reid's criteria (29), the data indicated that the elevation in AMP deaminase activity was a key change. It was observed, however, that hepatic AMP deaminase activity could be elevated in several systems unrelated to the induction of hepatic malignancies.

MATERIALS AND METHODS

Animals.—Female Holtzman strain rats, which weighed 130-180 gm. at the beginning of each experimental series, were used in this study. In experiments with partially hepatectomized rats animals were anesthetized with ether, and about 65 per cent of the liver was removed according to the procedure outlined by Higgins and Anderson (13). Hypophysectomized rats were purchased from Hormone Assay Laboratories, Chicago, Illinois.

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Diets.—When animals were fed azo dyes, α-napthyl isothiocyanate, or p-hydroxypropioephene, the basal diet was the semi-synthetic, riboflavin-deficient diet described by Medes, Friedmann, and Weinhoven (23). Three derivatives of 4-dimethylaminobenzene (DAB)—namely, 3'-Me-DAB, 4'-fluoro-DAB (4'-F-DAB), and 4'-Me-DAB—were synthesized from appropriate precursor molecules by the method of Giese, Miller, and Baumann (11). A fourth azo dye α-aminoazotoluene was purchased from a commercial source and was recrystallized twice before using. All four azo dyes were added to a semi-synthetic diet (23) in amounts to give a protein concentration of 0.06 per cent. p-Hydroxypropioephene1 was fed at 1.67 per cent (30), whereas α-napthyl isothiocyanate1 was fed at two concentrations, 0.04 and 0.08 per cent. Animals were always conditioned 7 days on the basal diet prior to initiation of any dietary regimen.

The basal diet used in experiments to determine whether the protein concentration of the diets affected hepatic AMP deaminase activity was the semi-synthetic diet described by Farber (8). The glucose content of this diet was altered to give either a 2 per cent or a 91 per cent protein concentration (27).

Preparation of tissue.—Animals were killed by cervical fracture, and livers were quickly excised and chilled in ice-cold buffer. The lobes were finely minced with scissors; the minced tissue was blotted and weighed. Citrate buffer (0.05 M, pH 6.0) was added to give 1 g tissue/10 ml buffer, and the tissue was thoroughly homogenized in a glass-Teflon homogenizer. Homogenates were centrifuged at 105,000 X g in a Spincos Model L preparative ultracentrifuge for 20 minutes. The clear supernatant fluid was assayed for AMP deaminase activity.

Enzyme assays.—The standard assay system for AMP deaminase activity contained AMP, 13.3 mmoles; sufficient 105,000 X g supernatant from homogenates to give a protein concentration of 1.5—2.5 mg/ml; and 0.05 M citrate buffer, pH 6.0 (18). Usually 5 ml. of 0.04 M AMP dissolved in buffer was added to 5 ml. of pH 6.0 citrate buffer, and the mixture was allowed to equilibrate to 38° C. in a Dubnoff metabolic shaker. Then 5 ml. of supernatant fluid, which had been equilibrated to 38° C., was added to the mixture. Aliquots of the reaction mixture were removed immediately after the addition of the supernatant fluid and after 30 minutes incubation, and the proteins in each aliquot were precipitated by the addition of an equal volume of 10 per cent trichloroacetic acid. Following centrifugation the clear supernatants were subjected to the various analytical procedures.

Analytical methods.—Our adaptations of two methods used to determine the concentration of ammonia—viz., the aeration method of Boyce (3) and the direct catalyzed indophenol reaction of Chaney and Marbach (7)—were described in detail previously (18). In this work the indophenol method (7) was used predominantly.

The concentration of inosinic acid (IMP) was determined spectrophotometrically after elution from Dowex-1 columns exactly as previously described (18). In every experiment the concentrations of ammonia and IMP in the reaction mixtures were determined. Generally, the proportionality between ammonia and IMP was excellent; however, occasionally proportionality was not good. These instances occurred randomly and appeared to be attributable to the analytical technic.

To assure that ammonia production in reaction mixtures was not mediated by prior dephosphorylation, the concentration of inorganic phosphate was determined at the start and finish of each incubation interval by the method of Fiske and SubbaRow (9). Irrespective of dietary or surgical treatment of the animals, the average amount of inorganic phosphate produced during the incubation interval was approximately 0.10 μmoles/mg protein; however, the maximum amount never exceeded 0.27 μmoles/mg protein. The protein concentration was determined by the method of Lowry et al. (21).

RESULTS AND DISCUSSION

The AMP deaminase activity of rats fed 0.06 per cent 3'-Me-DAB throughout 12 weeks is shown in Chart 1. For the first 2 weeks ingestion of 3'-Me-DAB caused no appreciable change in hepatic AMP deaminase activity; however, from the fourth through the 12th weeks, deaminase activity was elevated 1.5—2 times compared with that in the basal diet-fed control animals.

Since it had been observed that the AMP deaminase activity of both primary (5) and transplanted hepatomas (18) was elevated compared with that in normal liver, it was important to determine whether the elevated activity in precancerous liver was reversible. Accordingly, three groups of animals were fed 3'-Me-DAB for periods of 4, 8, or 12 weeks and then were fed the basal diet without 3'-Me-DAB. The deaminase activity was deter-
weeks upon the hepatic adenylic acid (AMP) deaminase activity of rats. 3'-Me-DAB was fed at 0.06 per cent. Each point represents averages of three to eight assays on individual rat livers. For assay conditions, see text or Chart 1.

Miller and Miller (24) established that both 3'-Me-DAB and 4'-F-DAB had relative hepatocarcinogenic activities of 10-12, but the relative activity of 4'-Me-DAB was less than 1. Badger and Lewis (2) considered that o-aminazoalotolene was approximately as hepatocarcinogenic in the rat as was 4'-Me-DAB. When we fed these azo dyes throughout 12 weeks, the data depicted in Chart 3 were obtained. After 4 weeks AMP deaminase activity of rats fed 4'-F-DAB was appreciably elevated and remained elevated for the entire feeding period. On the other hand, the AMP deaminase activity of rats fed o-aminazoalotolene or 4'-Me-DAB never significantly exceeded that of animals fed the basal diet. Reid (29) considered that any effect produced by diverse hepatocarcinogens, but not by noncarcinogenic analogs, was likely to be a key change. The data in Chart 3 appeared to fit this criterion and supported a contention that the elevation of hepatic AMP deaminase activity was a key change in azo dye-induced hepatocarcinogenesis.

Two procedures have been described for the retardation or prevention of 3'-Me-DAB hepatocarcinogenesis. These are removal of the pituitary (12, 28, 31) and feeding the estrogenic compound p-hydroxypropiophenone (1, 30). A comparison between the effects of 3'-Me-DAB upon the AMP deaminase activity in the liver of control rats and hypophysectomized rats is shown in Table 1. Throughout 6 weeks the AMP deaminase activity in the liver of hypophysectomized rats fed 3'-Me-DAB was not significantly increased; however, among control rats a substantial increase was observed at both 4 and 6 weeks.

When 1.67 per cent p-hydroxypropiophenone was added to the diet of animals ingesting 3'-Me-DAB, results depicted in Chart 4 were observed. 3'-Me-DAB alone caused elevation of the AMP deaminase activity, but p-hydroxypropiophenone alone caused no significant change in the enzyme activity. When both were fed simultaneously no elevation occurred in the first 4 weeks, but significant elevations were seen at 8 and 12 weeks. Here the magnitude of the elevation was less than that seen with 3'-Me-DAB.

Reid (29) considered that a treatment which retarded hepatocarcinogenesis would also retard the manifestation of key changes in precancerous liver. When considered from this standpoint, results observed with hypophysectomized rats supported a contention that the elevation of AMP deaminase activity in precancerous rat liver was a key change. The results with p-hydroxypropiophenone were less definitive. If it is assumed that the change in this enzyme activity was a key change, then the data in Chart 4 indicate that p-hydroxypropiophenone afforded only partial protection from tumor induction. In studies by Robertson, Griffin, and Richardson (30), however, a 16-week regimen of 3'-Me-DAB and p-hydroxypropiophenone yielded histologically normal livers. Despite data in Chart 1 suggested a correlation between the time for achievement of maximum levels of dye-binding (24) and the establishment of an elevated level of enzyme activity. In a like manner, the data in Chart 2 suggested a correlation between the clearance of bound dye from the liver (24) and the reversion to normal levels of enzyme activity.

For assay conditions, see text or Chart 1.
this observation, evidence from several groups might be interpreted as indicating that p-hydroxypropiophenone delayed rather than prevented hepatocarcinogenesis. For instance, 3'-Me-DAB hepatocarcinogenesis was not delayed by 0.83 per cent p-hydroxypropiophenone, and, with 1.67 per cent p-hydroxypropiophenone, dye binding persisted at a half-maximal level (30). Hepatocarcinogenesis with N-2-fluorenylaceticamide was not affected by p-hydroxypropiophenone, and ethionine carcinogenesis, although greatly inhibited, still persisted (32). In the latter case it was observed that cellular damage, especially cholangiofibrosis, was considerably greater in the ethionine-p-hydroxypropiophenone group than it was in the ethionine group (32).

Weber (35) described the histological alterations during 3'-Me-DAB carcinogenesis as a "turmoil of structural and cytological events" involving cell degeneration, bile duct proliferation, and hyperplasia of hepatic cells, to name but a few. In view of this, it was important to determine the status of AMP deaminase activity in livers showing histologic changes induced by agents or treatments unrelated to tumor induction.

Partial hepatectomy (13) was used to ascertain whether regeneration per se affected AMP deaminase activity, and the data are shown in Table 2. At none of the postoperative intervals was the enzyme activity significantly elevated above the activity observed immediately following the operation. Thus, it seemed unlikely that the elevated enzyme activity in precancerous liver was associated with cyclical regeneration.

Lopez and Mazzanti (20) described massive hyperplasia of biliary ducts following dietary administration of α-naphthyl isothiocyanate. Biliary proliferation with α-naphthyl isothiocyanate was adequately confirmed (22, 33), and data were reported showing it to be inactive as a hepatocarcinogen (22).

When α-naphthyl isothiocyanate was added to diets and fed for 12 weeks, data graphically presented in Chart 5 were obtained. After 2 weeks a marked increase in the hepatic AMP deaminase activity was observed, and the elevated activity persisted for 6 weeks. By the 8th week, Am

**TABLE 1**

<table>
<thead>
<tr>
<th>AMP Deaminase Activity (µmoles IMP formed/mg protein)</th>
<th>Weeks on diets</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Hypophysectomized rats:</td>
<td></td>
</tr>
<tr>
<td>Basal diet</td>
<td>(5) 0.60 ± 0.07</td>
</tr>
<tr>
<td>0.06% 3'-Me-DAB</td>
<td>(5) 0.69 ± 0.04</td>
</tr>
<tr>
<td>Control rats:</td>
<td></td>
</tr>
<tr>
<td>Basal diet</td>
<td>(20) 0.86 ± 0.06</td>
</tr>
<tr>
<td>0.06% 3'-Me-DAB</td>
<td>(14) 0.99 ± 0.09</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the number of assays on individual rat livers. The values reported are averages with standard error (±). For assay conditions, see text or Chart 1.
however, activity had declined to the normal range and remained in this range throughout the balance of the experiment. When α-naphthyl isothiocyanate was fed at 0.04 per cent for 12 weeks, the data were similar except that the values at 2 and 4 weeks were not as high as those observed with 0.08 per cent α-naphthyl isothiocyanate.

Previous histological descriptions (20, 33, 34) emphasized not only the extent of the biliary proliferation but also the progressive nature of this proliferation throughout 4 months of dietary administration of α-naphthyl isothiocyanate. In the present study dietary administration of α-naphthyl isothiocyanate for 6 weeks resulted in a marked increase in hepatic AMP deaminase activity, which then was followed by a decline to normal values. Since biliary proliferation must have proceeded unchecked throughout this interval, it appeared unlikely that there was any direct association between biliary proliferation and the elevation of AMP deaminase activity. Indirect support for this contention also can be drawn from experiments with 3'-Me-DAB and p-hydroxypropiophenone. As shown in Chart 4, AMP deaminase activity was elevated in the liver of animals fed p-hydroxypropiophenone and 3'-Me-DAB simultaneously. Robertson, Griffin, and Richardson (30) considered the livers of such animals to be normal except for a moderate fatty metamorphosis.

Dietary factors frequently exert marked effects upon hepatic enzyme activities. For example, dietary restriction caused some decrease in the hepatic 5-hydroxytryptophan decarboxylase activity (17), and liver phosphorylase activity decreased in fasting animals (25). Replacing the glucose content of diets with protein, fat, or several other sugars increased hepatic glucose-6-phosphatase activity (10). Both serine and threonine dehydrase activities of rat liver were markedly increased when high protein diets were fed (27). In view of these observations, experiments were initiated to determine whether dietary factors might alter AMP deaminase activity.

In the first experimental group the Rockland Chow diet was changed to semi-synthetic diets (8) which had protein contents of 2 per cent or 91 per cent (27). Neither diet was changed to semi-synthetic diets (8) which had support for this contention also can be drawn from experiments with 3'-Me-DAB and α-naphthyl isothiocyanate. Robertson, Griffin, and Richardson (30) considered the livers of such animals to be normal except for a moderate fatty metamorphosis.

The data from the experiments with azo dyes consistently indicated that the elevation of AMP deaminase activity during dye carcinogenesis was associated with restricted dietary intake. The elevation of AMP deaminase activity with a low (but not with a high) protein diet following fasting sets this phenomenon apart from that reported for the increase of serine and threonine dehydrase (27). In a later report (28) evidence was presented indicating enzyme induction, and the inductions could be inhibited with glucose.

The data from the experiments with azo dyes consistently indicated that the elevation of AMP deaminase activity in precancerous rat liver was a key change (29). Technics or procedures which were known to delay or prevent the appearance of azo dye-induced tumors also delayed or prevented the elevation of AMP deaminase activity in azo dye-treated precancerous livers. The technics or procedures used in this work for delay or prevention of tumorigenesis appeared to fit criteria 2 and 3 of Reid's key change definition (29). Thus, the increase in this enzyme activity should be considered a key change.

Hepatic AMP deaminase activity was increased in the dietary experiments and in the experiments with α-naphthyl isothiocyanate. Since neither of these experimental conditions is known to be hepatocarcinogenic, it is obvious that the elevation of hepatic AMP deaminase
activity is not a phenomenon exclusively characteristic of azo-dye precancerous liver. It is interesting to note one major difference between the increases seen in the precancerous livers and those seen following noncarcinogenic treatments. With the carcinogenic azo dyes AMP deaminase activity remained elevated as long as the dye was ingested by the animal. With noncarcinogenic treatments, a normal level of enzyme activity was ultimately observed, despite continued administration of the treatments. The mechanism(s) underlying this difference is undoubtedly a key change by Reid's definition (29), and it also seems possible that this mechanism(s) is more closely associated with hepatocarcinogenesis than the actual elevation of enzyme activity.

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