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KINETIC AND IMMUNOCHEMICAL EVIDENCE FOR INDUCTION OF HEPATIC ADENOSINE MONOPHOSPHATE DEAMINASE BY 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE OR THIOACETAMIDE

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Abstract—The objective in these experiments was to determine whether increases in hepatic AMP deaminase activity after ingestion or injection of the hepatocarcinogens, 3′-methyl-4-dimethylaminoazobenzene or thioacetamide, were associated with increased enzyme amount or alterations in allosteric modulation. Using crude 105,000 *g* supernatant fractions from liver homogenized in 0·25 M sucrose as the enzyme source, data were obtained which indicated that increased AMP deaminase activity was associated with increased enzyme amount. Data in support of this contention were: (1) activity of enzyme from livers of control or hepatocarcinogen-treated animals was modulated by alkali metal ions and by certain mono-, di- or triphosphonucleotides, but the extent and nature of these modulations were similar, irrespective of enzyme source; (2) when reaction mixtures contained ATP and alkali metal ions, plots of velocity versus substrate concentration were hyperbolic and activity of enzyme from hepatocarcinogen-treated animals always exceeded that of controls; and (3) reaction of enzyme from control or hepatocarcinogen-treated animals with antibody to partially purified AMP deaminase from rat liver resulted in complete loss of enzyme activity, irrespective of source, but titration curves indicated higher antigen levels in supernatants from hepatocarcinogen-treated rats.

Incubation of 105,000 *g* supernatants at 55° resulted in losses of AMP deaminase activity, but enzyme activity losses among supernatants from carcinogen-treated animals occurred at rates nearly 3-fold greater than losses from control supernatants. Thus, it appeared that, concomitant with induction of AMP deaminase by hepatocarcinogens, appreciable alterations in physical properties of the enzyme occurred.

THE ACTIVITY of AMP deaminase in precancerous rat liver increased 2- to 3-fold after the administration of various hepatocarcinogens, and this early change seemed well correlated with the later onset of hepatic cancer.[1–6] Stimulation of AMP deaminase activity in precancerous rat liver coincided with the stimulation of hepatic cell proliferation.[7] On the other hand, when proliferation was stimulated by partial hepatectomy, AMP deaminase activity was not appreciably altered.[7] It appeared, therefore, that stimulation of hepatic AMP deaminase was correlated with the onset of unique proliferative events which occurred early in the development of hepatic cancer. The purpose of experiments described in this communication was to elucidate mechanisms underlying stimulation of AMP deaminase activity in precancerous rat liver.

AMP deaminase from rat liver was partially purified and regulatory properties were described[8] which were similar to those described by Monod *et al.*[9] for allosteric

enzymes. Allosteric properties have been described for this enzyme from erythrocytes,[10–13] brain,[14–17] muscle,[18–20] ascites tumor cells,[21] heart and lung.[22] The activity of allosteric enzymes varies widely when levels of metabolites other than the catalytically active substrates are changed.[23] Since appreciable metabolic changes occur in target organs after the administration of chemical carcinogens,[24] it was possible that changes in the levels of various effectors resulted in the stimulation of AMP deaminase activity in precancerous liver.

Smith and Kizer[8] prepared antiserum to the partially purified AMP deaminase of rat liver. Incubation of the partially purified enzyme with antiserum resulted in essentially complete loss of enzyme activity, suggesting that the antiserum contained antibodies to the partially purified enzyme.[8] Thus, it was possible to determine whether increases in enzyme activity in precancerous rat liver were accompanied by increases in immunochemical titer.

Data are presented showing that in precancerous rat liver: (1) modulation of AMP deaminase activity by alkali metal ions or nucleotides was not altered; (2) both kinetic and immunochemical data indicated that increased enzyme activity was associated with increased enzyme amount; and (3) the new enzyme cohort possessed altered physical properties.

MATERIALS AND METHODS

Female Holtzman rats weighing 150–200 g were used in these experiments. They were fed Rockland mouse and rat diet, except when carcinogens were fed. Carcinogens were fed *ad lib.* in a semi-synthetic diet[25] at concentrations of 0·06% for azo dyes or 0·07% for thioacetamide; animals fed the semi-synthetic diet alone served as controls. When azo dyes were injected intra-abdominally, they were dissolved in corn oil and injected at 250 mg/kg of body weight. Thioacetamide was dissolved in 0·9% saline and injected at 50 mg/kg of body weight. Animals were given a single injection of azo dyes, but thioacetamide was injected once daily. Azo dyes were synthesized,[26] but thioacetamide was purchased from J. T. Baker Chemical Company, Phillipsburg, N. J. Animals were killed by cervical fracture. Livers were quickly removed and immersed in ice-cold 0·25 M sucrose.

Enzyme assays. Livers were finely minced, blotted dry and weighed to give a 10% homogenate (w/v) in 0·25 M sucrose. Homogenates were centrifuged at 105,000 *g* for 30 min. The supernatant was the enzyme source.

Three buffers were used in reaction mixtures for enzyme assay: 0·05 M sodium citrate buffer, pH 6·0; 0·05 M Tris-citrate buffer, pH 6·0; and 0·05 M Tris-maleate buffer at several pH levels between 6·0 and 7·4. When sodium citrate was the buffer, the sodium salt of nucleotides was used in reaction mixtures, but when Tris-citrate or Tris-maleate was the buffer, the Tris salt of nucleotides was used in reaction mixtures.

Reaction mixtures contained buffer, AMP, enzyme and additions of various nucleotides or alkali metal ions or of both, as indicated in the tables or figures. In one experiment, AMP-8-^{14}C was the substrate. The total volume was 0·6 ml. Incubations were for 30 min in stoppered tubes at 37° with continuous shaking. Reactions were stopped by the addition of 0·6 ml of 10% trichloroacetic acid. Precipitated proteins were removed by centrifugation. Reaction mixtures for assaying enzyme activity after immunochemical titration with antiserum contained 40 mM AMP, 4 mM ATP, 150 mM LiCl, and 0·5 M sodium citrate buffer, pH 6·0. When enzyme

activity was determined in heat stability studies, reaction mixtures contained 17·2 mM AMP, 4 mM ATP, 50 mM KCl and 0·05 M sodium citrate buffer, pH 6·0. The higher substrate concentration was used in the immunochemical studies to achieve the greatest possible sensitivity for detection of residual enzyme activity.

Tris-ATP was purchased from Sigma Chemical Company, St. Louis, Mo.; AMP-8-^{14}C was purchased from New England Nuclear, Boston, Mass.; all other nucleotides were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Tris-GTP was made by percolating sodium GTP through a Dowex-50 column at 4°. The pH of the eluate was then adjusted to 6·0 with Tris-base.

Analytical procedures. In most experiments, the extent of AMP deamination was estimated by determination of the evolved ammonia. Ammonia was determined by a direct colorimetric procedure[27] when reaction mixtures were buffered with sodium citrate, but when Tris ions were present, ammonia was diffused[28] prior to reaction with Nessler's reagent.

IMP was isolated from neutralized trichloroacetic acid extracts by embedding the extracts on 18 cm × 2 cm Dowex-1 formate columns and elution with gradient 4 M formic acid essentially in the manner described by Hurlbert *et al.*[29] The concentration was determined spectrophotometrically. When the substrate was AMP-8-^{14}C, aliquots of fractions eluted from Dowex-1 formate columns were added to a xylene–dioxane–cellosolve counting mixture[30] and the isotope concentration was determined in a liquid scintillation system.* Quenching was corrected by the channels ratio method.[31]

Immunochemical titration of AMP deaminase. Procedures for the preparation of antiserum to the partially purified enzyme and demonstration of antibody to the enzyme have been described.[8] Immunochemical titration was carried out essentially as described by Jost *et al.*[32] A 0·2-ml aliquot of the 105,000 *g* supernatant from rat liver homogenates was incubated with appropriate dilutions of antiserum or control serum (rabbits injected only with Freund's adjuvant) for 30 min at 37° and then for 24 hr at 4°. After centrifugation at 1500 *g* in the cold, enzyme activity in the supernatant was assayed.

RESULTS

We reported previously that feeding[3, 4] or injecting[4–6] 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) or thioacetamide caused increases in hepatic AMP deaminase activity, but all assays were done at a single substrate concentration. Since the purified enzyme from normal rat liver showed substrate cooperativity under appropriate conditions,[8] it was important to see whether enhanced enzyme activity persisted throughout a broad substrate range. As shown by velocity versus substrate plots in Figs. 1 and 2, both 3′-Me-DAB and thioacetamide stimulated hepatic AMP deaminase activity, irrespective of substrate concentration, between 3·4 and 45·7 mM. Therefore, our earlier conclusions[3–5] were not associated with cooperativity. On the other hand, all the plots in both Figs. 1 and 2 deviated from a hyperbola and points would not fit a single line when plotted in the double reciprocal manner. Thus, enzyme activity did not adhere to Michaelis–Menten kinetics, and an assumption of proportionality between enzyme activity and enzyme amount was not warranted.

Data in Figs. 1 and 2 demonstrated the progressive action of the two carcinogens with respect to stimulation of enzyme activity, but there were differences in the time of

* Mark I, Nuclear Chicago Corp. system.

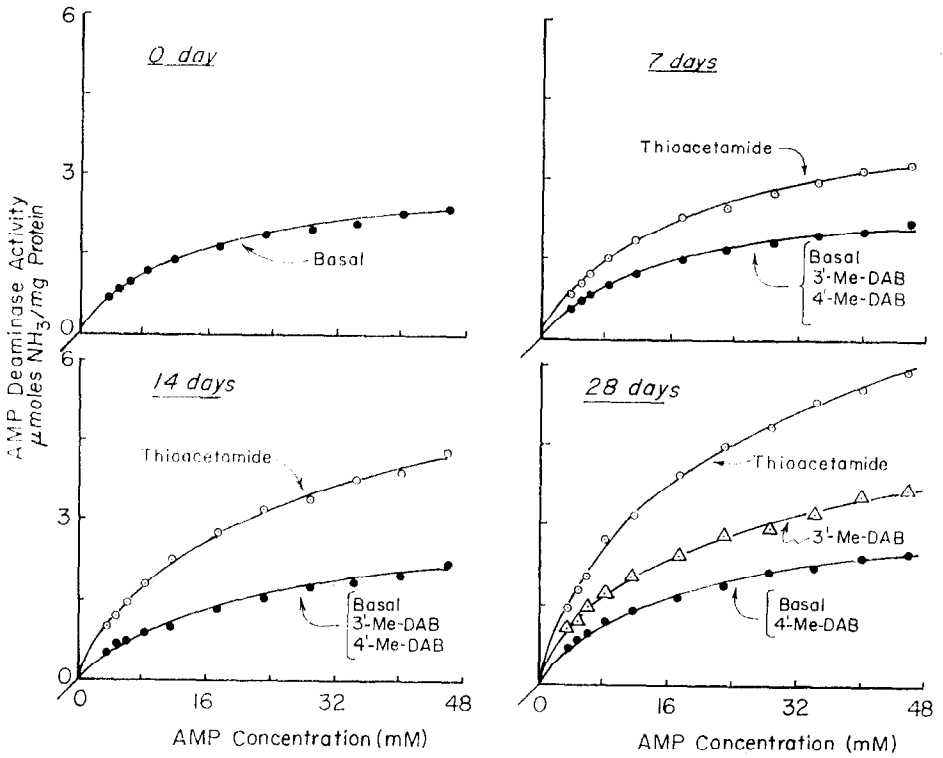

FIG. 1. Effect of feeding azo dyes or thioacetamide to female rats on hepatic AMP deaminase activity. The 3'-methyl (3'-Me) and 4'-methyl (4'-Me) derivatives of 4-dimethylaminoazobenzene (DAB) were fed *ad lib.* at 0·06% in a semi-synthetic diet.[25] Thioacetamide was fed at 0·07%. Livers were homogenized in 0·25 M sucrose and homogenates were centrifuged for 30 min at 105,000 *g*. Supernatants were the enzyme source. Reaction mixtures for the assay of AMP deaminase contained: AMP at concentrations indicated; sodium citrate buffer, pH 6·0; and enzyme. Sodium ion concentration contributed by the buffer was 42 mM, while that contributed by pH adjustment of substrate AMP ranged from 2·9 to 38·8 mM. Potassium ion concentration contributed by pH adjustment of substrate AMP ranged from 2·1 to 27·7 mM. Incubation was for 30 min at 37°. Reactions were terminated by addition of 10% trichloroacetic acid 1:1 with reaction mixture. Ammonia was determined by a direct colorimetric procedure.[27] Values plotted are averages of assays on five or six animals on each dietary regimen. Standard error terms about these averages did not exceed 10% of the mean value.

onset and magnitude of stimulatory response. For instance, when thioacetamide was fed (Fig. 1), detectable stimulation was observed as early as 7 days later, and further increases were recorded at 14 and 28 days. On the other hand, when carcinogens were injected (Fig. 2),both were equally stimulatory at 2 days but, after 3 days, stimulation with 3'-Me-DAB exceeded that seen with thioacetamide. Whether fed or injected, 4'-methyl-4-dimethylaminoazobenzene (4'-Me-DAB) caused no stimulation of enzyme activity.

Modulation of AMP deaminase activity by nucleotides has been demonstrated,[6, 8, 11, 12, 14–18, 20–22] and enzyme activity stimulated by thioacetamide injections was stimulated further by addition of ATP to the assay medium.[6] Summarized in

Table 1 are data from experiments in which we determined whether the activity of the hepatic enzyme from 3′-Me-DAB-injected animals was modulated by addition of nucleotides to the assay medium. In general, the extent of nucleotide activation or inhibition of the enzyme from carcinogen-treated animals did not differ appreciably from effects seen on the enzyme from control animals; this agreed with earlier findings on the enzyme from thioacetamide-injected rats.[6] Addition of two triphosphonucleotides, ATP and ITP, resulted in good activation, while inhibition was seen with 3′(2′)AMP. Less extensive activation was observed with GTP, CTP and ADP.

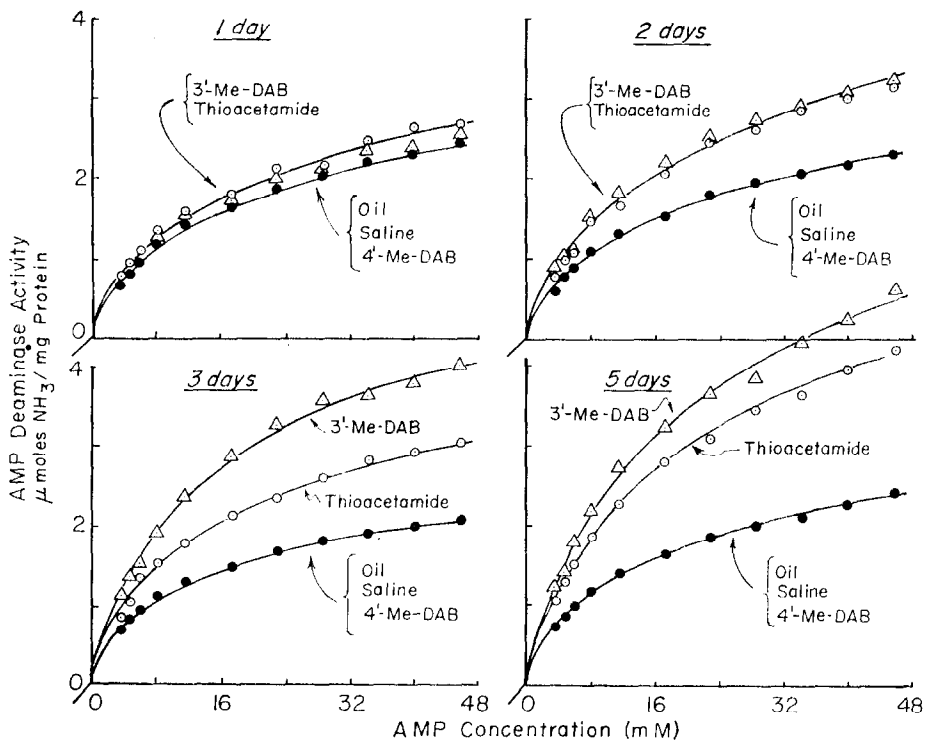

FIG. 2. Effect of injecting azo dyes or thioacetamide into female rats on hepatic AMP deaminase activity. 3′-Me-DAB and 4′-Me-DAB were dissolved in corn oil and injected once at 250 mg/kg of body weight, while thioacetamide was dissolved in 0·9% saline and injected once daily at 50 mg/kg of body weight. Controls received injections of the diluents. Animals were killed 1, 2, 3 and 5 days after a single injection of azo dye or oil, or 24 hr after 1, 2, 3 and 5 injections of saline or thioacetamide. All other conditions were as described in Fig. 1. Values plotted are averages of assays on four to eight rats per treatment group. Standard error terms about these averages did not exceed 10% of the mean value.

The effects of ATP and GTP on the velocity versus substrate plots of AMP deaminase activity in the liver of rats injected with oil or 3′-Me-DAB are shown in Fig. 3. ATP activated the enzyme from both control and carcinogen-treated rats and, in each case, velocity curves were hyperbolic. When GTP was added to reaction mixtures, velocity curves were sigmoidal in shape. Enzyme activity in the liver of rats injected with 3′-Me-DAB exceeded the activity in the liver of oil-injected rats, irrespective of the triphosphonucleotide composition of reaction mixtures.

TABLE 1. EFFECT OF NUCLEOTIDES ON THE HEPATIC AMP DEAMINASE ACTIVITY OF RATS INJECTED WITH 3′-ME-DAB*

Nucleotides added	Concn (mM)	Enzyme activity (%)	
		Oil	3′-Me-DAB
None		100	100
ATP	4	130	140
ITP	4	128	123
GTP	4	113	115
CTP	4	113	109
NAD-P	4	102	97
ADP	4	118	114
UMP	4	96	99
IMP	4	94	101
TMP	4	96	91
3′,2′-UMP	8	101†	103
3′,2′-CMP	8	97†	96
3′,2′-GMP	8	95†	85
3′,2′-AMP	8	72†	75

* Rats were injected as described in Fig. 2. Enzyme activity was assayed as described in Fig. 1, except that AMP was held constant at 17·2 mM. Sodium and potassium ion concentrations, resulting from pH adjustment of substrate AMP, were 18·2 and 13·0 mM respectively. Nucleotides were added as the Na–K salts. Values are averages of assays on three rats.

† These rats were not injected with the oil vehicle; values are averages of assays on four rats.

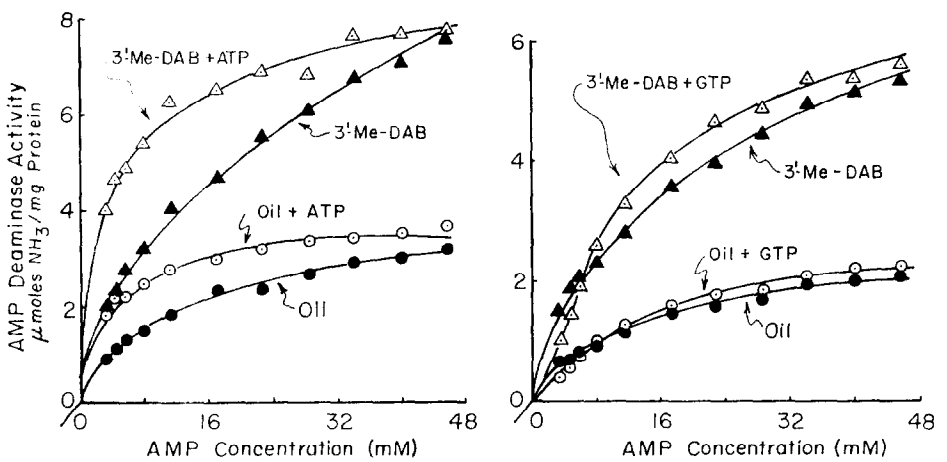

FIG. 3. Activation of hepatic AMP deaminase by ATP or GTP after injection of 3′-methyl-4-dimethyl aminoazobenzene (3′-Me-DAB). Oil or 3′-Me-DAB was injected as described in Fig. 2. ATP or GTP was added at 4 mM. All other conditions were as described in Fig. 1. Each point represents averages of assays on three rats per treatment group. The S. E. about each point did not exceed 15% of the mean value.

In preceding experiments, enzyme activity in crude supernatant fractions was determined by ammonia analysis. Since we could not be certain that AMP deaminase was the only enzyme catalytically active, it was appropriate to determine the stoichiometric relationship between the two products, IMP and ammonia. As shown in Fig. 4, over a broad range of substrate concentrations, the expected stoichiometry between IMP and ammonia was not altered by ATP or GTP. Therefore, ammonia evolution

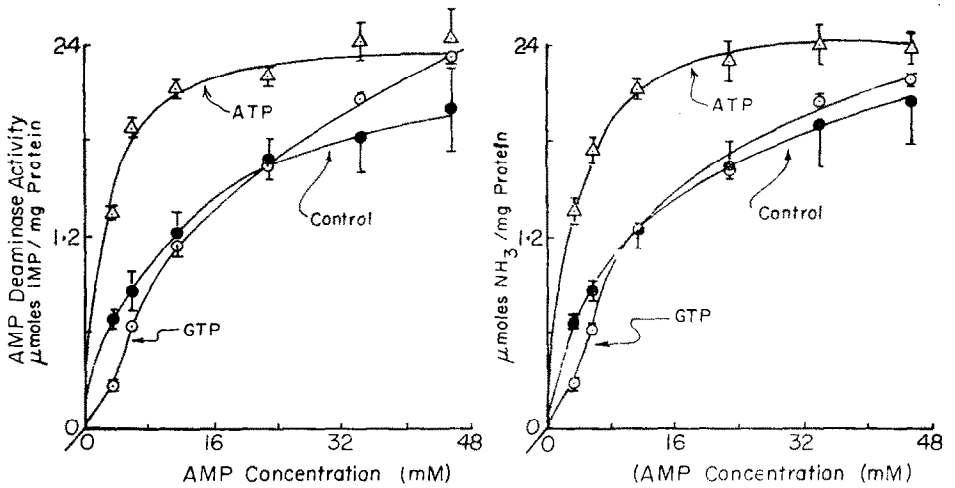

FIG. 4. Stoichiometry between the products, IMP and ammonia, when ATP or GTP was present during the assay of hepatic AMP deaminase. The enzyme source was the 105,000 *g* supernatant from livers of untreated female rats. ATP and GTP concentrations were 4 mM. IMP was determined spectrophotometrically after elution from Dowex-1 formate columns as described by Hurlbert *et al.*[29] Other conditions were as described in Fig. 1.

resulted primarily from the oxidative deamination of AMP. Velocity versus substrate curves, whether utilizing ammonia or IMP determinations, were hyperbolic in the presence of ATP and sigmoidal in the presence of GTP. In these experiments the substrate was AMP-8-^{14}C at 38,600 ± 900 dpm/μmole. The specific activity of IMP isolated from control, ATP or GTP reaction mixtures was 35,700 ± 1800, 44,000 ± 2000 and 35,200 ± 1000 dpm/μmole respectively. Thus, it seemed clear that the velocity plots in Figs. 3 and 4 resulted primarily from the action of AMP deaminase upon the substrate AMP without appreciable contributions from extraneous reactions.

Monovalent alkali metal ions activated AMP deaminase from liver,[8] erythrocytes,[10, 11] brain[15] and muscle.[18–20] In preceding experiments, sodium ion was present in reaction mixtures contributed by the buffer and by pH adjustment of substrate and modulator solutions. To determine whether stimulation of enzyme activity by carcinogens was associated with alkali metal ion activation, Tris-citrate buffer was used instead of sodium citrate, and pH adjustments were made with Tris-base. As shown in Table 2, Li^+, K^+ or Na^+ at 150 mM essentially doubled enzyme activity in supernatants from oil-injected rats. Velocity ratios between the enzyme from oil-injected and 3′-Me-DAB-injected rats were essentially constant, irrespective of the

TABLE 2. ACTIVATION OF HEPATIC AMP DEAMINASE BY ALKALI METAL IONS AFTER INJECTION OF 3′-ME-DAB*

	AMP deaminase activity (μmoles NH_3/mg protein)			
	Additions to reaction mixtures			
	None	Li^+	K^+	Na^+
Oil injected	1·1 ± 0·1	1·7 ± 0·1	2·0 ± 0·2	2·1 ± 0·1
3′-Me-DAB injected	2·2 ± 0·3	3·9 ± 0·5	4·3 ± 0·5	4·7 ± 0·7
$\frac{V_{oil}}{V_{3'\text{-me-DAB}}}$†	0·51	0·45	0·45	0·45

* Alkali metal ion concentration was 150 mM. 3′-Me-DAB was injected as described in Fig. 2 and livers were homogenized as described in Fig. 1. Reaction mixtures contained: 17·2 mM AMP; 0·05 M Tris-citrate buffer, pH 6·0; and alkali metal ions as indicated. Ammonia was determined by diffusion[28] and reaction with Nessler's reagent. Values reported are averages ± S.E. based on three (oil) or four (3′-Me-DAB) rats.

† Velocity ratio between the enzyme from oil-injected and 3′-Me-DAB-injected rats.

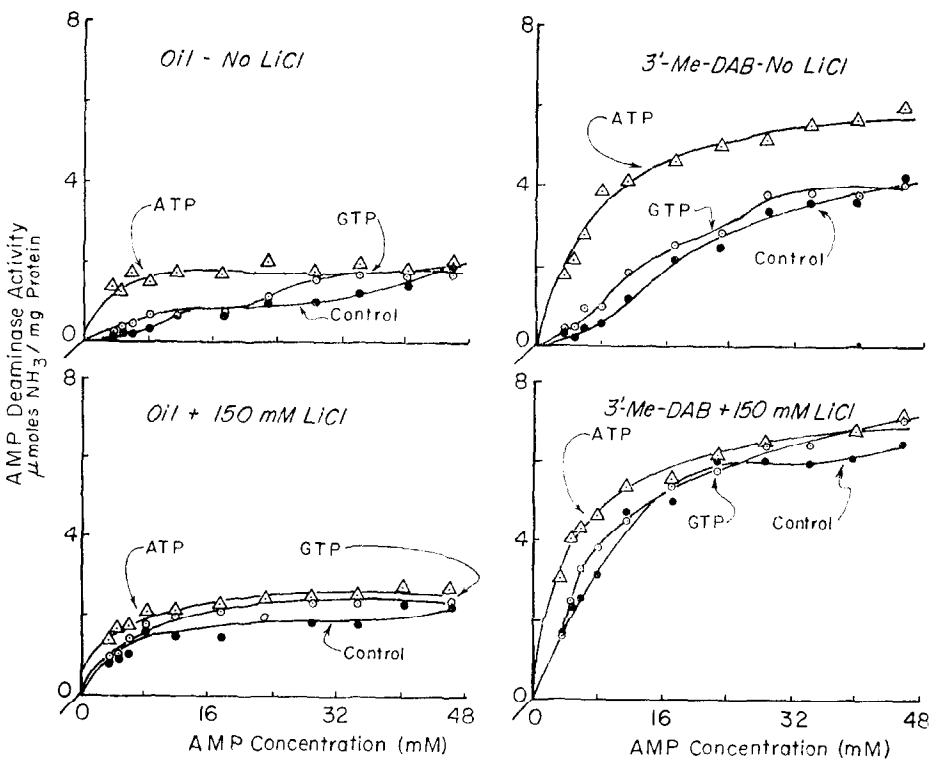

FIG. 5. Effect of alkali metal ions on the activation of hepatic AMP deaminase by ATP or GTP after injection of 3′-Me-DAB. Oil or 3′-Me-DAB was injected as described in Fig. 2, and the animals were killed after 5 days. Reaction mixtures contained: Tris-AMP at concentrations indicated; 0·05 M Tris-citrate buffer, pH 6·0; and, where indicated, 4 mM Tris-GTP or 4 mM Tris-ATP. Ammonia was diffused[28] from reaction mixtures and reacted with Nessler's reagent. Each point represents averages of assays on three to four rats per treatment group.

alkali ion status. Therefore, stimulation of enzyme activity by carcinogens did not appear to be associated with alkali metal ion activation.

Experiments to assess alkali metal ion–triphosphonucleotide interactions on enzyme from carcinogen-treated rats are summarized in Fig. 5. As in the previous experiment, Tris-citrate buffer was used and the two nucleotides were Tris salts. Here, as in preceding experiments, activity of AMP deaminase from 3′-Me-DAB-treated rats exceeded that of controls regardless of the alkali metal ion–triphosphonucleotide composition of reaction mixtures. When Li^+ was absent from reaction mixtures: ATP was a potent activator and velocity plots were essentially hyperbolic; control and GTP velocity plots were essentially sigmoidal in shape. When LiCl was added to reaction mixtures at 150 mM: ATP still activated and the velocity plot was essentially hyperbolic; both control and GTP velocity plots lost their sigmoidal shape, but appreciable deviation from a hyperbola persisted. Thus, in the presence of adequate concentrations of alkali metal ions and ATP, the enzyme in the crude supernatant fraction obeyed Michaelis–Menten kinetics, whether from normal or carcinogen-treated animals. Since under these conditions velocity is proportional to enzyme amount, these data supported a conclusion that AMP deaminase was induced after 3′-Me-DAB injections.

Smith and Kizer[8] showed that ATP activation of the purified liver enzyme was inhibited by GTP at concentrations approximately $\frac{1}{10}$ that of ATP. Therefore,

TABLE 3. HEPATIC AMP DEAMINASE ACTIVITY OF RATS FED 3′-ME-DAB OR THIOACETAMIDE AFTER PASSING THE 105,000 *g* SUPERNATANT THROUGH SEPHADEX G-25*

Diet	Sephadex treatment	AMP deaminase activity (μmoles NH_3/mg protein)
Basal	−	3·05 ± 0·29
Basal	+	3·07 ± 0·30
3′ Me-DAB	−	3·96 ± 0·52
3′-Me-DAB	+	4·00 ± 0·48
Thioacetamide	−	6·27 ± 0·38
Thioacetamide	+	6·92 ± 0·78

* Rats were fed 0·06% 3′-Me-DAB or 0·07% thioacetamide in the basal diet of Medes *et al.*[25] throughout 4 weeks. Supernatants prepared as described in Fig. 1 were passed through 10·2 cm × 2·5 cm Sephadex G-25 columns which had been equilibrated with 0·25 M sucrose. Enzyme activity was assayed as described in Table 2, except that the Na^+ salt of nucleotides was used and the buffer was 0·05 M sodium citrate, pH 6·0. Ammonia was determined by direct color reaction.[27] Values shown are averages ± S.E. of assays on four animals in each group.

stimulation by carcinogens could result from changes in nucleotide composition of the crude supernatant fraction. Accordingly, supernatant fractions were prepared from the liver of animals fed 3′-Me-DAB or thioacetamide throughout 4 weeks. Prior to assaying for AMP deaminase activity, the supernatants were passed through Sephadex G-25 columns to minimize possible differences in the endogenous levels of various effector molecules. The data are summarized in Table 3. When the supernatants were assayed in the presence of excess ATP and alkali metal ions, passage through Sephadex G-25 columns caused no appreciable change in enzyme activity, irrespective of the source of the fraction. Since the activity of the enzyme from animals fed either 3′-Me-

DAB or thioacetamide exceeded that of animals fed basal diet, these data also supported a conclusion that hepatocarcinogens induced hepatic AMP deaminase.

Earlier experiments with hepatic AMP deaminase indicated that the enzyme had a rather sharp pH optimum between 6·5 and 7·0.[2] The pH optimum was not appreciably changed by purification, but it was less sharp.[8] In view of the emergence of kinetic data supporting a conclusion that hepatocarcinogens caused induction of rat liver AMP deaminase, it was important to ascertain that this conclusion was not associated with some interaction between various effector ions and pH. Data showing the absence of such interactions are shown in Fig. 6. Irrespective of the pH (between pH 6·0 and

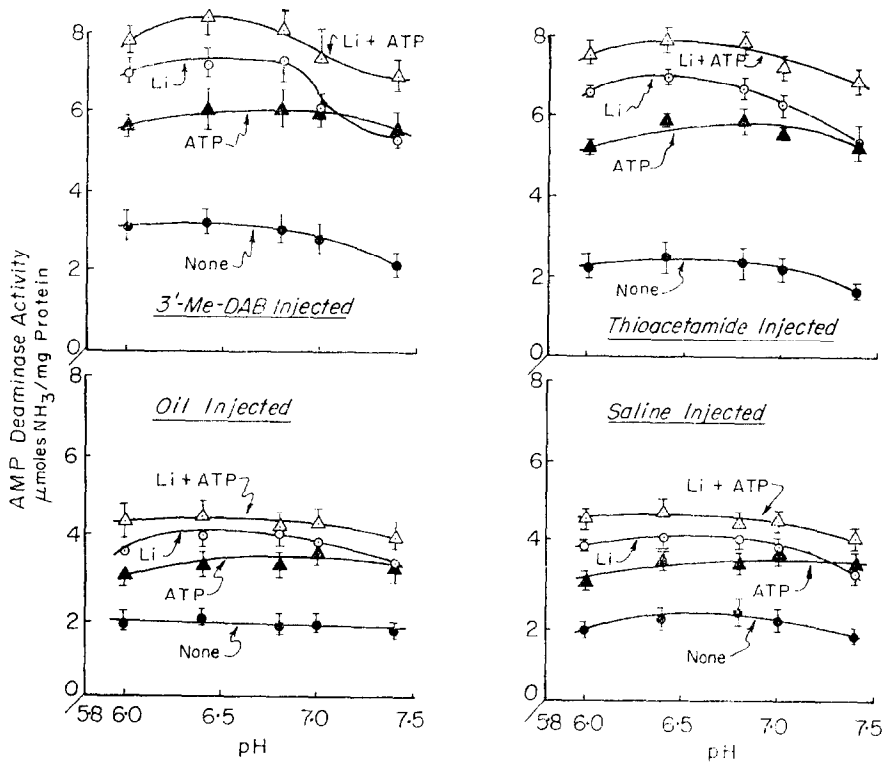

FIG. 6. Velocity plots of hepatic AMP deaminase from animals injected with 3′-Me-DAB or thioacetamide after variation of pH or effectors during assay *in vitro*. Hepatocarcinogens were injected as described in Fig. 2. Control animals received the oil or saline vehicles. The buffer used was 0·05 M Tris-maleate. The substrate was 17·2 mM AMP. When present, effector concentrations were: ATP, 4 mM; LiCl, 150 mM. Values plotted are averages ± S. E. of assays on six rats per treatment group.

7·5) or the status of effectors, enzyme activity among carcinogen-treated animals exceeded that of control animals. At a pH above 7·0, velocity was inhibited, but between pH 6·0 and 7·0 enzyme activity was readily measured.

Immunochemical titration of AMP deaminase from livers of rats injected with oil or 3′-Me-DAB is graphically depicted in Fig. 7. Although, in the absence of antiserum, activity in livers of 3′-Me-DAB rats exceeded that of oil-injected rats by 2-fold, reaction with antiserum resulted in essentially complete loss of catalytic activity in

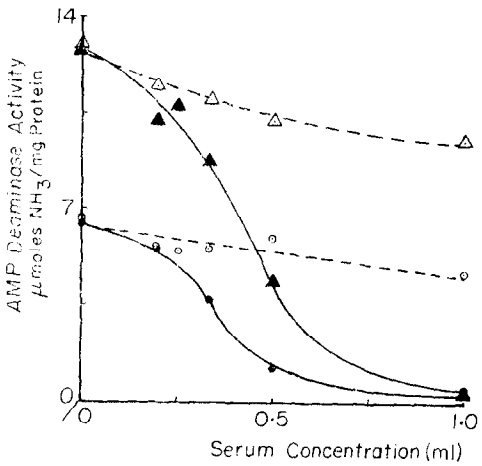

FIG. 7. Immunochemical titration of hepatic AMP deaminase from animals injected with oil or 3′-Me-DAB. Circles represent activity of supernatants from oil-injected rats; triangles, activity of supernatants from 3′-Me-DAB-injected rats. Solid circles and triangles represent levels of residual enzyme activity after reaction with antiserum; open circles and triangles represent residual activity after incubation with control serum. AMP deaminase activity represents activity in supernatants after: incubation with serum for 30 min at 37° and 24 hr at 4°; and centrifugation at 1500 *g* for 20 min.[32] Animals were injected with oil or 3′-Me-DAB exactly as described in Fig. 2. Details of immunochemical titration and assay of enzyme activity are given in the Materials and Methods section. Each point represents averages of duplicate immunochemical titrations and quadruplicate enzyme assays.

both groups. Titration curves suggested that the higher enzyme activity of the 3′-Me-DAB-injected animals was associated with a higher content of antigen. At all antiserum levels, except the highest level, higher proportions of activity remained in supernatants from 3′-Me-DAB-injected animals than in control supernatants. For instance, after reaction with an antiserum level of 0·3 ml, about 50 per cent of the control enzyme activity remained, whereas about 80 per cent of the 3′-Me-DAB enzyme activity remained. When animals were injected with thioacetamide or saline throughout 5 days and enzyme from both groups was reacted with antiserum, essentially identical results were obtained. Thus, these data also supported a contention that the two carcinogens, 3′-Me-DAB and thioacetamide, caused induction of hepatic AMP deaminase.

Effector molecules are often useful in the stabilization of allosteric enzymes.[23] ATP afforded protection against heat lability for the AMP deaminase of calf brain[15] and rat liver,[8] and this stabilization was useful in the purification of the enzyme from both tissues. Since the data in Table 3 and Fig. 7 indicated induction of hepatic AMP deaminase by hepatocarcinogens, it was important to determine whether heat lability of the new enzyme cohort was altered. As shown in Fig. 8, enzyme from both oil-injected and 3′-Me-DAB-injected rat livers was heat labile, but regression coefficients calculated from plots of velocity versus log time (see inset, Fig. 8) indicated that enzyme from oil-injected rats was inactivated at 0·73 units/unit log time, whereas enzyme from 3′-Me-DAB-injected rats was inactivated at 1·66 units/unit log time. Covariance analysis showed this difference to be significant at probability levels exceeding 0·01 per cent. ATP effectively stabilized the enzyme from both sources to such an extent that only slight velocity losses were observed throughout 90 min.

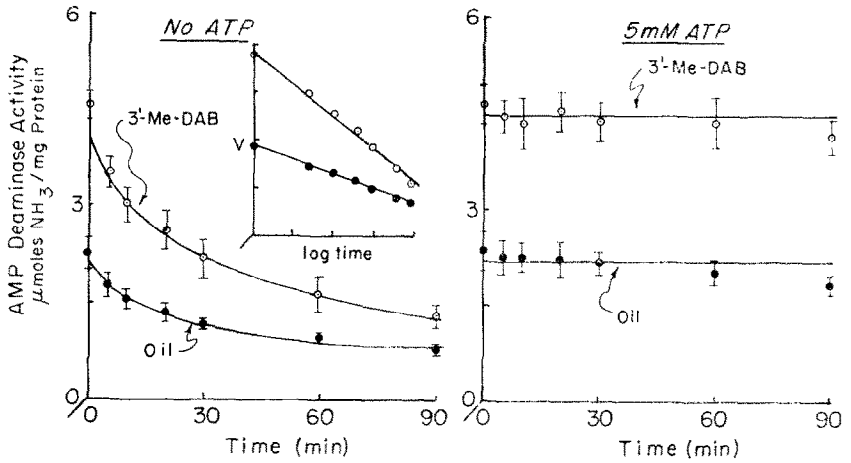

FIG. 8. Effect of 3′-Me-DAB injections upon heat lability of hepatic AMP deaminase. Oil or 3′-Me-DAB was injected as described in Fig. 2. Supernatants were heated at 55° for varying periods either in the absence (figure at left) or presence of 5 mM ATP (figure at right) and then plunged into ice-cold water. Enzyme activity was assayed in reaction mixtures containing: 17·2 mM AMP; 4 mM ATP; 50 mM KCl; and 50 mM sodium citrate buffer, pH 6·0. Values plotted are averages ± S. E. of assays on enzyme from 4 rats. Plots of velocity versus log time yielded regression coefficients of 0·73 units enzyme activity/unit log time for oil-injected and 1·66 units/unit log time for 3′-Me-DAB-injected animals. Covariance analysis indicated that the two slopes differed at a probability level greater than 0·01%.

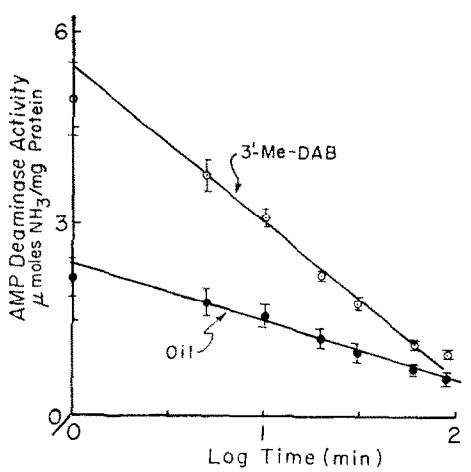

FIG. 9. Comparison of thermal labilities of AMP deaminase from oil-injected or 3′-Me-DAB-injected animals after removal of endogenous nucleotides. Conditions were as described in Fig. 6, except that, prior to heating, supernatants were passed through 10·2 cm × 2·5 cm Sephadex G-25 columns which had been previously equilibrated with 0·25 M sucrose. Values plotted are averages ± S. E. of assays on enzyme from four rats in each treatment group. Regression coefficients were calculated to be: oil-injected, 0·81 ± 0·06 units of enzyme lost/unit log time; 3′-Me-DAB, 2·14 ± 0·09 units of enzyme lost/unit log time. Covariance analysis showed this difference to be significant at a probability level of 0·01%.

It was possible that increased heat lability was associated with changes in the endogenous levels of various nucleotides in the supernatants. To test this, supernatants from oil-injected or 3′-Me-DAB-injected rats were passed through Sephadex G-25 prior to comparing the heat lability of the AMP deaminase. The data (Fig. 9) yielded regression coefficients of 0·81 ± 0·06 and 2·14 ± 0·09 units of enzyme activity lost/unit log time in supernatants from oil-injected or 3′-Me-DAB-injected animals respectively; covariance analysis indicated that these values differed significantly at the 0·01 per cent level of probability. Therefore, differences in the endogenous nucleotide levels of the two supernatant fractions did not contribute appreciably to differences in thermal lability. It appeared that the greater thermal instability of the AMP deaminase from 3′-Me-DAB-injected animals was associated with changes in physical properties of the enzyme.

DISCUSSION

The data from kinetic and immunochemical studies were consistent with a conclusion that interactions between rat liver and the two hepatocarcinogens, 3′-Me-DAB and thioacetamide, resulted in an increase in the level of hepatic AMP deaminase. Thus, the mechanism underlying increased hepatic AMP deaminase activity during hepatocarcinogenesis in rats with azo dyes or thioacetamide appeared to be induction. This conclusion should be considered a tentative one, having operational significance, but certainly not proven. Stadtman[23] stated that ". . . every allosteric enzyme studied in detail has been shown to possess a tertiary and in several instances also a quaternary structure". In addition to the usual factors which influence catalytic behavior of enzymes, activity of allosteric enzymes is profoundly influenced by effector status, state of association and state of aggregation.[23] Control of these factors in crude tissue preparations can be attempted, but rarely achieved.[33] Control was complicated further in these experiments by major shifts in liver cellularity which usually follow hepatocarcinogen administration.[24, 34, 35] In light of these considerations, a tentative conclusion concerning induction of this enzyme by hepatocarcinogens seems prudent.

Whether induction of hepatic AMP deaminase was associated with enhanced synthesis or decreased degradation of enzyme,[33] or alternatively with major shifts in liver cellularity,[24, 34, 35] kinetic properties of the new enzyme population were not altered appreciably; but data showing increased heat lability (Figs. 8 and 9) were consistent with a conclusion that physical properties of the new enzyme population were altered. Within parameters of the Monod–Wyman–Changeux model for allosteric enzymes,[9] this change suggested either an alteration in tertiary or quaternary structure or in both, or in the associated or aggregated state or in both states of the oligomer. Either kind of alteration might plausibly result from binding of azo dyes to protein. Covalent binding of aromatic azo dyes by substitution in the diamine ring or in the *N*-alkyl groups[36] to soluble protein, especially the h-proteins,[37] has been well documented. Recently, it has been shown that much of this binding involves methionine[38] and tyrosine[39] moieties of liver proteins. If AMP deaminase of the soluble liver proteins were a target for covalent binding of azo dyes, altered physical properties might result.

The physiological function of AMP deaminase remains obscure. It seems likely that this enzyme is ubiquitously distributed in mammalian tissues; the present study

together with previous studies (e.g.,[8, 13, 16, 17]) adequately demonstrated its exquisite regulation. As noted by Stadtman,[23] such regulation is usually reserved for enzymes located at critical points in interlocking or overlapping metabolic systems. Studies with bacterial systems indicated that the interconversion of AMP–IMP–GMP was regulated with respect to *de novo* purine biosynthesis[40] and histidine biosynthesis.[41] If subsequent studies should further substantiate induction of AMP deaminase by hepatocarcinogens, studies on the status of purine and histidine biosynthetic pathways in precancerous rat liver may be appropriate.

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