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THE USE OF CHRONICALLY ADMINISTERED LOW DOSES OF ETHIONINE AND PUROMYCIN TO STUDY INCREASES IN RAT LIVER ADENOSINE MONOPHOSPHATE DEAMINASE ACTIVITY CAUSED BY THIOACETAMIDE INJECTIONS

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Abstract—Rat liver AMP deaminase activity was increased 2-fold 24 hr after three once-daily injections of thioacetamide at 50 mg/kg body weight. Since higher doses of inhibitors, administered over several days, led to fatal toxicities, lower doses of ethionine (250 mg/kg body weight, once daily) or puromycin (80 mg/kg body weight/day in three divided doses) were administered chronically throughout 4 or 5 days to test whether increased enzyme activity involved hepatic translation mechanisms. Ethionine inhibited thioacetamide-induced increases through 4 days in both male and female rats, but was no longer inhibitory after 5 days. Puromycin was not inhibitory. Studies on the incorporation of leucine-U-^{14}C into liver proteins and on cortisone induction of tryptophan oxygenase established that neither ethionine nor puromycin inhibited hepatic protein biosynthesis. Hepatic ATP levels were depleted by ethionine alone or in combination with thioacetamide, but it was considered unlikely that this change was linked with ethionine inhibition of increased AMP deaminase activity. The possibility that ethionine inhibition was related to allosteric modulation or to inhibition of cellular proliferation was discussed.

A PREVIOUS investigation on increases in hepatic AMP deaminase (EC 3.5.4.6) activity after thioacetamide injections indicated that these increases were not dependent upon RNA synthesis, but mediation by a stable or long-lived messenger RNA could not be eliminated.[1] Therefore, it was appropriate to determine whether chemicals known to inhibit protein synthesis would interfere with these increases.

Induction of hepatic enzymes was inhibited by ethionine[2–5] and puromycin.[2,4,6–9] In most of these studies, however, induction occurred in relatively short periods, i.e. in several hours, and blockades of protein synthesis were not maintained longer than 9 hr. Under these circumstances, both puromycin and ethionine were especially effective, for both inhibitors affected translation directly, ethionine apparently by depleting ATP levels[10] and puromycin by premature release of peptides.[11]

Significant elevation of rat liver AMP deaminase activity required two or three thioacetamide injections spaced at 24-hr intervals.[12] This consequently required that blockade of hepatic protein synthesis be maintained for 48 hr or longer. Attempts to administer doses of ethionine or puromycin throughout this period in regimes similar to those used in acute studies quickly led to fatal toxicities. Results from studies by Weber and Singhal[13,14] suggested that lower multiple doses of these two inhibitors

effectively inhibited hepatic translational mechanisms for longer periods.[13,14] In their studies, induction of two gluconeogenic enzymes in alloxan diabetic or cortisonized rats was inhibited despite a necessity for maintaining blocks through 4 days.[13,14]

Data are presented showing that chronic administration of ethionine, but not puromycin, caused inhibition of thioacetamide-induced increases in AMP deaminase activity. Neither drug, however, inhibited incorporation of labeled leucine into hepatic proteins or inhibited cortisone induction of tryptophan oxygenase. Inhibition of enzyme activity with ethionine was accompanied by depletion of hepatic ATP levels.

MATERIALS AND METHODS

Male and female rats weighing 80–120 g were purchased from the Holtzman Company, Madison, Wis. They were fed Rockland Chow diet until they weighed 140–160 g, and then were used for experiments. Male rats, weighing 90–110 g when adrenalectomized, were held 7–9 days after operation and weighed 100–120 g when killed. During this interval they were given 1% NaCl as drinking water.[15]

Throughout this work, thioacetamide was injected once daily at 50 mg/kg body weight. Most of the regimes used for administration of ethionine or puromycin were adaptations of regimes used by Weber and Singhal:[13,14] viz. ethionine once daily at 250 mg/kg body weight; puromycin, 80 mg/kg body weight/day in three divided doses or at 5·86 mg/kg body weight every 2 hr for 24 doses. In one experiment, 1·22 m-moles ethionine was injected as described by Villa-Trevino *et al.*[16] Control animals in all experiments received injections of saline.

Tryptophan oxygenase (EC 1.13.1.12) was induced by injecting cortisone, 10 mg/kg body weight, 3 hr prior to killing the animals.[17] Ethionine (250 mg/kg body weight) was given simultaneously with the inducer, while puromycin (5 mg/kg body weight) was injected with the inducer and again 1 hr before killing the animals.

The assay of AMP deaminase activity has been described previously,[12,18] but where indicated, reaction mixtures were changed by increasing the substrate concentration from 13·3 mM to 17·2 mM and adding 4 mM ATP. Tryptophan oxygenase was assayed in whole homogenates of rat liver.[19] Hematin, 0·5 μM, was added during assay.[20]

Leucine-U-^{14}C was injected intra-abdominally at 3·0 μc/rat, and was allowed to incorporate for 30 min. Livers were homogenized in 0·25 M sucrose; homogenates were strained through four thicknesses of cheesecloth and diluted by addition of an equal volume of 0·25 M leucine. The extent of leucine-U-^{14}C incorporation was determined by the procedure of Wannemacher *et al.*[21] The isotope concentration was determined in a Mark I (Nuclear Chicago Corp.) liquid scintillation system using a xylene–dioxane–cellosolve counting cocktail.[22] Quenching was corrected by the channels ratio method of Bush.[23]

The concentration of free leucine was determined in livers of rats given 4 once-daily injections of saline, thioacetamide (50 mg/kg body weight), ethionine (250 mg/kg body weight) or the combination. Livers from five rats per treatment were pooled, homogenized in distilled water, and homogenates were centrifuged for 30 min at 105,000 *g*. Aliquots of the 105,000 *g* supernatants were deproteinized with picric acid[24–26] and the leucine concentration was determined by using a Technicon Corp. amino acid analyzer.

ATP levels in the liver were estimated by the luciferin–luciferase reaction described by Strehler and McElroy.[27] Liver cellularity was determined by counting nuclei in whole homogenates.[28] The protein in the 105,000 *g* supernatant fraction was determined by the method of Lowry *et al.*[29] and in whole homogenates by the method of Oyama and Eagle.[30] The concentration of DNA was determined by the method of Burton.[31]

Puromycin dihydrochloride was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Firefly lantern extract, DL-*p*-fluorophenylalanine and β-2-thienylalanine were purchased from Sigma Chemical Co., St. Louis, Mo. L-Leucine-U-^{14}C was purchased from New England Nuclear, Boston, Mass. Cortisone acetate was purchased from Merck Sharp & Dohme, Philadelphia, Pa.

RESULTS AND DISCUSSION

A time-course study of the effects of ethionine upon increases in AMP deaminase activity after once-daily injections of thioacetamide into female rats is shown in Table 1. Increased AMP deaminase activity after thioacetamide administration either

TABLE 1. EFFECT OF ETHIONINE AND PUROMYCIN UPON INCREASES IN AMP DEAMINASE ACTIVITY IN THE LIVER OF FEMALE RATS CAUSED BY THIOACETAMIDE*

Compounds injected	AMP deaminase activity (μmoles NH_3/mg protein)				
	No. of injections				
	1	2	3	4	5
Saline	(6) 0·76 ± 0·05	(6) 0·64 ± 0·07	(8) 0·58 ± 0·02	(18) 0·75 ± 0·05	(6) 0·79 ± 0·08
Thioacetamide (TA)	(6) 0·95 ± 0·05†	(6) 1·08 ± 0·08†	(8) 1·26 ± 0·06†	(20) 1·33 ± 0·07†	(6) 1·48 ± 0·10†
Ethionine	(6) 0·92 ± 0·05†	(6) 0·71 ± 0·04	(8) 0·84 ± 0·03†	(8) 0·88 ± 0·06	(6) 1·01 ± 0·08
Puromycin				(6) 1·14 ± 0·06	
TA + ethionine	(6) 0·86 ± 0·07	(6) 0·94 ± 0·04	(8) 0·92 ± 0·04‡	(14) 1·04 ± 0·05‡	(6) 1·46 ± 0·08
TA + puromycin				(6) 2·21 ± 0·19‡	

* Thioacetamide (50 mg/kg body weight) and ethionine (250 mg/kg body wt.) were injected once daily but puromycin (80 mg/kg body wt./day) was given in three divided doses. Animals were killed 24 hr after the once daily injections. Reaction mixtures contained 13·3 mM AMP, 0·05 M citrate buffer, pH 6·0, and supernatant protein to give 1–2·5 mg/ml of reaction mixture. Ammonia was determined by the method of Chaney and Marbach.[33] Numbers in parentheses represent numbers of animals assayed. Values are averages ± S.E.

†‡Values which exceeded corresponding saline or thioacetamide values, respectively, at $P = 0·05$ or greater.

by feeding in diets or by injection has been reported previously[1,12,32] and was observed again in these studies. Simultaneous injections of ethionine interfered with these increases, especially after three and four injections. After five simultaneous injections, however, inhibition dissipated and enzyme activities among these animals could not be distinguished from activities seen among animals which had received thioacetamide alone. Alone, puromycin caused no change in enzyme activity; in combination with thioacetamide, enzyme activity was higher than it was with thioacetamide.

This experiment was repeated using five male rats per treatment group. Experimental details were identical except that an additional injection of puromycin (30

mg/kg body weight) was given 1 hr before animals were killed and reaction mixtures in the enzyme assay contained 17·2 mM AMP and 4 mM ATP. ATP has been shown previously to be an activator of AMP deaminase.[1] Enzyme activities expressed as micromoles of NH_3 per milligram of protein were: saline, 1·30 ± 0·09; thioacetamide, 3·07 ± 0·32;* thioacetamide + ethionine, 1·61 ± 0·14.* Puromycin again was ineffective against these increases. In this experiment, other animals injected with thioacetamide also received once-daily injections at 250 mg per kg of body weight of *p*-fluorophenylalanine or β-2-thienylalanine, but neither of these compounds effectively inhibited thioacetamide-induced increases in AMP deaminase activity.

Assuming that ethionine inhibition resulted from interference with hepatic translational mechanisms, the ineffectiveness of puromycin may have been related to rapid excretion.[34] Since Weber and Singhal[13] apparently circumvented rapid losses by increasing the frequency of injections (viz., 14 mg puromycin per rat was administered in 28 doses spaced throughout 48 hr), we adapted this protocol to our experimental situation and obtained data as shown in Table 2. Regardless of whether activity was

TABLE 2. EFFECT OF PUROMYCIN GIVEN AT 2-hr INTERVALS THROUGHOUT 48 hr ON INCREASES IN HEPATIC AMP DEAMINASE ACTIVITY IN FEMALE RATS INJECTED WITH THIOACETAMIDE*

Injections	AMP deaminase activity (μmoles NH_3/mg protein)		AMP deaminase activity (μmoles $NH_3/10^6$ liver cells)	
	Saline	TA	Saline	TA
24 hr after 1 injection	2·84±0·13	3·10±0·23	0·93±0·04	1·20±0·11
1 hr after 3 injections	3·02±0·08†	3·85±0·31‡	1·22±0·07†	1·90±0·20‡
1 hr after 3 injections + 1 puromycin injection	2·97±0·11	3·89±0·15‡	1·47±0·05	2·42±0·11‡
24 hr after 4 injections	2·39±0·08	3·77±0·18‡	1·11±0·07	2·10±0·24‡
24 hr after 4 injections + 24 puromycin injections	2·86±0·18	4·04±0·16‡	1·26±0·13	2·08±0·08‡

* Thioacetamide was injected as described in Table 1. Puromycin was injected at 5·86 mg/kg body wt. every 2 hr beginning with the third injection of saline or thioacetamide. Enzyme assays were performed as described in Table 1, except that reaction mixtures contained 17·2 mM AMP and 4 mM ATP. Cell numbers were determined by nuclei counts in homogenates.[28] Values reported are averages ± S.E. based on six animals/group.

† Five animals in this group.

‡ Values greater than corresponding saline values at P = 0·05 or greater.

expressed as specific activity in the supernatant fraction or as activity per million liver cells, thioacetamide increased enzyme activity, but injection of puromycin caused no interference in these increases. Thus, ethionine inhibited thioacetamide-induced increases in hepatic AMP deaminase activity, but puromycin did not. In addition, both *p*-fluorophenylalanine and β-2-thienylalanine also were ineffective in this regard.

Mechanisms by which ethionine[10] and puromycin[11] interfered with protein synthesis have been described. In addition, both *p*-fluorophenylalanine and β-2-thienylalanine were effective inhibitors of hepatic protein synthesis in several mam-

* Values different from saline control values or thioacetamide values at P = 0·05 or greater.

malian species,[35–38] and of hemoglobin synthesis in rabbit reticulocytes.[39] In view of these considerations, we investigated the status of protein synthesis. Table 3 shows the protein content and extent to which leucine-U-^{14}C was incorporated into hepatic proteins of male rats treated through 4 days with thioacetamide, ethionine or puromycin. In none of the rats, irrespective of treatment, was there evidence of protein

TABLE 3. EFFECTS OF THIOACETAMIDE, ETHIONINE AND PUROMYCIN UPON LEUCINE-U-^{14}C INCORPORATION *IN VIVO* INTO RAT LIVER*

Compounds injected	Protein content (mg/g liver)	Leucine-U-^{14}C incorporation (dpm × 10^3/g liver)
Saline	(5) 181±3	(5) 121±6
Thioacetamide (TA)	(5) 180±3	(5) 119±13
Ethionine (Eth)	(5) 265±22†	(4) 182±35
Puromycin (Puro)	(2) 189±3	(2) 92±15
TA + Eth	(5) 203±4†	(5) 163±16†
TA + Puro	(4) 182±7	(4) 117±18

* Compounds were injected into male rats throughout 4 days as described in Table 1, except that a final injection of puromycin (30 mg/kg body wt.) was given 1 hr before rats were killed. Leucine-U-^{14}C (3 μc/rat) was injected 30 min prior to killing the rats. The protein content was determined by the method of Oyama and Eagle.[30] Proteins were extracted by the procedure of Wannemacher *et al.*[21] Isotope was determined by liquid scintillation in a xylene–dioxane–cellosolve cocktail[22] with quenching corrected by channels ratio.[23] Values are averages ± S.E. Numbers in parentheses represent number of animals.

† Values greater than saline values at P = 0·05 or grear.

depletions below levels seen in controls, nor was there evidence that incorporation of labeled leucine was inhibited. Since these data could have been compromised by changes in leucine pools, these were determined among animals given four injections of thioacetamide or ethionine or both. The data, expressed as micromoles of leucine per gram of liver were: saline, 0·46; thioacetamide, 0·42; ethionine, 0·67; and thioacetamide + ethionine, 0·47.* The pools were also determined relative to protein content and to DNA content. Regardless of the mode of expression, when changes were observed relative to controls, pool sizes increased. Since this would have favored diminution of labeled leucine deposition into hepatic proteins, data shown in Table 3 suggested that neither ethionine nor puromycin interfered with general protein synthesis in the liver.

To test whether an interaction between ethionine and thioacetamide decreased the effectiveness of ethionine as an inhibitor of leucine-U-^{14}C incorporation, the concentration of ethionine was increased (*ca.* 4-fold) to levels known to inhibit the incorporation of labeled leucine,[16] and it was administered alone and with thioacetamide. Control animals received saline injections. Five and 24 hr later, four to six female rats in each treatment group were given L-leucine-U-^{14}C (3 μc/rat) by intra-abdominal injection. They were killed 30 min later. Incorporation of labeled leucine into hepatic proteins, evaluated as dpm per milligram of protein, but expressed as per cent of

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control values, was: 5 hr, ethionine, 31 per cent;* ethionine + thioacetamide, 14 per cent;* 24 hr, ethionine, 71 per cent; ethionine + thioacetamide, 38 per cent.* Clearly, simultaneous administration of thoacetamide caused no interference with this inhibition, but may have extended the period of time during which ethionine was inhibitory. Therefore, the possibility of a deleterious interaction between ethionine and thioacetamide seemed remote.

Experiments with labeled leucine indicated that chronic administration of ethionine or puromycin did not inhibit hepatic protein biosynthesis. Inhibition might still be detected, however, if adjudged by the action of inhibitors on the synthesis of protein during enzyme induction rather than upon protein synthesis in general. Intact and adrenalectomized male rats were given cortisone injections either alone or in combination with ethionine or puromycin. Tryptophan oxygenase activity was determined 3 hr after cortisone injections. The data are summarized in Table 4. There was no

TABLE 4. EFFECT OF ETHIONINE OR PUROMYCIN ON CORTISONE INDUCTION OF TRYPTOPHAN OXYGENASE IN MALE RATS*

Rats	Tryptophan oxygenase activity (μmoles kynurenine/g liver/hr)			
	Control	Cortisone	Cortisone + ethionine	Cortisone + puromycin
Intact	(5) 2·56±0·14	(5) 6·31±0·55†	(5) 5·82±0·25†	(5) 7·30±0·45†
Adx‡	(7) 1·63±0·09	(7) 5·33±0·29†	(7) 4·89±0·24†	(6) 5·46±0·51†

* Ethionine and puromycin (250 mg and 5 mg respectively/kg body wt.) were injected with the inducer, cortisone (10 mg/kg body wt.). A second puromycin injection was given after 2 hr and the animals were killed after 3 hr. Tryptophan oxygenase was assayed by the method of Feigelson and Greengard,[19] except that hematin (0·5 μM) was added.[20] Values recorded are averages ± S.E. Numbers in parentheses represent number of rats.

† Values greater than control values at P = 0·05 or greater.

‡ Adrenalectomy (Adx) was performed 7–9 days prior to assay.

indication that either ethionine or puromycin at the concentrations used inhibited cortisone induction of tryptophan oxygenase. Since Greengard *et al.*[20] had demonstrated that puromycin at 35 mg/kg body weight effectively blocked cortisone induction of tryptophan oxygenase, the failure of puromycin here probably was associated with low dosage and rapid excretion.[34] Similar considerations also may underly the ineffectiveness of ethionine, but it was clearly demonstrated that neither inhibitor, when used at concentrations permitting chronic administration, interfered to any detectable extent with protein synthesis. Therefore, whether hepatic translational mechanisms were involved in the increase of AMP deaminase activity in the liver of thioacetamide-injected rats was not tested. It has been shown that thioacetamide administration stimulated biosynthesis of both RNA[1,12] and protein.[40] Therefore, metabolic potentiality for employment of both transcriptional and translational mechanisms apparently was available, but determining whether this potential was used will require other experimental approaches.

Since Farber[10] has shown that ethionine intoxication was accompanied by marked depletions in hepatic ATP levels, ethionine was injected throughout 5 days, either

* Values different from saline values at P = 0·05 or greater.

alone or in combination with thioacetamide. Hepatic ATP levels are shown in Table 5. ATP levels in ethionine-treated rats were consistently less than levels in saline-injected control animals. The level of ATP among animals injected with the combination of ethionine and thioacetamide was as low as the level among animals injected with ethionine alone. Whether ATP losses were causatively or coincidentally linked with inhibition of increased enzyme activity is not known, but a coincidental relationship

TABLE 5. ADENOSINE TRIPHOSPHATE LEVELS IN THE LIVER OF RATS GIVEN MULTIPLE INJECTIONS OF THIOACETAMIDE OR ETHIONINE*

Compounds injected	ATP concentration (μmoles/g liver protein)			
	1 injection	3 injections	4 injections	5 injections
Saline	(5) 6·1±0·5	(2) 6·6±0·2	(3) 7·1±0·5	(4) 7·0±0·5
Thioacetamide (TA)	(5) 4·3±0·3†	(2) 4·2±0·8†	(2) 5·4±0·7	(6) 5·6±0·3†
Ethionine (Eth)	(3) 3·7±0·3†	(2) 3·7±0·7†	(4) 4·9±0·2†	(3) 5·6±0·1†
TA + Eth	(4) 3·1±0·2†	(1) 2·3±	(2) 2·8±0·1†	(3) 3·4±0·9†

* Ethionine and thioacetamide were injected as described in Table 1. ATP was determined by the luciferin–luciferase reaction.[27] Values are averages ± S.E. Numbers in parentheses represent number of animals.

† Values less than saline values at P = 0·1 or greater.

seems more likely. We have shown previously that liver AMP deaminase was activated by ATP.[1] Therefore, inhibitions through 4 days (Table 1) could be related to ATP depletions, but inhibition of enzyme activity was relieved after 5 days, whereas ATP depletions persisted. Furthermore, ethionine was inhibitory even though enzyme activity was determined in reaction mixtures containing excess ATP.

It is possible that both thioacetamide-induced increases in AMP deaminase activity and transitory inhibition of this increase with ethionine resulted from allosteric modulation. Stadtman[41] concluded that properties described for the AMP deaminase of bacteria or of ox brain were similar to properties reported for a variety of allosteric enzymes. The enzyme from rat liver shared these characteristics[42] and was activated by ATP[1] but inhibited by 3′(2′)AMP.[42] Farber[10] observed that ethionine substituted for methionine in many metabolic reactions, and one of the key substitutions was its activation by ATP at the sulfur group, resulting in the formation of S-adenosylethionine. This compound entered into transethylation reactions, but at rates slower than rates for transmethylation reactions; therefore, S-adenosylethionine accumulated.[10] The presence of the adenine moiety in S-adenosylethionine might permit a fit on either substrate or effector sites of the AMP deaminase molecule resulting in deleterious effects on enzyme activity. On the other hand, ethionine inhibited DNA synthesis in regenerating liver,[37,43] and thioacetamide intoxication in rats was accompanied by hepatic DNA synthesis[44–46] and cellular proliferation.[46–48] Therefore, if increased enzyme activity in thioacetamide-treated rats was associated with proliferation, ethionine inhibition (Table 1) might be related to inhibited proliferation. We are currently studying this possibility.

Acute doses of ethionine inhibited hepatic protein[10] and RNA[49] synthesis. Chronic doses did not inhibit protein synthesis (Tables 3 and 4) and stimulated hepatic RNA synthesis.[50] Chronic doses of ethionine stimulated glucose 6-phosphate dehydro-

genase, had no effect on 6-phosphogluconate dehydrogenase, and inhibited pyruvate kinase.[51] Clearly, hepatic metabolism under chronic and acute intoxication with ethionine differs. Cellularity shifts accompanying chronic administration of ethionine[7] undoubtedly underly some of these differences; however, the need for further investigation seems well documented.

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