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THE EFFECTS OF INOSINE AND AMINOIMIDAZOLE CARBOXAMIDE UPON THE ETHIONINE FATTY LIVER*

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Abstract—The administration of inosine or 5-amino-4-imidazole carboxamide to female rats is as effective as adenine or ATP in preventing the fatty liver induced by ethionine. Hadacidin, an antibiotic known to prevent the amination of inosinic acid to adenylic acid, counteracts the effect of inosine. Inosine is also very effective in preventing the mortality of female rats after ethionine administration. Aminoimidazole carboxamide is as effective as adenine, ATP, or inosine in returning the hepatic ATP level to normal 2 hr after ethionine administration. The results of this study strengthen further the hypothesis that the ethionine-induced fatty liver is secondary to a more basic interference with ATP metabolism in the rat liver.

PREVIOUS work has shown that the administration of ethionine to female rats induces a rapid decrease in hepatic adenosine triphosphate concentration[1] which is followed, in turn, by an inhibition of synthesis of ribonucleic acid[2] and of protein.[3] Adenine and the adenine nucleotides, 5′-AMP,† 5′-ADP, and 5′-ATP, as well as methionine, when given at the same time as ethionine, prevent both the drop in ATP and the inhibition of protein synthesis.[1, 3] Adenine or ATP also protects rats against the ethionine-induced fatty liver.[4]

On the basis of these and other findings, the fatty liver is interpreted to result from the following pathogenetic sequence.[4, 5]

> Ethionine ——→ hepatic ATP deficiency ——→ inhibition of protein synthesis ——→ decreased synthesis of protein moiety of plasma lipoproteins ——→ triglyceride accumulation in the liver.

According to this hypothesis, methionine prevents the fatty liver by counteracting the activation of ethionine to S-adenosylethionine and, thereby, preventing ethionine from trapping the adenine moiety of ATP. In contrast, adenine and adenine derivatives are considered to protect against the fatty liver by rapidly generating ATP to compensate for the trapping action of ethionine. The mechanism of this would be

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† The following abbreviations are used: 5′-AMP, 5′-adenylic acid; 5′-ADP, 5′-adenosine diphosphate; 5′-ATP, 5′-adenosine triphosphate; AICA, 5-amino-4-imidazole carboxamide; PRPP, 5-phosphoribosyl pyrophosphate.

via the catalytic action of AMP phosphorylase with 5-phosphoribosyl pyrophosphate and thence to ADP which, in turn, can be converted to ATP by oxidative phosphorylation.

Since inosine and 5-amino-4-imidazole carboxamide are precursors of adenine nucleotides through the intermediate formation of inosinic acid[6] and since inosine counteracts the effects of ethionine upon the ATP level and protein synthesis,[7] it was of interest to observe whether inosine and AICA would have similar effects upon the ethionine-induced fatty liver and whether AICA would also protect against the decrease in ATP. In addition, since hadacidin appears to be a specific inhibitor of the conversion of inosinic to adenylic acid,[8] the possible effect of this antibiotic upon the efficacy of inosine was investigated. The results of such treatments upon the occurrence of fatty liver with ethionine together with the effects of inosine upon the mortality of ethionine-treated animals are the subject of this communication.

EXPERIMENTAL PROCEDURE

White female rats of the Wistar strain (Carworth Farms), maintained on Purina laboratory chow were used. Two experiments were performed with inosine in which the animals, weighing 160 to 174 g and deprived of food overnight, were divided into four groups of four animals each. One group received one dose of DL-ethionine (California Foundation) by stomach tube in aqueous solution (25 mg/ml) at a dosage of 1 mg/g body weight and 3·0 ml saline (0·9% NaCl, w/v) i.p. at 0, 3, 6, and 9 hr A second group received saline both singly by stomach tube and by i.p. injection every 3 hr for four doses (0, 3, 6, and 9 hr). A third group received ethionine by stomach tube and 3·0 ml (0·32 mmole) aqueous inosine solution (28·6 mg/ml, adjusted to pH 6·4) i.p. every 3 hr for four doses (0, 3, 6, and 9 hr). This dosage is similar to that used previously in the studies with ATP and adenine.[4] A fourth group received saline by stomach tube and inosine i.p. on the same schedule. In the second experiment, an additional fifth group of three animals received ethionine by stomach tube and inosine i.p. by the same schedule as the third group, but also received s.c. 44·3 mg (in 0·1 ml) hadacidin (N-formylhydroxyaminoacetic acid[9]) in aqueous solution with each inosine injection. (The hadacidin was generously supplied by Merck, Sharpe and Dohme Research Laboratories through the courtesy of Dr. Harold T. Shiguera). Two experiments were performed with AICA in which the animals, weighing 165 to 175 g and deprived of food overnight, were divided into four groups of four animals each. The groups were the same as used in the experiments with inosine except that AICA replaced inosine. The AICA as the hydrochloride was given i.p. as an aqueous solution (17 mg/ml adjusted to pH 6·0) every 3 hr for four doses (0, 3, 6, and 9 hr), 0·32 mmole (3·0 ml) per dose. The animals were sacrificed by decapitation 12 hr after the initial intubation.

Two further experiments were done to test the efficacy of inosine in protecting against death induced by ethionine. In the first experiment, two groups of six animals each, weighing from 170 to 230 g and appropriately matched with respect to body weight, received 1 mg ethionine/g body weight i.p. in aqueous solution at 0 and 6 hr and one half this dose at 24 hr. One group received i.p. 0·16 mmole (43 mg) inosine at 0, 3, 6, 24, 30, 48, and 54 hr while the appropriate survivors of the second group received the same dosage of saline. In the second experiment, two similarly matched groups of twelve animals, weighing from 205 to 340 g, were injected i.p. with 1 mg

ethionine/g body weight at 0 and 6 hr with one half this dose at 24 and 30 hr. One group received 0·32 mmole (86·0 mg) inosine (pH 6·7) i.p. at 0, 3, 6, 27, and 30 hr. The control group received saline in place of the aqueous inosine.

The liver was rapidly removed at the time of sacrifice and weighed, and a tared aliquot of about 1·5 g was frozen in liquid nitrogen for subsequent lipid analysis. The liver tissue was thawed, dried by grinding with anhydrous sodium sulfate, and extracted with hot chloroform for 24 hr. After evaporation of the solvent, the residue was dissolved in petroleum ether, the clear solution was evaporated after transfer to a tared weighing bottle, and the total lipids were determined gravimetrically. Hepatic ATP levels were determined by the luciferin–luciferase reaction.[10] The animals for these determinations were kept under the same conditions as those used for the lipid studies. The timing in the determination was critically controlled as previously described.[1] Pieces of liver and other organs were fixed in Stieve's solution, and paraffin sections were stained with hematoxylin and eosin.

RESULTS

The administration of inosine protects female rats against the rise in liver total lipid (Table 1) and is as effective in this regard as are adenine and ATP.[4] These findings

TABLE 1. EFFECT OF INOSINE AND INOSINE PLUS HADACIDIN UPON LIVER LIPIDS IN ETHIONINE-TREATED FEMALE RATS

Group	Total liver lipid*	
	(g/100 g liver)	(mg/100 g body w.)
Saline + saline	5·4 ± 0·2 (8)	156 ± 9 (8)
Saline + inosine	4·7 ± 0·1 (8)	136 ± 4 (8)
Ethionine + saline	13·6 ± 1·3† (7)	410 ± 38† (7)
Ethionine + inosine	5·4 ± 0·2† (8)	163 ± 4† (8)
Ethionine + inosine + hadacidin	9·5 ± 0·3† (3)	278 ± 2† (3)

* Mean ± standard error of the mean for Tables 1–3. The number in parentheses represents the number of animals.

† $P < 0{\cdot}001$ (highly significant) when compared with the saline–saline or saline–inosine group.

† $0{\cdot}02 > P > 0{\cdot}01$ (probably significant) when compared with the ethionine–saline group; $P < 0{\cdot}001$ (highly significant) when compared with the ethionine–inosine group.

were confirmed by histologic examination which revealed no lipid accumulation in the liver cells of animals receiving both inosine and ethionine. Inosine *per se* has no effect upon the level of total lipid in the control animals. It appears also from Table 1 that hadacidin probably interferes with the efficacy of inosine. Unfortunately, the supply of this antibiotic was such as to prevent more extensive testing. Even though the number of animals is small and the effect is only partial, the absence of any overlap of results and the clear-cut nature of the interference with inosine on the ethionine-induced fatty liver make it appear very likely that the effect would have been complete had the dosage been adequate.

The efficacy of AICA in protecting rats against the ethionine-induced fatty liver is shown in Table 2. This precursor of inosinic and adenylic acids is as effective as is inosine, adenine, or ATP in this respect. In addition, AICA is also very effective in counteracting the decrease in ATP concentration in the liver induced by ethionine

TABLE 2. EFFECT OF AMINOIMIDAZOLE CARBOXAMIDE UPON LIVER LIPIDS IN ETHIONINE-TREATED FEMALE RATS

Group	Total liver lipid	
	(g/100 g liver)	(mg/100 g body w.)
Saline + saline	6·2 ± 0·1 (6)	180 ± 13 (6)
Saline + AICA	6·1 ± 0·1 (8)	176 ± 6 (8)
Ethionine + saline	11·8 ± 0·7* (8)	352 ± 24* (8)
Ethionine + AICA	6·8 ± 0·4† (8)	211 ± 17† (8)

* $P < 0.001$ (highly significant) when compared to the saline–saline group.

† $P < 0.001$ (highly significant) when compared to the ethionine–saline group.

TABLE 3. THE EFFECTS OF ETHIONINE AND 5-AMINO-4-IMIDAZOLE CARBOXAMIDE (AICA) ON HEPATIC ATP LEVELS OF FEMALE RATS

Number of rats	Treatment			ATP Levels (μmoles/g liver)	Relative ATP‡
	Ethionine*	AICA (0)†	AICA (2)†		
			Experiment 1		
(5)	−	−	−	1·366 ± 0·089	100
(5)	+	−	−	0·266 ± 0·007	20
(5)	+	+	−	1·131 ± 0·106	83
(5)	+	−	+	1·288 ± 0·151	94
			Experiment 2		
(5)	−	−	−	0·961 ± 0·046	100
(5)	+	−	−	0·327 ± 0·022	34
(5)	+	+	−	0·931 ± 0·058	97
(5)	+	−	+	1·038 ± 0·086	108

All rats were sacrificed 5 hr after the injection of ethionine or saline.

* −, Saline-treated (6 ml of 0·9% NaCl solution).
+, One mg (6·1 μmoles)/g body weight of DL-ethionine (saline solution containing 25 mg/ml) given i.p. at time zero.

† −, Saline-treated (6 ml of 0·9% NaCl solution).
+, Fifty-two mg (0·32 mmole) per rat of 5-amino-4-imidazole carboxamide hydrochloride (saline solution containing 10 mg/ml adjusted to pH 6·0) given i.p. either at time zero [AICA (0)] or at time 2 hr [AICA (2)].

‡ Based upon 100 for rats treated with saline only.

(Table 3). It is noteworthy that this compound is also active even when given 2 hr after the administration of ethionine, at which time the ATP levels are minimal.[3] AICA thus resembles adenine and derivatives rather than methionine[2, 7] in their protective effects against ethionine-induced alterations in liver metabolism.

Inosine is also seen to be effective in protecting female rats against the lethal action of ethionine (Table 4). In both experiments the administration of inosine gave almost complete protection. It is especially noteworthy that the inosine-treated animals showed no delayed mortality when followed for several weeks and that the kidneys showed no lesions either grossly or microscopically. This is in sharp contrast to

TABLE 4. EFFECTS OF INOSINE UPON THE MORTALITY OF FEMALE RATS INJECTED WITH ETHIONINE

Experiment	Group and number of animals	Number dead at 72 hr
1	Ethionine + saline (6)	6
	Ethionine + inosine (6)	1
2	Ethionine + saline (12)	12
	Ethionine + inosine (12)	1*

* The remaining 11 rats were followed for several weeks thereafter without observing any further mortality.

adenine and to ATP which induce obvious severe renal damage in all animals within a period of 24 hr.

DISCUSSION

The results of the present study add further evidence in support of the thesis that the accumulation of triglycerides in the liver induced by ethionine is secondary to changes in ATP and/or protein synthesis.[4, 5] The parallelism between the effects of adenine, ATP, inosine, and aminoimidazole carboxamide upon hepatic ATP levels and protein synthesis and upon total lipid in the ethionine-treated female rat is evident.[1, 3, 4, 7] Since inosine is a precursor of adenine nucleotides and since this metabolic pathway from hypoxanthine to AMP is blocked by the antibiotic hadacidin, the conclusion is almost inescapable that the conversion of inosine to adenylic acid is an essential reaction in the preventive effect of inosine upon the fatty liver. Although the data with AICA are not as extensive as with inosine or adenine, the results so far are completely consistent with this formulation.

Alternative explanations for the efficacy of adenine or ATP in protecting animals against the decrease in hepatic ATP and protein synthesis induced by ethionine were considered previously,[3] namely the acceleration of the excretion of ethionine from the whole organism or the prevention of its uptake by the liver. These alternatives are considered to be unlikely because of the following considerations:

(i) Female rats had the same distribution of radioactive ethionine (ethyl-1-^{14}C) in the plasma and in the acid-soluble fraction of liver, kidney, pancreas, adrenal, and skeletal muscle at the end of 5 hr after the injection of toxic doses of ethionine with or without ATP. The urinary excretion of ethionine during the 5-hr experiment was about equal in the two groups of animals.[3]

(ii) The concentration of free ethionine and of its important derivative, S-adenosylethionine, for many hours after the injection of ethionine is usually larger in

the livers of animals treated with ethionine plus adenine or ATP than with ethionine alone.[11]

(iii) Adenine, ATP,[3, 12] and inosine[7] are very effective in reversing the inhibition of protein synthesis or the drop in ATP even when administered at a time (2 to 5 hr) when the ATP and protein synthesis are maximally depressed. AICA, according to the results of the present study, appears to be similar.

(iv) Adenine is very effective in removing excess triglyceride accumulation inside the endoplasmic reticulum of the liver even when administered 4 to 5 hr after ethionine.[13]

(v) Adenine or ATP is selective in its protective effect upon the biochemical lesions induced by ethionine. Those effects related to the decrease in ATP (inhibition of ribonucleic acid and protein synthesis and fatty liver) are all readily prevented or cured by adenine or ATP, while those effects due to competition between S-adenosylethionine and S-adenosylmethionine are not prevented by adenine or ATP but only by methionine.[14]

It is obvious from this study that inosine is an effective protective agent against the lethal properties of ethionine.[15] If inosine protects against ethionine effects by virtue of its ability to act as a precursor of adenylic acid and ATP, it would appear that the mortality of female rats given ethionine is related to an effect on adenine nucleotide metabolism. It remains for future studies to pinpoint the locus of this effect as to metabolic pathway and organ.

The ability of inosine to protect against some of the effects of ethionine facilitates the study of the role of ATP in the genesis of other lesions induced by ethionine (cf. Ref. 16). Many attempts in our laboratory to study the possible protective effect of adenine or of ATP in ethionine-treated animals beyond 24 hr were always seriously handicapped by the induction or renal lesions with these compounds.[17, 18] Inosine administration does not appear to lead to this undesirable side effect, even when administered for many days. The explanation for this is readily available from the current knowledge of the metabolism of purines and their derivatives. Adenine *per se* does not appear to be present in the tissues of higher animals but only in the form of its nucleoside and nucleotides. During the usual intermediary metabolism of these compounds, they are first deaminated to inosinic acid or derivatives and thence converted to hypoxanthine, xanthine, and uric acid. In the case of the free base adenine, there are apparently no enzymes present in higher organisms for its deamination. It becomes oxidized, therefore, to 2,8-dioxyadenine, a highly insoluble compound which precipitates out in the kidney and thereby induces severe renal damage (cf. Refs. 17, 18). The administration of large amounts of ATP must generate a small amount of this adenine derivative as well, since it also leads to renal damage with crystals but to a much lesser degree than in the case of adenine.

Even though the results of this investigation further emphasize the probable role of ATP deficiency in the pathogenesis of ethionine-induced fatty liver, they throw no new light upon the problem of how the changes in ATP lead to the accumulation of excess triglyceride in the liver.[4, 5] Although a mechanism through inhibition of protein synthesis is still a most attractive one,[3–5] an alternative mechanism through altered permeability of the cell membrane[4] has not been ruled out. Hopefully, the further study of the biochemical pathology of hepatic lesions induced by ethionine will resolve this problem.

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