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FATTY LIVERS, ADENOSINE TRIPHOSPHATE AND ASPARAGINE

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Abstract—The effects of L-asparagine on the levels of triglyceride and adenosine triphosphate (ATP) in rat liver after the administration of ethionine, CCl_4 or ethanol have been investigated. The results indicate that L-asparagine prevents the decrease in hepatic ATP produced by each of these agents and protects against the fatty infiltration of the liver that follows the administration of ethionine or small doses of CCl_4, but not against that produced by large doses of CCl_4 or ethanol. These observations suggest that hepatic ATP depletion may not play a key role in the pathogenesis of the fatty liver produced by these agents.

A REDUCTION in adenosine triphosphate (ATP) concentration accompanies the accumulation of triglyceride that occurs in rat livers after the administration of ethionine,[1–3] carbon tetrachloride (CCl_4)[4,5] or ethanol.[3] The i.p. injection of ATP prevents both effects of these agents.[4–6]

Previous reports that L-asparagine also prevents the increase in hepatic triglyceride produced by ethionine,[7] CCl_4[7] or ethanol[8] prompted us to investigate the possibility that L-asparagine protects against fatty infiltration of the liver by preventing hepatic ATP depletion. Our findings indicate that L-asparagine prevents the depletion of hepatic ATP produced by ethionine, CCl_4 and ethanol and protects against fatty infiltration of the liver after the administration of ethionine or small doses of CCl_4, but does not prevent the fatty infiltration produced by large doses of CCl_4 or ethanol.

MATERIALS AND METHODS

Sprague–Dawley (CS) rats, 150–250 g, obtained from the Charles River Rat Farm, North Wilmington, Mass., were housed in individual cages and maintained on Purina lab chow and water *ad libitum*. Female animals were used in all experiments, except where otherwise indicated.

As in the experiments[6] of Farber *et al.*, DL-ethionine, 0·1 g (0·6 m-mole)/100 g body wt., was injected i.p. as a 0·15 M aqueous solution in two equally divided doses 2 hr apart. The animals were allowed free access to water but had been fasted for 16 hr prior to treatment and were sactificed 5 hr later. At this time the hepatic ATP concentration has reached a minimum and there is considerable fatty infiltration.[6]

In one experiment, a total of 0·2 ml CCl_4/100 g body wt. was administered s.c. in two equally divided doses 2 hr apart. In a second experiment, CCl_4 was diluted

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with an equal volume of mineral oil and was administered by stomach tube in a single dose of 0·5 ml/100 g body wt. (This dose is equivalent to 0·25 ml CCl_4.) These animals had been fasted for 5 hr prior to treatment and were sacrificed 16 hr later.

Ethanol was administered by stomach tube as a single dose at two levels, 0·5 and 0·75 g/100 g body wt., to female rats and at a level of 0·5 g/100 g body wt. to male rats. The animals had been fasted for 5 hr prior to treatment and were sacrificed 16 hr later. Controls received a caloric equivalent of glucose, 0·96 g/100 g body wt. in the case of the smaller dose of ethanol or 1·44 g/100 g body wt. in the case of the larger dose.

In the ethionine and CCl_4 experiments, L-asparagine, 0·2 g/100 g body wt., was administered i.p. in two equally divided doses 2 hr apart. The 0·311 M solution employed was prepared by dissolving L-asparagine in water heated to 80° and then cooled to 37°.

In the ethanol experiments, L-asparagine in a dose of 0·2 or 0·4 g/100 g body wt. was administered by stomach tube as a suspension of 0·1 g/100 ml water.

Animals were sacrificed by decapitation. Within 1 min, a 2·0 to 2·5 g wedge of liver was removed and promptly frozen in liquid nitrogen for subsequent ATP analysis. The extraction procedure was that of Lamprecht and Trautschold.[9] Frozen tissue was pulverized in a porcelain mortar and, after the addition of 6% perchloric acid (3·25 ml/g liver), was homogenized in a Potter–Elvehjem glass homogenizer for 30 sec. The homogenate was centrifuged for 15 min at 1000 *g*, after which the supernatant fraction was neutralized with 5 M K_2CO_3 with methyl orange as the indicator. An aliquot was diluted 100-fold and analyzed for ATP by the Strehler and Totter luciferin-luciferase method.[10] With this method, a linear response was obtained over the range of 0·3–2·0 μg ATP and 95 per cent of the ATP added to liver homogenates was recovered.

Another wedge of fresh liver was homogenized with 9 vol. of 0·067 M, pH 7·0 phosphate buffer, and analyzed for triglyceride by the method of Butler *et al.*,[11] glycerol being used as the standard instead of corn oil. A triglyceride concentration of 1 μmole/g is equivalent to about 0·9 mg/g.

All results were expressed as the means $\pm$ S. E. M., and the statistical significance of differences between groups was assessed by the *t* test.

RESULTS

Ethionine. As shown in Table 1, i.p. injection of DL-ethionine was followed within 5 hr by a 3·5-fold increase in hepatic triglyceride and a 70 per cent decrease in hepatic ATP. Simultaneous injection of L-asparagine prevented both of these effects almost completely. Control animals injected with L-asparagine alone or with an NaCl solution equivalent in volume and osmolarity to that of DL-ethionine plus L-asparagine showed no significant changes in hepatic triglyceride or ATP.

CCl_4. As shown in Table 2, subcutaneous injection of 0·2 ml CCl_4/100 g body wt. increased hepatic triglyceride 3-fold and reduced hepatic ATP by 40 per cent. Intraperitoneal injection of L-asparagine prevented both of these effects. Control animals given a subcutaneous injection of NaCl plus an i.p. injection of NaCl equivalent in volume and osmolarity to that of L-asparagine showed no significant changes in hepatic triglyceride or ATP. Though not presented in Table 2, it was observed that L-asparagine prevented ATP depletion for as long as 16 hr after CCl_4 (s.c.).

The reason for the variability of ATP in the control groups of Tables 1 and 2 is not known, although it is noteworthy that other investigators[2, 12] have also obtained similar variability. The data in each table were derived from animals received in one shipment from the supplier, but a different shipment was used for each of the different tables.

TABLE 1. EFFECT OF L-ASPARAGINE ON HEPATIC TRIGLYCERIDE AND ATP AFTER DL-ETHIONINE ADMINISTRATION IN FEMALE RATS*

Treatment	Triglyceride (μmoles/g liver)	ATP (μmoles/g liver)
None (5)	7·8 ± 1·3	1·49 ± 0·21
DL-Ethionine, 0·1 g/100 g b.w., i.p. (7)	27·8 ± 4·0 (P < 0·001)†	0·43 ± 0·04 (P < 0·001)
DL-Ethionine 0·1 g/100 g b.w., i.p. + L-Asparagine, 0·2 g/100 g b.w., i.p. (7)	12·7 ± 1·2	1·04 ± 0·09
L-Asparagine, 0·2 g/100 g b.w., i.p. (5)	6·1 ± 1·2	1·15 ± 0·06
NaCl (0·155 M), 8·9 ml/100 g b.w., i.p. (5)	11·3 ± 2·2	1·46 ± 0·11

* DL-Ethionine (0·155 M) was injected intraperitoneally (i.p.) in 2 equally divided doses 2 hr apart, totalling 4 ml/100 g body wt. (b.w.). L-Asparagine (0·311 M) was similarly injected in 2 doses totalling 4·9 ml/100 g b.w. The control NaCl injection was equal in volume and osmolarity to the combined injection of DL-ethionine and L-asparagine. The animals had been fasted for 16 hr prior to treatment and were sacrificed 5 hr later. The numbers in parentheses refer to the number of animals in each group.

† Statistical comparisons were made with the group receiving none.

TABLE 2. EFFECT OF L-ASPARAGINE ON HEPATIC TRIGLYCERIDE AND ATP AFTER S.C. INJECTION OF CCL_4 IN FEMALE RATS*

Treatment		Triglyceride (μmoles/g liver)	ATP (μmoles/g liver)
None (6)		15·3 ± 3·9	0·96 ± 0·10
CCl_4, 0·2 ml/100 g b.w., s.c. + NaCl (0·155 M), 4·9 ml/100 g b.w., i.p.	(13)	46·9 ± 5·6 (P < 0·001)†	0·58 ± 0·07 (P < 0·05)
CCl_4, 0·2 ml/100 g b.w., s.c. + L-Asparagine, 0·2 g/100 g b.w., i.p.	(7)	20·4 ± 3·6	1·45 ± 0·22
NaCl (0·155 M), 0·2 ml/100 g b.w., s.c. + NaCl (0·155 M), 4·9 ml/100 g b.w., i.p.	(7)	14·6 ± 0·1	1·03 ± 0·09

* CCl_4 was injected s.c. in animals fasted for 16 hr. L-Asparagine (0·311 M) was injected i.p. in 2 equally divided doses 2 hr apart, totalling 4·9 ml/100 g body wt. (b.w.). The volume and osmolarity of the control NaCl injection were equivalent to those of L-asparagine. Animals were sacrificed 5 hr after treatment. The numbers in parentheses refer to the number of animals in each group.

† Statistical comparisons were made with the group receiving none.

When the dose of CCl_4 was increased to 0·5 ml/100 g body wt. and was administered by stomach tube, there was a 6-fold increase in hepatic triglyceride and a 50 per cent decrease in hepatic ATP (Table 3). Intraperitoneal injection of L-asparagine prevented the fall in hepatic ATP, but had no effect on the striking increase in hepatic

triglyceride induced by CCl_4. Neither oral administration of mineral oil nor i.p. injection of L-asparagine alone affected the concentration of hepatic triglyceride or ATP.

TABLE 3. EFFECT OF L-ASPARAGINE ON HEPATIC TRIGLYCERIDE AND ATP AFTER ORAL ADMINISTRATION OF CCL_4 IN FEMALE RATS*

Treatment		Triglyceride (μmoles/g liver)	ATP (μmoles/g liver)
None (4)		19·6 ± 6·2	1·03 ± 0·16
Mineral oil, 0·5 ml/100 g b.w., p.o. (4)		21·7 ± 2·8	0·90 ± 0·15
CCl_4, 0·5 ml/100 g b.w., p.o. (4)		119·3 ± 10·0 (P < 0·001)†	0·54 ± 0·99 (P < 0·05)
CCl_4, 0·5 ml/100 g b.w., p.o. + L-Asparagine, 0·2 g/100 g b.w., i.p.	(4)	112·8 ± 10·0 (P < 0·001)	0·92 ± 0·15
NaCl (0·155 M), 4·9 ml/100 g b.w., i.p. (4)		20·2 ± 6·3	0·88 ± 0·17

* CCl_4 was administered by stomach tube (p.o.) after dilution with an equal volume of mineral oil. L-Asparagine (0·311 M) was injected i.p. in 2 equally divided doses 2 hr apart, totalling 4·9 ml/100 g body wt. (b.w.). The volume and osmolarity of the control NaCl injection were equivalent to those of L-asparagine. The animals had been fasted for 5 hr before treatment and were sacrificed 16 hr later. The numbers in parentheses refer to the number of animals in each group.

† Statistical comparisons were made with the group receiving none.

Ethanol. In female rats given a single, large dose of ethanol by stomach tube, the liver showed, at the end of 16 hr, a striking increase in triglyceride and a significant fall in ATP as compared to controls given a caloric equivalent of glucose (Table 4). Triglyceride increased 6-fold in animals given 0·5 g ethanol/100 g body wt. and 9-fold

TABLE 4. EFFECT OF L-ASPARAGINE ON HEPATIC TRIGLYCERIDE AND ATP AFTER ORAL ADMINISTRATION OF ETHANOL IN FEMALE RATS*

Treatment		Triglyceride (μmoles/g liver)	ATP (μmoles/g liver)
Glucose, 0·96 g/100 g b.w., p.o. (19)		7·3 ± 2·0	1·23 ± 0·11
Ethanol, 0·5 g/100 g b.w., p.o. (19)		46·6 ± 6·5 (P < 0·001)†	0·67 ± 0·06 (P < 0·05)
Ethanol, 0·5 g/100 g b.w., p.o. + L-Asparagine, 0·2 g/100 g b.w., p.o.	(6)	36·8 ± 7·4 (P < 0·001)	0·86 ± 0·10
Glucose, 0·96 g/100 g b.w., p.o. + L-Asparagine, 0·2 g/100 g b.w., p.o.	(4)	9·8 ± 1·5	1·06 ± 0·03
Glucose, 1·44 g/100 g b.w., p.o. (7)		7·5 ± 1·6	0·92 ± 0·12
Ethanol, 0·75 g/100 g b.w., p.o. (6)		66·7 ± 13·7 (P < 0·001)	0·24 ± 0·07 (P < 0·001)
Ethanol, 0·75 g/100 g b.w., p.o. + L-Asparagine, 0·2 g/100 g b.w., p.o.	(6)	60·2 ± 8·8 (P < 0·001)	0·48 ± 0·08 (P < 0·05)

* Ethanol was administered by stomach tube (p.o.) as a single dose of 2 ml of a 31·5% or 47·25% (v/v) aqueous solution/100 g body wt. (b.w.). A caloric equivalentof D-glucose, 2 ml of a 48% or 72% (w/v) aqueous solution, was similarly administered. L-Asparagine was administered as a suspension of 100 mg/ml in water. Animals had been fasted for 5 hr prior to treatment and were sacrificed 16 hr later. The number in parentheses refer to the number of animals in each group.

† Statistical comparisons were made with the group receiving glucose only.

in those given 0·75 g/100 g body wt. while ATP fell 36 and 77 per cent, respectively, in the two groups. L-Asparagine prevented the decline in hepatic ATP completely with the smaller dose of ethanol and partially with the larger. However, in neither case did L-asparagine prevent the accumulation of hepatic triglyceride. In control animals receiving glucose alone, L-asparagine had no effect on either hepatic triglyceride or ATP.

For more direct comparison with the results of Lansford *et al.*,[8] a similar experiment was carried out in male rats. As in females, ethanol raised the level of hepatic triglyceride and depressed hepatic ATP. Again, 0·4 g L-asparagine/100 g body wt. prevented the fall in ATP, but failed to protect against the accumulation of triglyceride. When the dose of L-asparagine was halved, the hepatic ATP concentration was only partially reversed and the fatty liver persisted (Table 5).

TABLE 5. EFFECT OF L-ASPARAGINE ON HEPATIC TRIGLYCERIDE AND ATP AFTER ORAL ADMINISTRATION OF ETHANOL IN MALE RATS

Treatment		Triglyceride (μmoles/g liver)	ATP (μmoles/g liver)
Glucose, 0·96 g/100 g b.w., p.o. (4)		16·0 ± 2·2	1·80 ± 0·2
Ethanol, 0·5 g/100 b.w., p.o. (4)		31·0 ± 1·0 ($P < 0·05$)†	0·50 ± 0·1 ($P < 0·001$)
Ethanol, 0·5 g/100 g b.w., p.o. + L-Asparagine, 0·2 g/100 g b.w., p.o.	(4)	27·4 ± 1·9 ($P < 0·05$)	0·99 ± 0·2
Ethanol, 0·5 g/100 g b.w., p.o. + L-Asparagine, 0·4 g/100 g b.w., p.o.	(4)	24·6 ± 3·7 ($P < 0·05$)	2·25 ± 0·2
Glucose, 0·96 g/100 g b.w., p.o. + L-Asparagine, 0·2 g/100 g b.w., p.o.	(4)	8·9 ± 2·8 ($P < 0·05$)	1·69 ± 0·2

* Ethanol was administered by stomach tube (p.o.) as a single dose of 2 ml of a 31·5% (v/v) aqueous solution/100 g body wt. (b.w.). A caloric equivalent of D-glucose, 2 ml of a 48% (w/v) aqueous solution/100 g b.w., was similarly administered. L-Asparagine was administered by stomach tube as a suspension of 100 mg/ml in water. Animals had been fasted for 5 hr prior to treatment and were sacrificed 16 hr later. The numbers in parentheses refer to the number of animals in each group.

† Statistical comparisons were made with the group receiving glucose only.

DISCUSSION

The data presented confirm previous reports that ethionine,[1–4,13–17] CCl_4[4,5,18] and ethanol[4,5] increase hepatic triglyceride and depress hepatic ATP. Neither Buttner *et al.*[19] nor French [12] observed a fall in the hepatic ATP level after the administration of a single dose of ethanol. Indeed, French[12] found that under such conditions there was a transient but significant increase in hepatic ATP. The dose of ethanol employed by Buttner *et al.*,[19] 0·1 g/100 g body wt., may have been too small to elicit a response, but French[12] administered 0·6 g/100 g body wt., a dose which in the present study and in that of Hyams and Isselbacher[4] depressed hepatic ATP. Possibly, the discrepancy can be attributed to the fact that the strain of rats differed, the Wistar type being used in French's experiments and the Sprague–Dawley type in the others. However, French[12] did observe a decrease in hepatic ATP in Wistar rats when they were given 30% ethanol (w/v) in lieu of drinking water for a period of several months.

In the present study, L-asparagine prevented the decline in hepatic ATP concentration produced by ethionine, CCl_4 or ethanol. Also, it protected against the fatty infiltration of the liver induced by ethionine or a small dose of CCl_4 or ethanol.

Since L-asparagine prevented the accumulation of hepatic triglyceride when the dose of CCl_4 was small, and failed to do so when the dose was large, it would appear that the protective action of L-asparagine against fatty infiltration is abolished when the stimulus for triglyceride accumulation induced by CCl_4 is sufficiently intense. However, the effectiveness of L-asparagine in preventing depletion of hepatic ATP is unaffected by a change in the dose of CCl_4.

In contrast to our own observations, Lansford *et al.*[8] found that L-asparagine prevented the increase in hepatic triglyceride induced by ethanol. The dose of ethanol employed and the degree of fatty infiltration attained were similar in the two studies, but the source of the rats in the two experiments differed. Whether or not this difference accounts for the discrepancy between our results is uncertain.

Available data do not permit any conclusions regarding the mechanism involved in the protective action of L-asparagine in preventing the depletion of hepatic ATP induced by ethionine, CCl_4 or ethanol. However, considering the diverse chemical nature of these agents, it appears highly improbable that L-asparagine spares hepatic ATP by any direct effect on their metabolic pathways. The possibility that L-asparagine protected against a fatty liver by inducing hypothermia[5,20,21] has not been excluded in the present experiments, since body temperatures were not measured.

It has been postulated that depletion of hepatic ATP, by inhibiting lipoprotein systhesis and thus blocking the transport of triglyceride out of the liver, plays a key role in the pathogenesis of ethionine-induced fatty infiltration of the liver.[3,22,23] This hypothesis is based on evidence indicating that ethionine, in addition to raising the level of triglyceride in the liver, also lowers hepatic ATP,[1] inhibits protein synthesis[3,4,22,23] and depresses the level of triglyceride in the plasma[5,17] and that all of these effects can be prevented by intraperitoneal injection of ATP[3,4,6] or its precursors.[2,4,18] Our own observation that triglyceride accumulation was prevented when the ATP depletion was blocked is consistent with this hypothesis.

Although the fatty liver produced by the administration of either CCl_4 or ethanol is also associated with a decrease in hepatic ATP, several lines of evidence suggest that the depletion of hepatic ATP and inhibition of lipoprotein synthesis do not account for the accumulation of hepatic triglyceride under these conditions. Thus, in our own experiments, L-asparagine protected against hepatic ATP depletion, but did not prevent fatty infiltration after the administration of either a larger dose of CCl_4 or ethanol. In the study of Hyams *et al.*,[4,5] i.p. injection of ATP protected against CCl_4-induced fatty infiltration and ATP depletion in the liver, but did not influence the associated inhibition of protein synthesis or depression of plasma triglyceride. Moreover, the latter investigators found that in the case of both CCl_4 and ethionine administration, the accumulation of fat in the liver was accompanied by an increase in plasma free fatty acids, presumably due to enhanced mobilization from adipose tissue, and that this effect was abolished by injection of ATP. A similar increase in plasma free fatty acid concentration has been observed after alcohol administration.[24] These observations are consistent with the view that mobilization of fatty acids may be required for the development of the fatty liver produced not only by CCl_4 and ethanol, but also by ethionine and that the protection afforded by ATP

injection is related more closely to its inhibition of fatty acid mobilization than to its maintenance of a normal hepatic ATP level. (However, our data do not exclude other hypotheses that have been proposed to explain fatty infiltration of the liver; see the review by Recknagel[25] on CCl_4 hepatotoxicity.) Conceivably, L-asparagine prevents fatty infiltration of the liver by a similar mechanism. Consistent with this interpretation is our previously reported observation that, when a small dose of CCl_4 is administered and accumulation of hepatic triglyceride is prevented, the liver no longer shows the characteristic change in the hepatic fatty acid pattern indicative of lipid mobilization.[7] The failure of L-asparagine to prevent the fatty infiltration of the liver induced by ethanol does not necessarily rule out the possibility that L-asparagine blocks the mobilization of fatty acids from adipose tissue, since ethanol has a number of actions that lead to the accumulation of triglyceride in the liver.[26]

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