Studies on Ethionine-Induced Fatty Liver

By P. PUDDU, C. M. CALDARERA AND M. MARCHETTI

Istituto di Chimica Biologica dell'Università di Bologna, Bologna, Italy

(Received 5 April 1966)

1. The effects of the administration of DL-ethionine on some aspects of lipid and nucleotide metabolism in rat liver were studied. 2. In ethionine-treated animals neutral fat was increased, whereas phospholipids and cholesterol were unchanged. Lipogenesis *in vitro* was inhibited. 3. The concentration of nicotinamide nucleotides, purine nucleotides and pyrimidine nucleotides was decreased. The decrease was due to free adenine nucleotides, inosine nucleotides, uridine nucleotides and cytidine nucleotides. Also, the protein-bound biotin content was lower. 4. In biotin-deficient rats the development of ethionine-induced fatty liver was inhibited. 5. The possibility was considered that ethionine might produce an inhibition of the synthesis of biotin-dependent acetyl-CoA carboxylase.

It has been observed that a variety of agents which produce a fatty liver, ethionine included, also lead to a rapid decrease in hepatic concentration of ATP (Marchetti, Puddu & Caldarera, 1962; Farber, Shull, Villa-Trevino, Lombardi & Thomas, 1964; Hyams & Isselbacher, 1964). Since the administration of ATP together with ethionine prevents the development of fatty liver, it has been suggested that the ethionine-induced accumulation of triglycerides in the liver is related to ATP deficiency, since ATP may participate in releasing hepatic triglycerides into the plasma (Farber, Lombardi & Castillo, 1963; Farber *et al.* 1964).

However, besides the increase of triglycerides, a marked inhibition of fatty acid synthesis (Doering & Natori, 1965) and a decrease of [32 P]phosphate incorporation into phospholipids (Olmsted, 1955; Robinson & Harris, 1961) have been observed in the livers of ethionine-treated rats. It is possible, therefore, that different mechanisms may be involved in the altered metabolism of lipids in the livers of rats treated with ethionine.

The present experiments are concerned with the relationships between acid-soluble nucleotide and nicotinamide nucleotide contents and lipid metabolism in the liver of ethionine-treated rats. The results of comparative studies in biotin-deficient ethionine-treated rats are also given. Previous studies have shown that in biotin-deficient animals the development of ethionine-induced fatty liver was inhibited (Marchetti, Ottani & Puddu, 1966).

EXPERIMENTAL

Materials. DL-Ethionine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sodium [$1\text{-}^{14}\text{C}$]acetate (10.5 mCi/m-mole) was obtained from The

Radiochemical Centre, Amersham, Bucks. D-Biotin was purchased from Hoffmann-La Roche and Co. Ltd., Basle, Switzerland. All the other chemicals used were of the highest purity available commercially.

Animals. Female weanling albino rats of the Wistar strain weighing 40–45 g. were divided into two groups, housed in cages with wire bottoms and fed *ad libitum* on a biotin-free diet and a biotin-supplemented diet respectively (Marchetti, Pasquali & Landi, 1965). All animals were given water *ad libitum*. The first group developed severe biotin deficiency after 60 days. After 60 days animals of each group were injected intraperitoneally with 75 mg. of DL-ethionine/100 g. body wt. in three equal doses at intervals of 2.5 hr. The other animals of each group were injected with 0.9% NaCl. Both ethionine-treated rats and controls were killed 24 hr. after the first injection and the livers were saved for chemical analysis and for experiments *in vitro*.

Analytical methods. Total lipids were measured gravimetrically after extraction by a method described previously (Marchetti, Puddu & Caldarera, 1964); cholesterol was analysed by the method of Sperry & Webb (1950) and phospholipids by phosphorus analysis in total lipids (Bartlett, 1959). Protein-bound biotin was assayed microbiologically with *Lactobacillus arabinosus* 17–5 A.T.C.C. 8014 on samples of liver homogenate autoclaved with 6N-H₂SO₄ at 121° for 60 min. The growth was measured titrimetrically after an incubation period of 72 hr. at 30°. Determinations of nicotinamide nucleotides were carried out by the fluorimetric method outlined by Dianzani (1955).

The free nucleotides of liver acid-soluble fraction were determined by chromatography on Dowex 1 (formate form) as described by Hurlbert, Schmitz, Brumm & Potter (1954).

Liver protein was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as a standard.

Experiments in vitro. Liver slices (500–600 mg.) were incubated in 25 ml. Erlenmeyer flasks containing 5 ml. of calcium-free Krebs–Ringer phosphate medium, pH 7.4, 5 μ moles of α -oxoglutarate and 0.5 μ mole of sodium [$1\text{-}^{14}\text{C}$]acetate. The flasks were shaken at 37° for 1 hr. in air.

Determination of radioactivity. After incubation the reaction was stopped by placing the flasks on ice, decanting the medium, rinsing the slices three times with ice-cold buffer, and homogenizing in 20 vol. of ethanol-diethyl ether (3:1, v/v). The extract was evaporated to dryness and the lipid residue was dissolved first in light petroleum (b.p. 30–50°) and then in 5 ml. of chloroform. Samples of this solution were taken for radioactivity measurements in a windowless gas-flow counter.

Statistical treatment. The results are given as means $\pm$ s.e.m. Fisher's *P* values are given and are considered significant if *P* is greater than 0.05. Where *P* is greater than 0.1 the values are quoted as not significant (N.S.).

RESULTS

As shown in Table 1 the total lipid content was significantly increased in the livers of ethionine-treated rats (group 2) when compared with the control rats on biotin-supplemented diet (group 1). This increase was entirely due to the neutral lipid fraction, no significant changes being found in phospholipid and cholesterol. The fat content of liver of ethionine-treated rats maintained on a biotin-free diet (group 4) was not significantly increased when compared either with the control (group 1) or with the biotin-deficient rats (group 3). The rate of incorporation of [14 C]acetate into total lipids of slices from livers of ethionine-treated rats (group 2) was lower than that of control rats (group 1). The rate of incorporation of [14 C]-acetate in biotin-deficient rats (group 3) and in biotin-deficient ethionine-treated rats (group 4) was significantly lower when compared with the controls (group 1).

Table 2 shows that the protein-bound biotin content of the liver of ethionine-treated rats (group 2) was significantly lower than that in the livers of controls (group 1). The lowest protein-bound biotin contents were observed in the liver of biotin-deficient rats (group 3) and of biotin-deficient rats receiving ethionine (group 4).

The concentration of nicotinamide nucleotides in the liver of ethionine-treated rats (group 2) was decreased when compared with the control rats (group 1). A marked decrease of liver nicotinamide nucleotides was also noted in biotin-deficient rats treated with ethionine (group 4).

The administration of ethionine resulted in significant changes in the content of liver free nucleotides (Tables 3 and 4). Purine nucleotides, and in particular adenine nucleotides and inosine nucleotides, showed remarkable decreases in the liver of ethionine-treated rats (group 2) when compared with the control animals (group 1). The decrease of adenine nucleotides was due to ATP or to ADP or to AMP. A decrease of pyrimidine nucleotides and in particular of uridine nucleotides and cytidine nucleotides was also noted. No

Table 1. *Effect of ethionine on the composition of liver lipids and on the incorporation of [14 C]acetate in vitro into the liver lipids of the rat*

Group	Animals in experiment	Liver lipids (mg./g. of wet tissue)				Total lipids	Incorporation of [14 C]-acetate into liver lipids (counts/min./g. of wet tissue)
		Cholesterol	Phospholipids	Neutral lipids			
1	Control	2.55 $\pm$ 0.22	29.52 $\pm$ 1.46	24.58 $\pm$ 2.82	56.69 $\pm$ 2.73	2023 $\pm$ 356	
2	Control + ethionine	3.44 $\pm$ 0.24 (<0.05)	32.78 $\pm$ 2.30 (N.S.)	77.74 $\pm$ 5.54 (<0.001)	113.97 $\pm$ 4.69 (<0.01)	479 $\pm$ 35 (<0.001)	
3	Biotin-deficient	2.53 $\pm$ 0.30 (N.S.)	24.77 $\pm$ 0.67 (N.S.)	29.32 $\pm$ 1.81 (N.S.)	56.64 $\pm$ 2.11 (N.S.)	428 $\pm$ 37 (<0.001)	
4	Biotin-deficient + ethionine	2.39 $\pm$ 0.30 (N.S.)	35.94 $\pm$ 2.57 (N.S.)	22.33 $\pm$ 2.19 (N.S.)	60.66 $\pm$ 3.85 (N.S.)	445 $\pm$ 41 (<0.001)	

The animals were injected intraperitoneally with 75 mg. of DL-ethionine/100 g. body wt. in three equal doses at intervals of 2.5 hr. The controls were injected with 0.9% NaCl. All animals were killed 24 hr. after the first injection. Each value is given as the mean $\pm$ s.e.m. of six determinations on different animals. Fisher's *P* values are given in parentheses. N.S., Not significant. Experimental details are given in the text.

Table 2. *Effect of ethionine on the hepatic concentrations of protein-bound biotin and nicotinamide nucleotides in the rat*

The animals were injected intraperitoneally with 75 mg. of DL-ethionine/100 g. body wt. in three equal doses at intervals of 2.5 hr. The controls were injected with 0.9% NaCl. All animals were killed 24 hr. after the first injection. Each value is given as the mean $\pm$ S.E.M. of six determinations on different animals. Fisher's *P* values are given in parentheses. N.S., Not significant. Experimental details are given in the text.

Group	Animals in experiment	Liver protein-bound biotin (μ g./g. of defatted tissue)	Liver nicotinamide nucleotides (μ g./g. of defatted tissue)
1	Control	3.12 $\pm$ 0.11	1450 $\pm$ 64
2	Control + ethionine	1.68 $\pm$ 0.15 (< 0.001)	1160 $\pm$ 57 (< 0.001)
3	Biotin-deficient	0.50 $\pm$ 0.15 (< 0.001)	1730 $\pm$ 273 (N.S.)
4	Biotin-deficient + ethionine	0.53 $\pm$ 0.05 (< 0.001)	978 $\pm$ 95 (< 0.001)

significant changes in purine and pyrimidine nucleotides were observed between biotin-deficient ethionine-treated rats (group 4) and biotin-deficient rats (group 3). On the contrary, a marked decrease of adenine nucleotides either in biotin-deficient or in biotin-deficient ethionine-treated rats was noted when compared with control rats (group 1); in particular ATP was significantly lower both in the animals of group 3 and in those of group 4. No significant differences in the concentrations of guanine nucleotides and inosine nucleotides in the liver were noted in biotin-deficient animals of both groups 3 and 4. Cytidine nucleotides were decreased in biotin-deficient rats when compared with biotin-treated control animals.

DISCUSSION

It has been suggested that the ethionine-induced fatty liver is probably due to a block in the release of liver lipid into the blood. The decreased secretion of lipid from the liver has been considered secondary to an inhibition of the synthesis of the protein moiety of lipoprotein (Robinson & Harris, 1961). In this connexion, the cellular depletion of ATP produced by ethionine, which is thought to trap ATP to form *S*-adenosylethionine (Stekol, Mody, Bedrak, Keller & Perry, 1960), was suggested to be the basic metabolic disturbance, since ATP is known to play a part in protein and hence in lipoprotein synthesis (Farber *et al.* 1964). However, the cellular depletion of ATP may not be in all instances directly related to the fatty infiltration of the liver. In fact, in biotin-deficient rats the development of ethionine-induced fatty liver was inhibited and yet the concentration of hepatic ATP was significantly decreased. It seems possible that the decrease of ATP in both biotin-deficient groups may have occurred gradually over the 60 days on the biotin-deficient diet, which may explain the absence of fatty liver in both biotin-deficient groups, even though the concentrations of ATP

are lower than in the controls. Since neither ATP concentrations nor total adenine nucleotides are significantly different in the livers of biotin-deficient rats of groups 3 and 4, the possibility cannot be excluded that the conversion of ethionine into *S*-adenosylethionine may be inhibited in the biotin-deficient rats.

On the other hand, in addition to the decrease in the concentration of adenine nucleotides, there may be other possible relationships between ethionine and induction of fatty liver. The possibility cannot be excluded that the observed changes in the rate of mobilization of fatty acids to the liver (Rees & Shotlander, 1963), or their rate of oxidation in that organ (Artom, 1959), may also contribute to the increase in liver triglyceride concentration.

The decrease in the liver content of cytidine nucleotides in ethionine-treated rats must also be considered, since cytidine nucleotides are required as coenzymes for phospholipid synthesis. In fact, evidence for a decreased rate of liver phospholipid synthesis was obtained with the same experimental conditions (Olmsted, 1955; Robinson & Harris, 1961).

In the liver of ethionine-treated rats a marked decrease in the concentration of nicotinamide nucleotides and protein-bound biotin was observed (Table 2). In addition, ethionine has been reported to interfere with the synthesis of CoA in the liver (Stekol, Anderson, Weiss & Levin, 1958). These changes cannot be interpreted as a consequence of an increased consumption of these coenzymes for lipid synthesis because lipogenesis is inhibited in ethionine-induced fatty liver (Doering & Natori, 1965) and similar results were obtained in the present experiment. Since it was found that the ethionine effect on lipogenesis was not due to a deficiency in the availability of ATP, NADPH or CoA, Doering & Natori (1965) considered the possibility that ethionine might produce an inhibition of the synthesis of acetyl-CoA carboxylase as this enzyme is a biotin protein. The observed decrease

Table 3. *Effect of ethionine on the free nucleotides of rat liver*

The animals were injected intraperitoneally with 75 mg. of DL-ethionine/100 g. body wt. in three equal doses at intervals of 2.5 hr. The controls were injected with 0.9% NaCl. All animals were killed 24 hr. after the first injection. Each value is given as the mean $\pm$ s.e.m. of eight determinations on different animals. Experimental details are given in the text.

Group	Animals in experiment	Free nucleotides (μ moles/100 g. of defatted liver)												
		CMP	AMP	GMP	UMP	IMP	ADP	UDPAG*	UDPG†	GDP‡	CTP	UDP	ATP	UTP
1	Control	93 $\pm$ 11.2	205 $\pm$ 23.4	44 $\pm$ 3.5	75 $\pm$ 9.7	78 $\pm$ 6.9	90 $\pm$ 5.6	76 $\pm$ 12.1	51 $\pm$ 10.6	39 $\pm$ 3.0	3 $\pm$ 0.4	36 $\pm$ 3.9	33 $\pm$ 3.1	11 $\pm$ 0.3
2	Control + ethionine	63 $\pm$ 6.8	94 $\pm$ 8.7	38 $\pm$ 4.2	66 $\pm$ 7.3	44 $\pm$ 5.0	44 $\pm$ 2.9	42 $\pm$ 5.6	28 $\pm$ 5.2	27 $\pm$ 4.0	5 $\pm$ 0.6	28 $\pm$ 3.4	14 $\pm$ 1.7	7 $\pm$ 0.4
3	Biotin-deficient	54 $\pm$ 6.1	136 $\pm$ 11.5	39 $\pm$ 3.0	78 $\pm$ 10.1	85 $\pm$ 8.3	90 $\pm$ 6.6	61 $\pm$ 9.5	71 $\pm$ 3.2	28 $\pm$ 3.2	trace	21 $\pm$ 2.4	17 $\pm$ 3.1	trace
4	Biotin-deficient + ethionine	75 $\pm$ 7.3	128 $\pm$ 11.2	61 $\pm$ 5.1	65 $\pm$ 8.0	63 $\pm$ 5.4	53 $\pm$ 4.0	63 $\pm$ 7.3	51 $\pm$ 5.6	44 $\pm$ 7.4	trace	24 $\pm$ 2.0	20 $\pm$ 2.2	trace

* UDPAG, Uridine diphosphate acetylglucosamine.

† UDPG, Uridine diphosphate glucose.

‡ This fraction, besides GDP, contains a mixture of unidentified adenosine polyphosphates.

Table 4. *Effect of ethionine on the free nucleotides of rat liver*

The animals were injected intraperitoneally with 75 mg. of DL-ethionine/100 g. body wt. in three equal doses at intervals of 2.5 hr. The controls were injected with 0.9% NaCl. All animals were killed 24 hr. after the first injection. Each value is given as the mean $\pm$ s.e.m. of eight determinations on different animals. Fisher's *P* values are given in parentheses. N.S., Not significant. Experimental details are given in the text.

Group	Animals in experiment	Free nucleotides (μ moles/100 g. of defatted liver)						Total purine nucleotides
		Uridine nucleotides	Cytidine nucleotides	Total pyrimidine nucleotides	Adenine nucleotides	Guanine nucleotides	Inosine nucleotides	
1	Control	245 $\pm$ 26.6	97 $\pm$ 9.3	342	328 $\pm$ 29.0	93 $\pm$ 7.8	77 $\pm$ 11.9	498
2	Control + ethionine	165 $\pm$ 17.1 (< 0.02)	68 $\pm$ 6.7 (< 0.01)	233	151 $\pm$ 13.2 (< 0.001)	72 $\pm$ 7.7 (N.S.)	44 $\pm$ 6.1 (< 0.05)	267
3	Biotin-deficient	231 $\pm$ 21.3 (N.S.)	55 $\pm$ 6.3 (< 0.01)	286	243 $\pm$ 22.4 (< 0.05)	68 $\pm$ 4.5 (N.S.)	77 $\pm$ 10.3 (N.S.)	408
4	Biotin-deficient + ethionine	204 $\pm$ 19.2 (N.S.)	76 $\pm$ 7.1 (N.S.)	280	202 $\pm$ 21.6 (< 0.01)	105 $\pm$ 9.3 (N.S.)	63 $\pm$ 5.1 (N.S.)	370

in the content of protein-bound biotin (Table 2) in the liver of ethionine-treated animals may be consistent with this hypothesis.

REFERENCES

- Artom, C. (1959). *J. biol. Chem.* **234**, 2259.
Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
Dianzani, M. U. (1955). *Biochim. biophys. Acta*, **17**, 391.
Doering, C. H. & Natori, Y. (1965). *Proc. Soc. exp. Biol., N.Y.*, **118**, 957.
Farber, E., Lombardi, B. & Castillo, A. E. (1963). *Lab. Invest.* **12**, 873.
Farber, E., Shull, K. H., Villa-Trevino, S., Lombardi, B. & Thomas, M. (1964). *Nature, Lond.*, **203**, 34.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Hyams, D. E. & Isselbacher, K. J. (1964). *Nature, Lond.*, **204**, 1196.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Marchetti, M., Ottani, V. & Puddu, P. (1966). *Arch. Biochem. Biophys.* **115**, 84.
Marchetti, M., Pasquali, P. & Landi, L. (1965). *Biochem. J.* **94**, 763.
Marchetti, M., Puddu, P. & Caldarera, C. M. (1962). *Biochim. biophys. Acta*, **59**, 475.
Marchetti, M., Puddu, P. & Caldarera, C. M. (1964). *Biochem. J.* **92**, 46.
Olmsted, C. A. (1955). *Fed. Proc.* **14**, 375.
Rees, K. R. & Shotlander, V. L. (1963). *Proc. Roy. Soc. B*, **157**, 517.
Robinson, D. S. & Harris, P. M. (1961). *Biochem. J.* **80**, 361.
Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **234**, 466.
Stekol, J. A., Anderson, E. I., Weiss, S. & Levin, H. (1958). *Abstr. Pap. Amer. chem. Soc., 134th Meet., Chicago*, p. 47c.
Stekol, J. A., Mody, U., Bedrak, E., Keller, S. & Perry, J. (1960). *Fed. Proc.* **19**, 37.