Ethionine Administration in the Rat

2. EFFECTS ON THE INCORPORATION OF [^{32}P]ORTHOPHOSPHATE AND DL-[1-^{14}C]LEUCINE INTO THE PHOSPHATIDES AND PROTEINS OF LIVER AND PLASMA

BY D. S. ROBINSON AND PAMELA M. HARRIS
External Staff of the Medical Research Council, Sir William Dunn School of Pathology, Oxford

(*Received* 22 *November* 1960)

The administration of ethionine to experimental animals results in a reduction in the activity of a number of biochemical systems in the body (see Tarver, 1954; Meister, 1957; Matthews, 1958; Chantrenne, 1958; Shive & Skinner, 1958). Several of these systems involve, either directly or indirectly, the essential amino acid methionine, and it is generally accepted that the action of ethionine at the biochemical level is related to its ability to act as a methionine antagonist.

It has proved difficult, however, to relate the effects of administered ethionine on particular biochemical reactions to the changes which are produced in the intact animal. The present study was carried out to obtain further information on the mechanism of ethionine action in raising the liver-triglyceride concentration and lowering the plasma-lipid concentration in the rat (see Harris & Robinson, 1961).

MATERIALS AND METHODS

The animals and the method of ethionine injection were as described by Harris & Robinson (1961). Additional details are given in the descriptions of individual experiments.

Incorporation of [^{32}P]*orthophosphate into the liver and plasma lipids* in vivo

Several procedures have been described for the measurement of the incorporation of [^{32}P]orthophosphate into the tissue lipids *in vivo* (see Hevesy, 1948). That used in this study is based on the findings of Fishler, Taurog, Perlman & Chaikoff (1941), Hahn & Tyrèn (1946) and Johnson & Dutch (1951).

Rats were injected intraperitoneally with 4 μc of [^{32}P]-orthophosphate (supplied as orthophosphate in iso-osmotic solution containing phosphate buffer, pH 7) in 0·9% NaCl soln. Four hr. later each rat was bled by aortic cannulation and its plasma was separated as described by Harris & Robinson (1961). The liver was removed, rinsed in 0·9% NaCl soln, dried on hard filter paper, weighed and stored at 0° until all the rats in a particular group had been killed.

In experiments in which the incorporation of [^{32}P]-orthophosphate into the plasma lipids was determined, each plasma sample was treated further as described by Friedlander, Chaikoff & Entenman (1945). Samples of the final trichloroacetic acid and ether extracts were taken for total phosphorus estimation and for counting.

A sample (between 0·5 and 1 g.) of each liver was homogenized in a Potter–Elvehjem glass homogenizer at 0° with 5 ml. of a soln. of 10% (w/v) trichloroacetic acid containing $MgCl_2$ (0·4 M). The homogenate was left for 10 min. at 0° and then centrifuged at 2500*g* for 10 min. The supernatant soln. was retained and the ppt. was dispersed in 7 ml. of a solution of 5% (w/v) trichloroacetic acid containing $MgCl_2$ (0·4 M). The suspension was centrifuged again and the washing procedure was repeated until the final supernatant soln. contained no detectable radioactivity. Up to eight washings were required. All the operations were carried out in a cold room at 4°.

Liver acid-soluble phosphorus. The combined supernatant solutions were centrifuged again to remove any traces of ppt. and suitable samples were taken for counting and for total phosphorus estimation.

Liver-lipid phosphorus. The washed ppt. was dispersed in 200 ml. of ethanol–diethyl ether (3:1, v/v), extracted for 2 hr. at 55° and filtered. The residue on the filter paper was redispersed in a further 100 ml. of ethanol–ether, re-extracted for 1 hr. at 55° and refiltered. The filtrates were combined and the solvent was removed *in vacuo* at 55° on a rotary evaporator. Air in the evaporator was replaced by N_2 before the system was evacuated. The brown oily residue which remained after evaporation was extracted three times with 20 ml. of diethyl ether at room temperature and the total ether extract was washed by shaking it in a separating funnel with 20 ml. of a soln. of 0·1 N-HCl and 0·01 N-NaCl. The ether phase which separated on standing was retained, together with the interfacial material, and the washing procedure was repeated five times. During the washing diethyl ether was added to keep the volume of the ether phase above 50 ml. The final ether layer was clear and was recovered free from interfacial material. In one experiment the amount of radioactive material in the discarded interfacial layer was measured and was found to be less than 1% of the total radioactivity of the ether phase.

The rigorous washing procedure described above was carried out to ensure that non-lipid material containing ^{32}P was removed from the ether extract. As an added precaution, in some experiments the ether phase was also washed with a saturated soln. of Na_2HPO_4 (Table 1, Expts. 2 and 3) and in others it was shaken with solid Na_2HPO_4 (Table 1, Expt. 1). In both cases it was subsequently re-washed several times with the soln. of 0·1 N-HCl and 0·01 N-NaCl.

The final ether extract was concentrated to a volume of 5 ml. and left overnight in a conical centrifuge tube at 0°. A small amount of water usually separated at the bottom of the tube: this was removed and the ether extract was made up to a known volume. Samples were taken for counting and for total phosphorus estimation. The specific

activities of lipid extracts from replicate samples of the same liver agreed within 3%.

Liver residue phosphorus. The ppt. remaining after the extraction with ethanol–ether contained some organic phosphorus, presumably in the form of nucleic acid and phosphoprotein. The amount of radioactivity associated with this fraction was measured in some experiments by the method described by Cornatzer, Gallo & Davison (1953). The ppt. was dissolved in 20 ml. of N-NaOH with heating and suitable samples of the soln. were taken for counting and for total phosphorus estimation. The samples were neutralized with N-HCl.

Experiments were carried out to determine the extent of incorporation of [^{32}P]orthophosphate into the individual phosphatides of the rat liver. The experimental procedure was as described above except that the livers from each group of animals, injected with either ethionine or 0·9% NaCl soln., were combined and treated together. They were homogenized in a Waring Blendor and the quantities of trichloroacetic acid, ethanol–ether etc. that were used were increased appropriately. The ether extracts were not treated with Na_2HPO_4.

Fractionation of rat-liver phosphatides on silicic acid columns. In the experiments to determine the extent of incorporation of [^{32}P]orthophosphate into the individual phosphatides of the rat liver the total phosphatides were first separated on a silicic acid column from the other lipids in the ether extract (Borgström, 1952). More than 95% of the lipid phosphorus added to this column was recovered in the final methanol eluate. The phosphatides were then fractionated further on a second column (Marinetti, Erbland & Kochen, 1957; Hanahan, Dittmer & Warashina, 1957). All the silicic acid used in these procedures had been employed previously in the fractionation of liver phosphatides so that breakdown of any plasmalogens present, which may occur on columns of fresh silicic acid (Gray & Macfarlane, 1958), was presumably minimal.

Preparation of silicic acid columns. The columns (15 mm. diam.) were fitted with a sintered-glass filter end-piece and on this was placed a plug of glass wool, approx. 20 mm. thick. Each column was loaded with between 10 and 20 g. of silicic acid (Mallinckrodt's analytical reagent, 100 mesh, not activated by heat) in $CHCl_3$. A solvent reservoir, coupled to a cylinder of N_2, was fitted at the top of the column for overnight fractionations. Up to 25 mg. of lipid/g. of silicic acid was added to each column. Flow rates were between 0·5 and 1·0 ml. of solvent/min. and were maintained, where necessary, by slight N_2 pressure. All the fractionations were carried out at 4° and were completed in 48 hr.

Separation of the individual liver phosphatides. The total methanol eluate from the preliminary column separation was taken to dryness in a rotary evaporator at 37° and the phosphatide residue was redissolved in 15 ml. of $CHCl_3$. The $CHCl_3$ soln. was added to a second silicic acid column and was washed on to the column with $CHCl_3$. The phosphatides were then fractionated with the following solvent mixtures, used in succession: $CHCl_3$–methanol (4:1, v/v), $CHCl_3$–methanol (1:1, v/v) and methanol. The progress of the fractionation was followed by phosphorus analyses on the eluate fractions. More than 95% of the lipid phosphorus added to the column was recovered in the eluate fractions.

The fractionation pattern is shown in Fig. 1. Although no complete analysis was attempted the main fraction (A) eluted by $CHCl_3$–methanol (4:1, v/v) had an esterified fatty acid:phosphorus ratio of 2·04:1 and a high content of amino nitrogen. It did not contain inositol or choline. Hydrolysates of the first component (B) eluted by $CHCl_3$–methanol (1:1, v/v) contained inositol as a major constituent. The second component (C) eluted by $CHCl_3$–methanol (1:1, v/v) had an esterified fatty acid:phosphorus:choline ratio of 1·92:1:0·95. It contained 3% of plasmalogen but no detectable amino nitrogen. The fractions eluted by methanol were not studied. On the basis of the above data the main components were identified as: A, a mixture of ethanolamine and serine phosphatides; B, inositol phosphatide; C, lecithin. This pattern of elution agrees with that described by Hanahan *et al.* (1957) for a similar fractionation system.

Reagents. For most of the extractions and analytical estimations $CHCl_3$, methanol, ethanol and anaesthetic diethyl ether were used as supplied. The $CHCl_3$ used in the silicic acid-column fractionations was purified by washing with water, drying over anhydrous Na_2SO_4 and redistillation, and was finally stabilized with 2% methanol (v/v).

Incorporation of DL-[1-^{14}C]*leucine and* [^{32}P]*orthophosphate into the liver and serum proteins and lipids* in vitro

Liver slices were incubated in serum containing either DL-[1-^{14}C]leucine or [^{32}P]orthophosphate to determine the extent of incorporation of these substances into either the liver and serum proteins (see Radding, Bragdon & Steinberg, 1958) or the liver lipids.

Preparation of liver slices and incubation procedure. Rats were killed and their livers were removed and rinsed in ice-cold Hanks (1948) soln. (16 g. of NaCl, 0·8 g. of KCl, 0·8 g. of $CaCl_2$,$6H_2O$, 0·4 g. of $MgSO_4$,$7H_2O$, 0·2 g. of Na_2HPO_4 and 0·2 g. of KH_2PO_4/2 l. of water). Liver slices, approx. 0·5 mm. thick, were cut by hand by the method of Deutsch (1936) as described by Umbreit, Burris & Stauffer (1957). The livers and the slicer were kept moist with ice-cold Hanks soln. A sample of liver was retained for determination of the esterified fatty acid concentration.

The slices were put immediately into 5 ml. of ice-cold serum containing either 6 μc of DL-[1-^{14}C]leucine or 1·2 μc of [^{32}P]orthophosphate in a 25 ml. Erlenmeyer flask. About 500 mg. of tissue was used from each liver. The serum was obtained by allowing blood from normal rats to clot and then separating the serum by centrifuging. Air was displaced from the flasks by O_2 and the flasks were sealed and gently shaken in a water bath at 37°. After 4 hr. the serum was poured off from the slices and centrifuged for 10 min. at 2500*g* to remove tissue fragments. The liver slices were rinsed in Hanks soln., dried on hard filter paper and weighed.

In the experiments with DL-[1-^{14}C]leucine the serum was fractionated by high-speed centrifuging as described below. Subsequent treatment of the liver slices depended upon the nature of the experiment. If the incorporation of [^{32}P]-orthophosphate into the liver lipids was being studied acid-soluble and lipid phosphorus fractions were prepared as already described for the experiments *in vivo*. If the incorporation of DL-[1-^{14}C]leucine into the liver proteins was under investigation the slices were homogenized in a Potter–Elvehjem glass homogenizer with 7 ml. of 10% (w/v) trichloroacetic acid soln. The homogenate was centri-

fuged at 2500*g* for 10 min. at 0° and the ppt. was washed repeatedly with 5% (w/v) trichloroacetic acid soln. until the washings contained no detectable radioactivity. Up to 12 washings were required. The ppt. was then extracted with three successive portions of hot acetone–ethanol (1:1, v/v) and once with diethyl ether and was stored as a suspension in water.

Separation of serum fractions. A centrifuging technique was used for the separation of the serum fractions (see Bragdon, Havel & Boyle, 1956; Korn, 1959). After the removal of cell debris by centrifuging for 10 min. at 2500*g*, the density of the serum was adjusted to 1·063 by the addition of a salt soln. of *d* 1·35 (NaCl–KBr mixture). The serum was then centrifuged at 114 000*g* for 22 hr. at 0° in the no. 40 head of the Spinco (model L) ultracentrifuge. Serum lipoproteins of $d < 1{\cdot}063$ were concentrated at the top of the Lusteroid centrifuge tube (capacity 6·5 ml.) and could be recovered by slicing the tube at a suitable level (2·1 cm. from the bottom of the tube cap) with a slicer similar to that described by Randolph & Ryan (1950). Sufficient solid KBr was then added to the serum fraction in the bottom segment of the centrifuge tube to raise *d* to 1·21, and this soln. was transferred to a second tube. This was filled with a salt soln. of *d* 1·21 and centrifuged at 114 000*g* for 48 hr. at 0°. Lipoproteins of *d* 1·063–1·21 were concentrated at the top of the tube and were recovered by slicing as before. The serum proteins of $d > 1{\cdot}21$ in the bottom of the tube were retained.

Precipitation and extraction of serum proteins. Each centrifugal fraction, or a suitable sample of the proteins of $d > 1{\cdot}21$, was transferred quantitatively to a Pyrex centrifuge tube (15 ml.). Ethanol and trichloroacetic acid were added to final concentrations of 70% (v/v) and 5% (w/v) respectively. The contents of each tube were mixed, left for 24 hr. at 0° and centrifuged at 2500*g* for 10 min. at 0°. The protein ppt. was washed with 70% (v/v) ethanol containing 5% (w/v) of trichloroacetic acid until the washings contained no detectable radioactivity and was then extracted with three successive portions of hot acetone–ethanol (1:1, v/v).

These procedures for washing the lipoprotein fractions and for lipid extraction were adopted after several other methods had been tried. With trichloroacetic acid alone the lipoprotein ppt. was difficult to centrifuge and much was lost. Extraction of the lipoprotein ppt. with further organic solvent mixtures did not extract any additional lipid.

Acid hydrolysis of protein fractions. The serum-protein ppts. were dried in air at 37°. Samples of the liver-protein suspension were also dried in Pyrex centrifuge tubes in air at 37°. Hydrochloric acid (6N, 0·5 ml./2 mg. of protein) was then added, the tubes were sealed and the protein was hydrolysed for 18 hr. at 105°. The hydrolysates were taken to dryness over P_2O_5 in a vacuum desiccator containing solid KOH. A little water was added to each residue and removed again *in vacuo* to ensure the removal of excess of HCl. The hydrolysates were then dissolved in 0·5 ml. of citrate buffer, pH 5·0 [0·2M-NaOH–0·2M-citric acid soln. (65:35, v/v)]. After centrifuging, suitable samples of the supernatant soln. were taken for the measurement of amino nitrogen and radioactivity.

Two experiments were carried out to determine whether DL-[1-^{14}C]leucine that was not incorporated into protein would be removed from the protein ppts. by the procedure described. In one, 5 ml. of serum containing 6 μc of DL-[1-^{14}C]leucine was incubated alone for 4 hr. at 37° and, in the other, liver slices were incubated for 1 min. only at 37° in 5 ml. of serum containing 6 μc of DL-[1-^{14}C]leucine. In neither case did the activity of any of the serum- and liver-protein fractions which were isolated exceed 2 counts/min./100 μg. of amino nitrogen. In similar expts., in which liver slices were incubated for 5 min. at 37° in 5 ml. of serum containing 1·2 μc of [^{32}P]orthophosphate, the activity of the liver-phosphatide fraction was never greater than 1·5 counts/min./μg. of phosphorus.

The concentration of free amino acid in the serum was between 4 and 5 μmoles/ml. initially. After incubation with liver slices from either normal or ethionine-treated rats it rose to between 8 and 10 μmoles/ml./500 mg. of wet tissue. Thus the increase in the free amino acid concentration during incubation was not influenced by the origin of the slices.

Counting procedure

The counting procedure was as described by Harris & Robinson (1961). Counts for replicate samples agreed within 3% except for those on material containing acid-soluble phosphorus in trichloroacetic acid, where replicate samples agreed within 5%.

In the experiments with DL-[1-^{14}C]leucine a self-absorption curve for DL-[1-^{14}C]leucine in the presence of citrate buffer and a suitable amino acid mixture was prepared and the counts were corrected to zero mass.

Analytical methods

The methods used to prepare lipid extracts of plasma and liver and to estimate total esterified fatty acids, total cholesterol and phosphatide phosphorus therein have been described by Harris & Robinson (1961). The method of Allen (1940) was used to measure acid-soluble phosphorus.

Acid hydrolysis of the phosphatides eluted from the silicic acid columns was carried out by refluxing with 6N-HCl for 6 hr. the residues remaining after evaporation of the eluting solvent. After hydrolysis water was added to lower the HCl concentration to 2N and the free fatty acids were extracted with light petroleum. Samples of the aqueous phase were then taken to dryness over P_2O_5 in a vacuum desiccator containing solid KOH. A little water was added to each residue and removed again *in vacuo* to ensure the removal of excess of HCl.

Choline in the hydrolysates was determined by the method of Appleton, La Du, Levy, Steele & Brodie (1953), as modified by Dawson (1956). Inositol in the hydrolysates was assessed by the paper-chromatographic method described by Olley (1956). The amino-nitrogen content of the phosphatide fractions was determined before hydrolysis by the method of Lea & Rhodes (1954). Plasmalogens were determined as described by Gray & Macfarlane (1958).

Free amino acid nitrogen in protein hydrolysates and in serum was measured by the method of Yemm & Cocking (1955). The serum proteins were first removed as described by Dunn, Schott, Frankl & Rockland (1945).

RESULTS

Effect of ethionine treatment on the incorporation of [^{32}P]*orthophosphate into liver and plasma phosphatides* in vivo. In Tables 1 and 2 are shown the

Table 1. *Effect of ethionine on the incorporation of* [^{32}P]*orthophosphate into the liver phosphatides of the female rat* in vivo

Female rats, which had received subcutaneous injections of either 2 ml. of ethionine (25 mg./ml.) in 0·9 % NaCl soln. (ethionine group) or 2 ml. of 0·9 % NaCl soln. (control group), at the times specified below, were injected intraperitoneally with [^{32}P]orthophosphate (4 μc/rat) and killed 4 hr. later. *Expt.* 1: ethionine injections at 0, 24, 48 and 72 hr.; $^{32}PO_4$ injection at 72 hr. *Expt.* 2: ethionine injections at 0, 24, 48, 56 and 72 hr.; $^{32}PO_4$ injection at 72 hr. Rats were fasted from 56 hr. *Expt.* 3: ethionine injections at 0, 8 and 24 hr.; $^{32}PO_4$ injection at 24 hr. Rats were fasted from 12 hr.

			Total esterified fatty acids		Specific activity (counts/min./μg. of P)		Relative specific activity*		
Expt. no.	Group	Rat no.	Plasma (mg./ml.)	Liver (mg./g. wet wt.)	Liver acid-soluble phosphorus	Liver phosphatide	Liver phosphatide	Liver residue phosphorus	Plasma phosphatide
1	Ethionine	1	—	96	39·5	6·1	0·15	—	—
		2	—	78	41·8	3·8	0·09	—	—
		3	—	113	37·0	9·4	0·25	—	—
	Control	1	—	45	42·0	12·0	0·29	—	—
		2	—	54	39·6	13·5	0·35	—	—
		3	—	48	28·0	9·3	0·33	—	—
2	Ethionine	1	0·48	122	45·0	6·6	0·15	0·08	0·03
		2	0·30	79	49·5	10·2	0·21	0·10	0·04
		3	0·33	114	35·0	6·7	0·19	0·08	0·05
	Control	1	1·45	38	48·8	13·0	0·27	0·07	0·35
		2	1·14	41	48·6	14·9	0·31	0·06	—
		3	1·23	48	45·5	12·1	0·27	0·08	0·30
3	Ethionine	1	0·48	121	33·6	7·8	0·23	—	0·03
		2	0·31	139	36·1	6·8	0·19	—	0·12
		3	—	115	33·5	6·8	0·20	—	0·07
	Control	1	1·13	54	41·5	14·5	0·35	—	0·32
		2	0·99	41	41·8	13·8	0·33	—	0·33
		3	1·26	43	39·0	16·0	0·41	—	0·39

* Ratio of the specific activity of phosphorus in the form specified to that of the acid-soluble phosphorus.

Table 2. *Effect of ethionine on the incorporation of* [^{32}P]*orthophosphate into the liver phosphatides of the male rat* in vivo

Conditions were as described in Table 1. *Expt.* 1: ethionine injections at 0, 24, 28, 32 and 48 hr.; $^{32}PO_4$ injection at 48 hr. *Expt.* 2: ethionine injections at 0, 4, 8 and 24 hr.; $^{32}PO_4$ injection at 24 hr. Rats were fasted from 8 hr.

			Total esterified fatty acids		Specific activity (counts/min./μg. of P)		Relative specific activity*		
Expt. no.	Group	Rat no.	Plasma (mg./ml.)	Liver (mg./g. wet wt.)	Liver acid-soluble phosphorus	Liver phosphatide	Liver phosphatide	Liver residue phosphorus	Plasma phosphatide
1	Ethionine	1	0·44	115	31·0	1·6	0·05	0·04	—
		2	0·23	77	32·0	3·6	0·11	0·05	0·05
	Control	1	1·07	48	39·5	11·2	0·28	0·09	0·32
		2	1·26	37	26·6	8·1	0·30	0·08	0·26
		3	1·03	40	30·5	8·3	0·27	0·07	0·32
2	Ethionine	1	—	54	37·3	6·9	0·18	0·05	—
		2	—	82	43·1	5·7	0·13	0·05	—
		3	—	101	32·0	4·3	0·13	0·05	—
		4	—	86	37·7	5·1	0·13	0·06	—
	Control	1	—	29	45·9	10·0	0·22	0·06	—
		2	—	34	43·5	9·3	0·21	0·06	—
		3	—	35	38·0	10·2	0·27	0·06	—
		4	—	39	38·7	12·0	0·31	0·05	—

* Ratio of the specific activity of phosphorus in the form specified to that of the acid-soluble phosphorus.

specific activities and relative specific activities of various liver and plasma fractions 4 hr. after the intraperitoneal injection of [^{32}P]orthophosphate into rats previously injected with either ethionine or 0·9% sodium chloride solution. The extent of incorporation of [^{32}P]orthophosphate into the fractions shows considerable individual variation. However, under these experimental conditions there is in both male and female rats a decrease in the extent of [^{32}P]orthophosphate incorporation into the liver phosphatides after ethionine treatment. Incorporation into plasma phosphatides is also markedly lowered.

The relative specific activity of the liver residue protein fraction, which presumably contains nucleoprotein and phosphoprotein, is much less than that of the liver phosphatides in the normal rat: no significant change occurs after ethionine treatment.

Effect of ethionine treatment on the incorporation

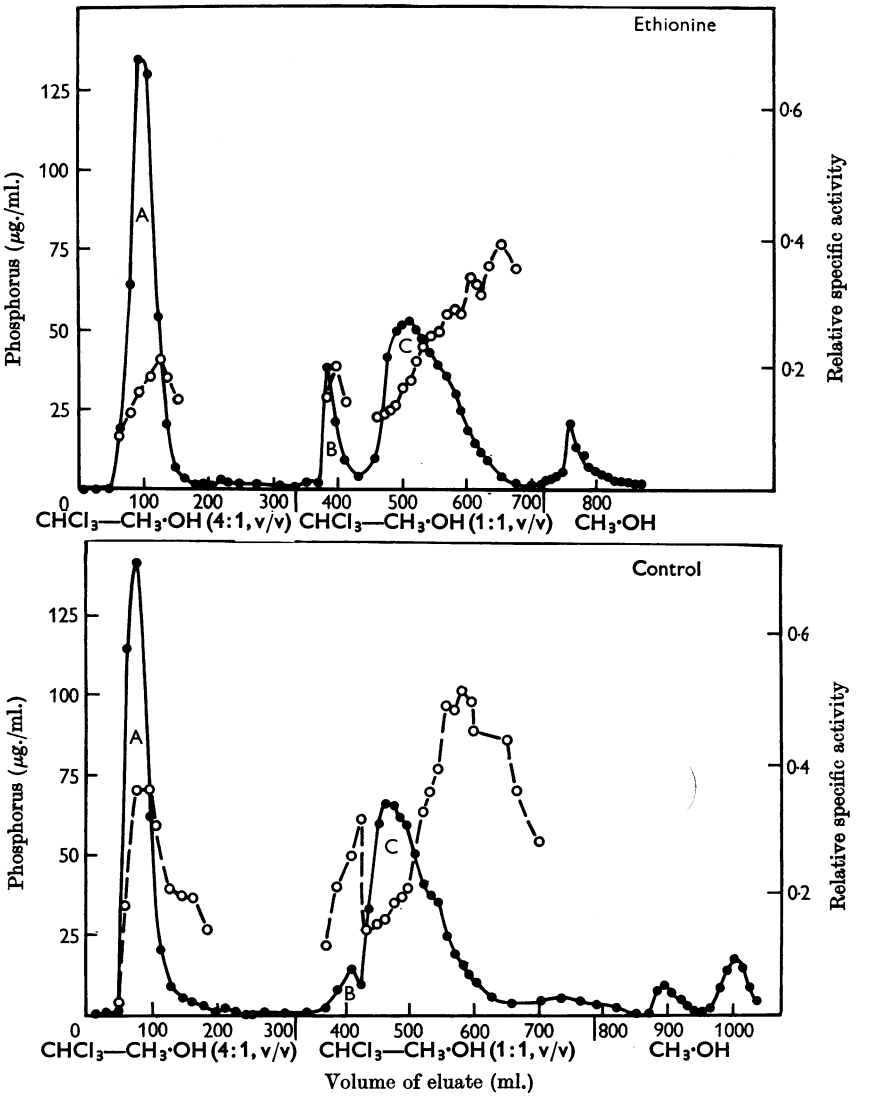

Fig. 1. Fractionation of the liver phosphatides from control and ethionine-treated female rats injected with [^{32}P]orthophosphate. A, Mixed serine and ethanolamine phosphatides; B, inositol phosphatide; C, lecithin. The identification of these components is based on data given in the Methods section. The relative specific activity (○), as well as the phosphorus content (●), of certain of the eluate fractions is shown. Relative specific activity = ratio of the phosphatide phosphorus specific activity to the acid-soluble phosphorus specific activity.

of [^{32}P]*orthophosphate into individual liver phosphatides* in vivo. To determine whether the decrease in incorporation of [^{32}P]orthophosphate after ethionine administration was restricted to particular liver-phosphatide components, the liver phosphatides from rats which had been injected with [^{32}P]orthophosphate, after being given either ethionine or 0·9 % sodium chloride solution, were fractionated on silicic acid columns. The relative specific activities of the total phosphatide extracts before their fractionation were 0·29 for the animals injected with 0·9 % sodium chloride and 0·19 for the animals injected with ethionine.

The results of the fractionations and the relative specific activities of certain of the individual phosphatide components are shown in Fig. 1: the relative specific activities of all the components could not be measured as there was not enough material. After ethionine treatment there was a decrease in the relative specific activities of all the phosphatides studied.

In both the normal and ethionine-treated group, there was a progressive rise in the relative specific activities of the fractions collected from the column as the liver lecithin was eluted. With respect to the rate of incorporation of [^{32}P]orthophosphate therefore the liver-lecithin component appears to be heterogeneous. The mixed serine and ethanolamine phosphatide and the inositol phosphatide components may have a similar heterogeneity since the data in Fig. 1 show that the eluted fractions of these phosphatides with maximum relative specific activity were not those of maximum phosphorus content. These findings, together with evidence that the heterogeneity of the liver-lecithin component is associated with differences in the fatty acid composition of the eluted fractions, have been presented elsewhere (Harris, Robinson & Getz, 1960).

Effect of ethionine treatment on the incorporation of [^{32}P]*orthophosphate into liver phosphatides* in vitro. Liver slices from rats that had been injected with either ethionine or 0·9 % sodium chloride solution were incubated for 4 hr. at 37° in serum containing [^{32}P]orthophosphate and the relative specific activities of the phosphatide fractions isolated from the liver slices after the incubation were measured (Table 3). No change could be detected in the relative specific activity after ethionine administration.

The conditions of this experiment differed from those of the studies *in vivo* since ethionine was given only a few hours before the rats were killed, although the total amount of ethionine administered was comparable with that in Expt. 3, Table 1. The conditions were similar to those in the experiments described below on the incorporation of DL-[1-^{14}C]leucine into the rat-liver proteins.

Effect of ethionine treatment on the incorporation of DL-[1-^{14}C]*leucine into rat liver and serum proteins* in vitro. Ethionine administration in the rat reduces the rate of incorporation of labelled amino acids into the hepatic proteins, both *in vivo* and in cell-free preparations *in vitro*, and lowers the activity of certain liver enzymes (Simpson, Farber & Tarver, 1950; Lee & Williams, 1952; Bollag & Gallico, 1952; Younathan, Frieden & Dittmer, 1956; Farber & Corban, 1958; Freedland & Harper, 1958). On the basis of these findings it has been suggested that ethionine interferes with some stage of hepatic-protein synthesis (see Farber, 1959). If this is so, the synthesis of the serum proteins and lipoproteins, which are formed in the liver, should also be inhibited after ethionine administration. This possibility was investigated by incubating liver slices from normal and ethionine-treated rats in normal rat serum containing DL-[1-^{14}C]leucine.

Table 3. *Effect of ethionine on the incorporation of* [^{32}P]*orthophosphate into rat-liver phosphatides* in vitro

Female rats were injected subcutaneously with 3, 1·5 and 1·5 ml. of ethionine (25 mg./ml.) in 0·9 % NaCl soln. at 0, 1·5 and 3 hr. respectively (ethionine group). Other female rats were given corresponding injections of 0·9 % NaCl soln. (control group). The rats were killed 5 hr. after the initial injection and slices (approx. 500 mg. wet wt.) from each rat liver were incubated for 4 hr. at 37° in 5 ml. of rat serum containing 1·2 μc of [^{32}P]-orthophosphate. Incorporation of the ^{32}P into the liver phosphatides was measured.

Group	Rat no.	Liver total esterified fatty acids (mg./g. wet wt.)	Specific activity of liver acid-soluble phosphorus (counts/min./ μg. of P)	Specific activity of liver phosphatide (counts/min./ μg. of P)	Relative specific activity of liver phosphatide*
Ethionine	1	56	543	40	0·07
	2	52	606	32	0·05
	3	41	588	26	0·04
Control	1	40	470	24	0·05
	2	36	384	18	0·05
	3	39	450	23	0·05

* Ratio of specific activity of phosphatide to that of acid-soluble phosphorus.

Table 4. *Effect of ethionine on the incorporation of* DL-[1-^{14}C]*leucine into rat liver and serum proteins* in vitro

Female rats were injected subcutaneously at 0, 1·5 and 3 hr. with either 3, 1·5 and 1·5 ml. of ethionine (25 mg./ml.) in 0·9 % NaCl soln. (ethionine group) or equivalent amounts of 0·9 % NaCl soln. (control group). The rats were killed 5 hr. after the initial injection and slices (approx. 500 mg. wet wt.) from each rat liver were incubated for 4 hr. at 37° in 5 ml. of rat serum containing 6 μc of DL-[1-^{14}C]leucine. Incorporation of ^{14}C into the liver and serum proteins was determined. Radioactivity values are expressed as the means ± S.D. Each group consisted of four rats.

Group	Liver total esterified fatty acids (mg./g. wet wt.)	Radioactivity in protein hydrolysates (counts/min./100 μg. of amino N)			
		Serum lipoproteins of $d < 1·063$	Serum lipoproteins of d 1·063–1·21	Serum proteins of $d > 1·21$	Liver proteins
Ethionine	54 ± 11	99 ± 23	48 ± 6·3	22·5 ± 2·2	265 ± 59
Control	39 ± 5	291 ± 42	112 ± 41	32 ± 13	507 ± 57

Under these conditions Radding *et al.* (1958) have shown that with liver slices from normal rats ^{14}C was incorporated into the serum proteins and lipoproteins. This represented the release of newly synthesized serum protein by the liver, since incorporation did not occur in the absence of liver and was decreased in the presence of known inhibitors of protein synthesis such as dinitrophenol and fluorophenylalanine.

In the present experiments the incorporation of ^{14}C into the serum lipoproteins of $d < 1·063$ (low-density lipoproteins) and d 1·063–1·21 (high-density lipoproteins) and into the serum-protein fraction of $d > 1·21$, which consists mainly of serum albumin, was reduced in animals pretreated with ethionine (Table 4). Incorporation into the liver proteins was also reduced. The reductions in incorporation into the liver-protein fraction and into the two serum-lipoprotein fractions were significant at a 1 % level of confidence and those into the serum-protein fractions of $d > 1·21$ at a 5 % level of confidence, as measured by the Behrens test of the significance of differences between two means (Fisher & Yates, 1957).

It would be of interest to determine whether the extent of the reduction in incorporation of ^{14}C after ethionine treatment differed for the different serum-protein groups since the rates of synthesis of these proteins by the liver could be affected by ethionine to different extents (see Discussion). The data in Table 4 are not adequate to test this possibility. The percentage reduction in the number of counts in the serum-lipoprotein fraction of $d < 1·063$ is not significantly different at a 5 % level of confidence from the reduction in the fraction of d 1·063–1·21: and, although the percentage reduction in the number of counts in each of the lipoprotein fractions is significantly greater at a 5 % level of confidence than that in the fraction of $d > 1·21$, the latter contains several distinct protein species.

DISCUSSION

Effect of ethionine on the incorporation of [^{32}P]-*orthophosphate into the liver and serum phosphatides.* The development of fatty livers in experimental animals on a diet deficient in choline is associated with a reduction in the rate of phosphatide synthesis in the liver (Best, 1956). The present studies suggest that under certain conditions *in vivo* a reduced rate of hepatic-phosphatide synthesis may accompany the development of a fatty liver after ethionine administration. Since ethionine reduces the ability of the essential amino acid, methionine, to provide methyl groups for endogenous choline synthesis (Simmonds, Keller, Chandler & du Vigneaud, 1950), it might be supposed that the fatty liver induced by ethionine arises from a lack of available choline for lecithin synthesis.

However, there are important differences between the fatty liver caused by ethionine and that due to choline deficiency. Dietary or injected choline does not prevent the development of the former (Farber, Simpson & Tarver, 1950), and the visible liver fat is said to be periportal in distribution in ethionine-treated animals but centrolobular in choline deficiency (Koch-Weser, Farber & Popper, 1951). The reduction in the rate of phosphatide synthesis by the liver in animals on a choline-deficient diet is restricted to the lecithin fraction (Artom & Cornatzer, 1948), whereas the fall in the rate of [^{32}P]orthophosphate incorporation into the liver phosphatides observed in the present study was not limited to this fraction. In view of these differences the decrease after ethionine administration in the rate of incorporation of [^{32}P]orthophosphate into the liver phosphatides *in vivo* observed in the present study seems unlikely to be due to a reduction in the availability of choline for lecithin synthesis. It may rather be secondary to inhibition of the synthesis of the protein moiety of the serum lipoproteins in the

liver (see below). Such an inhibition might be expected to result in a fall in the rate of formation in the liver of those phosphatides which are combined in the serum lipoproteins. The virtually complete inhibition of [^{32}P]orthophosphate incorporation into the serum phosphatides after ethionine administration, as compared with the moderate fall in the rate of incorporation into the total liver phosphatides, would be consistent with this view.

Effect of ethionine on the incorporation of DL-[1-^{14}C]*leucine into the liver proteins and the serum proteins and lipoproteins.* When rat-liver slices are incubated in serum containing DL-[1-^{14}C]leucine, ^{14}C is incorporated into the liver proteins; the rate of this incorporation is reduced when the liver slices are obtained from animals that have been treated with ethionine. This result, though not itself constituting proof of inhibition of protein synthesis in the liver by ethionine (see Tarver, 1954; Loftfield, 1957; Campbell, 1958), is consistent with the evidence for such an effect obtained by other workers (see Results). The further observation that the rate of ^{14}C incorporation into the serum proteins and lipoproteins was markedly reduced within 5 hr. of ethionine administration suggests that synthesis in the liver of the serum proteins, and of the protein moieties of the serum lipoproteins, is also inhibited. An effect on the serum lipoproteins would be of interest since it might explain the action of ethionine in lowering the serum-lipid concentration and raising the liver-triglyceride concentration. If the formation of the serum lipoproteins in the liver is inhibited the concentration of lipids in the serum will fall; and since triglyceride is normally carried from the liver as part of the serum lipoproteins any reduction in the rate of serum-lipoprotein formation could lead to an accumulation of liver triglyceride.

Although data are not available for the rat, measurements of the rates of turnover of the protein moieties of the low- and high-density plasma lipoproteins have been made in man and in the rabbit (Volwiler *et al.* 1955; Gitlin & Cornwell, 1956; Avigan, Eder & Steinberg, 1957). The circulating half-life of the low-density lipoprotein is between 2·0 and 3·5 days and that of the high-density lipoprotein is between 4 and 5 days. These values are of the order that would be required for a decrease in the plasma lipid to be observable *in vivo* within 24 hr. of giving ethionine, if the reduction was mediated through an inhibition of plasma-lipoprotein synthesis.

The present results are therefore consistent with the view that a reduction in the rate of synthesis of the protein moieties of the serum lipoproteins occurs at an early stage after the administration of ethionine to the rat, and that this reduction may contribute towards the changes in the concentration of serum and liver lipids that were found in the intact animal (Harris & Robinson, 1961). Further factors contributing towards the increase in liver-triglyceride concentration after ethionine may be a mobilization of fatty acids to the liver (Harris & Robinson, 1961) and a reduced rate of fatty acid oxidation in the liver as reported by Artom (1959).

A possibly analogous situation, in which the development of a fatty liver is accompanied by a decrease of serum lipid, occurs in a variety of conditions; for example, in protein malnutrition, as in man in kwashiorkor (Trowell, Davis & Dean, 1954), or in experimental animals fed with diets low in protein or deficient in certain essential amino acids (Olson, 1958–59). It can also occur in man in toxic hepatitis and advanced cirrhosis of the liver (Man, Kartin, Durlacher & Peters, 1945). It may be that a common factor in these conditions is an impaired hepatic synthesis of serum lipoproteins, caused by either a deficiency of amino acids reaching the liver or by extensive tissue damage.

SUMMARY

1. The administration of ethionine (150–250 mg./animal) to either male or female rats for periods of between 24 and 72 hr. reduces the rate of incorporation of [^{32}P]orthophosphate *in vivo* into the liver phosphatides by between 40 and 50 % and into the plasma phosphatides by between 80 and 90 %. The reduction in the rate of incorporation into the liver phosphatides is not restricted to the liver-lecithin fraction.

2. Ethionine administration (150 mg./animal) for a shorter period of 5 hr. does not change the rate of incorporation of [^{32}P]orthophosphate into the liver phosphatides when rat-liver slices are incubated in serum *in vitro*; under the same conditions, the rate of incorporation of DL-[1-^{14}C]leucine into the liver and serum proteins and lipoproteins is reduced.

3. The significance of these observations is discussed in relation to the action of ethionine in lowering the serum lipid and raising the liver-triglyeride concentration in the intact animal.

The authors thank Miss S. Such for careful and expert technical assistance.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.

Appleton, H. D., La Du, B. N., jun., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953). *J. biol. Chem.* **205**, 803.

Artom, C. (1959). *J. biol. Chem.* **234**, 2259.

Artom, C. & Cornatzer, W. E. (1948). *J. biol. Chem.* **176**, 949.

Avigan, J., Eder, H. A. & Steinberg, D. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 429.

Best, C. H. (1956). *Proc. Roy. Soc.* B, **145**, 151.
Bollag, W. & Gallico, E. (1952). *Biochim. biophys. Acta*, **9**, 193.
Borgström, B. (1952). *Acta physiol. scand.* **25**, 101.
Bragdon, J. M., Havel, R. J. & Boyle, E. (1956). *J. Lab. clin. Med.* **48**, 36.
Campbell, P. N. (1958). *Advanc. Cancer Res.* **5**, 97.
Chantrenne, H. (1958). *Annu. Rev. Biochem.* **27**, 35.
Cornatzer, W. E., Gallo, D. G. & Davison, J. P. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 103.
Dawson, R. M. C. (1956). *Biochem. J.* **64**, 192.
Deutsch, W. (1936). *J. Physiol.* **87**, 56P.
Dunn, M. S., Schott, H. F., Frankl, W. & Rockland, L. B. (1945). *J. biol. Chem.* **157**, 387.
Farber, E. (1959). *Arch. Path.* **67**, 1.
Farber, E. & Corban, M. S. (1958). *J. biol. Chem.* **233**, 625.
Farber, E., Simpson, M. V. & Tarver, H. (1950). *J. biol. Chem.* **182**, 91.
Fisher, R. A. & Yates, F. (1957). *Statistical Tables for Biological, Agricultural and Medical Research.* Edinburgh: Oliver and Boyd Ltd.
Fishler, M. C., Taurog, A., Perlman, I. & Chaikoff, I. L. (1941). *J. biol. Chem.* **141**, 809.
Freedland, R. A. & Harper, A. E. (1958). *J. biol. Chem.* **233**, 1041.
Friedlander, H. D., Chaikoff, I. L. & Entenman, C. (1945). *J. biol. Chem.* **158**, 231.
Gitlin, D. & Cornwell, D. (1956). *J. clin. Invest.* **35**, 706.
Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.
Hahn, L. & Tyrèn, H. (1946). *Ark. Kemi Min. Geol.* **21** A, no. 11.
Hanahan, D. J., Dittmer, J. C. & Warashina, E. (1957). *J. biol. Chem.* **228**, 685.
Hanks, J. H. (1948). *J. cell. comp. Physiol.* **31**, 235.
Harris, P. M. & Robinson, D. S. (1961). *Biochem. J.* **80**, 352.
Harris, P. M., Robinson, D. S. & Getz, G. (1960). *Nature, Lond.*, **188**, 742.
Hevesy, G. (1948). *Radioactive Indicators.* New York: Interscience Publishers Inc.
Johnson, R. M. & Dutch, P. H. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 662.
Koch-Weser, D., Farber, E. & Popper, H. (1951). *Arch. Path.* **51**, 498.
Korn, E. D. (1959). *Meth. biochem. Anal.* **7**, 145.
Lea, C. H. & Rhodes, D. N. (1954). *Biochem. J.* **56**, 613.
Lee, N. D. & Williams, R. H. (1952). *Biochim. biophys. Acta*, **9**, 698.
Loftfield, R. B. (1957). *Progr. Biophys.* **8**, 347.
Man, E. B., Kartin, B. L., Durlacher, S. H. & Peters, J. P. (1945). *J. clin. Invest.* **24**, 623.
Marinetti, G. V., Erbland, J. & Kochen, J. (1957). *Fed. Proc.* **16**, 837.
Matthews, R. E. F. (1958). *Pharmacol. Rev.* **10**, 359.
Meister, A. (1957). *Biochemistry of the Amino Acids.* New York: Academic Press Inc.
Olley, J. (1956). *2nd int. Conf., Biochemical Problems of Lipids, Ghent*, p. 49. Ed. by G. Popják & E. LeBreton. London: Butterworths Scientific Publications.
Olson, R. E. (1958–59). *Perspectives Biol. Med.* **2**, 84.
Radding, C. M., Bragdon, J. H. & Steinberg, D. (1958). *Biochim. biophys. Acta*, **30**, 443.
Randolph, M. L. & Ryan, R. R. (1950). *Science*, **112**, 528.
Shive, W. & Skinner, C. G. (1958). *Annu. Rev. Biochem.* **27**, 643.
Simmonds, S., Keller, E. B., Chandler, J. P. & du Vigneaud, V. (1950). *J. biol. Chem.* **183**, 191.
Simpson, M. V., Farber, E. & Tarver, H. (1950). *J. biol. Chem.* **182**, 81.
Tarver, H. (1954). In *The Proteins*, vol. 2B, p. 1199. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Trowell, H. C., Davis, J. N. P. & Dean, R. F. A. (1954). *Kwashiorkor.* London: Edward Arnold Ltd.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques.* Minneapolis: Burgess Publishing Co.
Volwiler, W., Goldsworthy, P. D., MacMartin, M. P., Wood, P. A., Mackay, I. R. & Fremont-Smith, K. (1955). *J. clin. Invest.* **34**, 1126.
Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.
Younathan, E. S., Frieden, E. & Dittmer, K. (1956). *J. biol. Chem.* **219**, 531.

Biochem. J. (1961) **80**, 369

The Isolation, Purification and some Properties of the Alkaline Phosphatase of Human Leucocytes

BY S. TRUBOWITZ, D. FELDMAN, S. W. MORGENSTERN AND V. M. HUNT
Medical Service and Medical Research Laboratories, Veterans Administration Hospital, East Orange, New Jersey, U.S.A.

(*Received* 9 *January* 1961)

Valentine & Beck (1951), Wachstein (1946), Wiltshaw & Moloney (1958) and Williams & Mendel (1954) have reported significant alterations in the alkaline phosphatase of the leucocyte in some maladies. Large increases in the content of this enzyme in the human leucocyte have been found in infection, polycythaemia vera and in leukaemoid states, and sharp decreases have been noted in chronic myelocytic leukaemia. These changes have been sufficiently well documented by both biochemical and histochemical techniques to justify their use as diagnostic aids in the study of