The Biosynthesis of Glycerides by Mitochondria from Rat Liver

THE REQUIREMENT FOR A SOLUBLE PROTEIN

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1. The synthesis of glycerides from L-3-glycerophosphate and palmitic acid by mitochondrial preparations from rat liver was shown to be stimulated markedly by a soluble factor from the supernatant fraction of the liver. 2. That the soluble factor was a protein was indicated by its inactivation after treatment with papain and after boiling for 3 min. at 100°, its precipitation by ammonium sulphate and its behaviour on Sephadex G-200. The soluble factor was purified by ammonium sulphate fractionation and gel filtration. 3. Bovine serum albumin and lipoprotein fractions from rat and human serum also stimulated glyceride biosynthesis but the stimulations were one-twentieth to one-third of that obtained with the soluble factor. 4. The function of the soluble factor could not be explained by assuming a leakage of acyl-CoA synthetase, phosphatidate phosphatase or diglyceride acyltransferase from the mitochondria into the supernatant during preparation of the mitochondrial fraction. 5. Palmitic acid, in the presence of the soluble factor and optimum amounts of ATP and CoA, was a more effective substrate than palmitoyl-CoA or palmitoylcarnitine for the biosynthesis of glycerides by mitochondria.

The biosynthesis of glycerides by subcellular fractions of mammalian liver is known to proceed by the pathway postulated by Kennedy (1957) which involves L-3-glycerophosphate and long-chain fatty acids as precursors. The investigations of Tietz & Shapiro (1956), Stein & Shapiro (1957) and Stein, Tietz & Shapiro (1957) showed that biosynthesis by mitochondria of rat liver proceeded at maximum rates only in the presence of small amounts of the microsomal and soluble components of the cell. They found that two factors, one heat-stable and the other heat-labile, were involved. Hübscher, Smith & Gurr (1964) showed that in rat liver the soluble factor(s) of the supernatant fraction stimulated both mitochondrial and microsomal biosyntheses of glycerides from L-3-glycerophosphate and palmitate. The stimulating effect of a soluble factor was also observed with particulate preparations from rat mammary gland (Dils & Clark, 1962), from rat liver and from the intestinal mucosa of the cat, guinea pig and rabbit (Hübscher *et al.* 1964). The present work was undertaken to investigate the nature and possible function of the soluble factor.

MATERIALS AND METHODS

Preparation of subcellular fractions. Preparation of the mitochondrial and soluble fractions from rat liver was carried out according to Sedgwick & Hübscher (1965).

Livers from albino rats weighing between 150 and 250 g. were homogenized in 0.3 M-sucrose containing 2 mM-EDTA. The homogenate was centrifuged for 10 min. at 1000 g to sediment nuclei and cell debris and then for 10 min. at 4300 g to sediment the mitochondrial fraction, which was washed four times and finally suspended in 0.3 M-sucrose containing 2 mM-EDTA. The supernatant obtained after the removal of mitochondria was centrifuged for 60 min. at 104000 g and the sediment discarded. The final supernatant obtained was called the 'supernatant fraction'. In some experiments it was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the sediments obtained after centrifugation were dissolved in 0.3 M-sucrose.

Special preparations. Phosphatidic acid was prepared from phosphatidylcholine as described by Hübscher & Clark (1960). Analysis of the purified preparation gave molar proportions phosphate:ester:nitrogen 1:1.9:0.03.

1,2-Diglyceride was prepared as described by Gurr, Brindley & Hübscher (1965).

Low-density lipoproteins were isolated from pooled citrated human plasma by the method of Oncley, Walton & Cornwell (1957) and a high-density lipoprotein fraction was prepared from human plasma as described by Briner, Riddle & Cornwell (1959). A crude lipoprotein fraction was prepared from rat serum by layering 11 ml. of serum over 18 ml. of 30% (w/v) sucrose and centrifuging for 22 hr. at 27000 g. After centrifugation, the upper 8 ml. was collected and dialysed against 0.066 M-Sørensen phosphate buffer, pH 7.4, before use. The preparation contained albumin as well as lipoprotein.

[1- ^{14}C]Palmitoyl-CoA was prepared by a slight modification of the method of Kornberg & Prier (1953). A mitochondrial fraction of rat liver was used as the source of

acyl-CoA synthetase and 40 mg. of protein of this fraction was incubated for 1 hr. at 37° in final volume 30 ml. containing 0.66 mM-CoA, 6.6 mM-MgCl₂, 3.3 mM-glutathione, 20 mM-ATP, 120 mM-KCl, 4.8 mM-potassium palmitate (24 μ C) and 100 mg. of bovine serum albumin. The pH was adjusted to 7.4 by the addition of dilute alkali. The reaction was stopped by adding 3 ml. of a 50% (w/v) solution of trichloroacetic acid. The precipitate was collected by centrifugation and washed three times each with water, acetone and ether. The palmitoyl-CoA was extracted from the washed precipitate with propan-2-ol-ethanol-water (1:1:1, by vol.) which had been adjusted to pH 7.4 by the addition of ammonia. The extract was evaporated to dryness under reduced pressure at 37° and the dry residue redissolved in 0.025 M-tris-HCl buffer, pH 7.4. The palmitoyl-CoA was again precipitated with trichloroacetic acid and the precipitate treated as described above except that the solvent extraction was omitted. Analysis of the final preparation gave molar ratio thio ester bond:phosphate 1.0:3.1 and specific radioactivity 1 μ C/6 μ moles.

[¹⁴C]Palmitoylcarnitine was prepared according to the method of Bremer (1962).

L-3-[¹⁴C]Glycerophosphate was prepared enzymically with glycerokinase by a method devised to give a good yield in terms of the costly [¹⁴C]glycerol used. The incubation system contained, in a final volume of 56 ml., 36 mM-[1-¹⁴C]-glycerol, 45 mM-ATP, 20 mM-MgCl₂ and 5 mg. of glycerokinase. The pH of the medium was adjusted to 9.8 with KOH. After incubation for 90 min. at 37° the reaction was stopped by adding an equal volume of a solution containing 5% (w/v) trichloroacetic acid and 12.5% (w/v) of Norit A. The charcoal and the precipitated protein were removed by filtration and washed with water, which was combined with the original filtrate. Trichloroacetic acid was extracted from the filtrate with 6 vol. of diethyl ether and the excess of ether was removed with a stream of air. The glycerophosphate was purified on a column of Dowex 1 (X8), 20 cm. $\times$ 1.5 cm., with a formic acid gradient for elution. For this purpose the mixing chamber contained 1 l. of 0.5 N-formic acid and the reservoir 1 l. of 1.5 N-formic acid. All the eluted fractions which contained organic phosphate were pooled and most of the water was removed by rotary evaporation under reduced pressure at 37°. The syrup obtained was left overnight in a desiccator over NaOH and then dissolved in water. The yield was 67% in terms of glycerol.

Analysis of the labelled product showed that the molar ratio of bound phosphate to primary hydroxyl group, as determined by periodate oxidation according to Burton (1957), was unity. On paper chromatography (Hübscher & Clark, 1960) the compound moved as a single spot with *R_f* identical with that of authentic DL-3-glycerophosphate. The specific activity was found to be 0.36 μ C/ μ mole.

Enzyme methods

Measurement of glyceride biosynthesis. The basic technique used was that described by Clark & Hübscher (1961), in which the amount of labelled palmitic acid incorporated into higher glycerides is determined. The incubation system contained, in final volume 3 ml., 11 mM-Sörensen phosphate buffer, pH 7.4, 6.6 mM-ATP, 0.033 mM-CoA, 20 mM-DL-3-glycerophosphate, 6.6 mM-MgCl₂, 6.6 mM-KF, 2.25 mM-

reduced glutathione, 0.8 mM-potassium palmitate (1 μ C/6 μ moles) and mitochondrial protein. In some experiments labelled palmitic acid and non-radioactive DL-3-glycerophosphate were replaced by non-radioactive palmitic acid and labelled L-3-glycerophosphate. After incubation for 40 min. at 37° the glyceride fraction was isolated and dissolved in phosphor. Its radioactivity was then determined with a Nuclear-Chicago three-channel liquid-scintillation counter. All experiments were done in duplicate and those in which the duplicate radioactive assays differed by more than 20% were disregarded and repeated. All values were corrected for the amount of radioactivity recovered in the glyceride fraction when the assay system was not incubated (zero-time control).

In some experiments, the nature of the glycerides formed was investigated. After its separation from other lipids by chromatography on alumina columns (Clark & Hübscher, 1961), the neutral lipid fraction was fractionated into tri-, di- and mono-glycerides by chromatography on silicic acid columns (Barron & Hanahan, 1958). Samples of the fractions eluted were tested for purity by thin-layer chromatography with light petroleum (b.p. 40–60°)-diethyl ether-acetic acid (60:40:1, by vol.) as the solvent (Mangold, 1965) and were shown to be pure. About two-thirds of the palmitic acid that was incorporated was recovered in the triglyceride fraction, and the remainder was mainly in the diglyceride fraction. The ratio of label in the tri- and di-glycerides did not change significantly when the supernatant fraction was added.

Acyl-CoA synthetase (EC 6.2.1.3). This enzyme was assayed essentially as described by Kornberg & Pricer (1953). The assay system contained, in final volume 1.5 ml., 6.6 mM-ATP, 2.6 mM-CoA, 4.0 mM-potassium palmitate, 2.3 mM-glutathione, 26 mM-MgCl₂, 11 mM-Sörensen phosphate buffer, pH 7.4, and 2 mg. of mitochondrial protein. After incubation for 20 min. at 37° the reaction was stopped by adding 2 ml. of a 7% (w/v) solution of HClO₄ and the amount of thio ester formed was determined as described below.

Phosphatidate phosphatase (EC 3.1.3.4). This enzyme was assayed as described by Coleman & Hübscher (1962), except that 15 μ moles of phosphatidate were present in the incubation system.

Diglyceride acyltransferase (EC 2.3.1.20). This was assayed by incubating, in final volume 1.5 ml., 0.5 mM-[¹⁴C]palmitoyl-CoA, 10 mM-1,2-diglyceride, 6 mM-MgCl₂, 2.5 mM-glutathione, 10 mM-Sörensen phosphate buffer, pH 7.4, and about 5 mg. of mitochondrial protein. The diglyceride was emulsified in water containing 0.1 mg. of Tween 20/ μ mole of diglyceride. After incubation at 37° for 45 min., the reaction was stopped by adding trichloroacetic acid to final concentration 5%. The extraction of the reaction products and the determination of the radioactivity was carried out as in the measurement of glyceride biosynthesis.

Analytical determinations

Protein, phosphate and nitrogen were determined as described previously (Hübscher, West & Brindley, 1965; Hawthorne & Hübscher, 1959). Carboxylic acid esters were determined as described by Stern & Shapiro (1953). Thio ester bonds of palmitoyl-CoA were determined by incubating samples of the thio ester for 30 min. with 0.5 ml. of

2M-hydroxylamine-HCl, pH 7.4, at 37°; 2 ml. of a 7% (w/v) solution of HClO₄ was added and the precipitated hydroxamic acid was collected by centrifugation and estimated as described by Kornberg & Pricer (1953).

CoA, ATP, CTP, GTP, UTP, glutathione, glycerokinase and bovine serum albumin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions of nucleoside triphosphates and of glutathione were adjusted to pH 7.4 by adding solid KHCO₃. DL-3-Glycerophosphate was purchased from Fluka A.-G., Buchs, Switzerland. Palmitic acid was bought from L. Light and Co. Ltd., Colnbrook, Bucks., and [1-¹⁴C]palmitic acid and [1-¹⁴C]glycerol were from The Radiochemical Centre, Amersham, Bucks. Solutions of potassium palmitate contained a 0.5 molar excess of KOH and were warmed to 60° before use.

Silicic acid (100 mesh) was obtained from Mallinckrodt Chemical Works, New York, N.Y., U.S.A., and alumina (100–200 mesh) from Hopkin and Williams Ltd., Chadwell Heath, Essex. Sephadex was purchased from Pharmacia, Uppsala, Sweden, and papain from the British Drug Houses Ltd., Poole, Dorset. Tween 20 was obtained from Honeywell and Stein Ltd., London, Dowex resin from the Dow Chemical Co., Midland, Mich., U.S.A., and Norit A from Pfanstiel Laboratories Inc., Waukegan, Ill., U.S.A. All other reagents were of A.R. purity.

RESULTS

Cofactor requirements of glyceride biosynthesis by rat-liver mitochondria. Previous work (Hübscher, Clark, Webb & Sherratt, 1963; Hübscher *et al.* 1964) has shown that the biosynthesis of glycerides by mitochondrial or microsomal fractions of rat liver is greatly enhanced by the addition of the supernatant fraction of the liver. Since a number of cofactors are known to be required for the biosynthesis of glycerides it was thought essential to determine the optimum concentrations of these, both in the absence and in the presence of the supernatant fraction. The results of such experiments should provide evidence on whether the stimulating activity of the supernatant fraction could be due to the presence in this fraction of any of the known cofactors of glyceride biosynthesis.

The results in Fig. 1 show that in the absence of the supernatant fraction CoA is not required and that optimum reaction rates are obtained with 6.6 mM-ATP, 6.0 mM-DL-3-glycerophosphate and 5.0 mM-magnesium chloride. In the presence of the supernatant fraction, the corresponding values are 6.6 mM, 13 mM and 6.6 mM. In the presence of the supernatant fraction, high concentrations of ATP, CoA or magnesium chloride brought about marked inhibitions. These results show that the stimulating effect of the supernatant fraction cannot be explained by the presence in this fraction of the cofactors tested or of the substrate, L-3-glycerophosphate.

In the absence of the supernatant fraction, or of ATP, DL-3-glycerophosphate or magnesium

Fig. 1. Effect of varying the concentration of ATP, CoA, DL-3-glycerophosphate and MgCl₂ on glyceride biosynthesis. The standard system contained, in final vol. 3 ml., 11 mM-Sörensen phosphate buffer, pH 7.4, 6.6 mM-ATP, 0.033 mM-CoA, 20 mM-DL-3-glycerophosphate, 6.6 mM-KF, 2.25 mM-reduced glutathione and 0.8 mM-palmitate (1 μC/6 μmoles). Incubations were carried out for 40 min. at 37°. The concentrations of ATP (a), CoA (b), DL-3-glycerophosphate (c) and MgCl₂ (d) were varied as indicated. ●, Mitochondrial protein (4 mg.); ▲, mitochondrial protein (4 mg.) + 0.45% saturated ammonium sulphate precipitate from the supernatant fraction (2 mg.).

chloride, very little glyceride synthesis occurred (Fig. 1). Nor has glyceride formation been observed when the supernatant fraction has been incubated in the absence of mitochondria. The requirement for CoA for glyceride synthesis when both mitochondria and the supernatant fraction are incubated is not absolute, however, presumably because of the endogenous CoA of mitochondria. By extrapolating the values shown in Fig. 1, the amount of endogenous CoA in the incubation mixture may be estimated to be approximately 45 μmoles, giving a true optimum concentration of CoA of approximately 145 μmoles/3 ml. Since almost 600 μmoles of palmitic acid were incorporated at this concentration, CoA probably played the role of an intermediate which turned over at least four times during the course of the reaction.

Although a fairly specific requirement for ATP as source of energy was observed in the presence of the supernatant fraction, the addition of a small

Table 1. *Effect of nucleoside triphosphates on glyceride biosynthesis*

The incubation system was that described in Fig. 1 except that the nucleoside triphosphates were added as indicated and 4 mg. of protein of the supernatant fraction was present.

Nucleotides added	Palmitic acid incorporated (m μ moles/40 min.)
ATP (6.6 mM)	298
ATP (8.0 mM)	272
ATP (6.6 mM) + CTP (1.4 mM)	402
ATP (6.6 mM) + UTP (1.4 mM)	264
ATP (6.6 mM) + GTP (1.4 mM)	328
CTP (1.4 mM)	57
UTP (1.4 mM)	44
GTP (1.4 mM)	20
ATP (1.4 mM)	123

amount of CTP to a system already containing optimum amounts of ATP caused a significant increase in the amount of palmitic acid incorporated (Table 1).

The effect of varying the concentration of mitochondrial protein or of the supernatant fraction is shown in Fig. 2(a). In the absence of the supernatant fraction, the amount of palmitic acid incorporated into glycerides was not proportional to the amount of mitochondrial protein present. The shape of the curve obtained suggested that there was either an inhibitor in one of the solutions used in the assay system, the inhibitor being progressively removed as more mitochondrial protein was added, or, alternatively, that the mitochondrial fraction, though it had been washed several times during preparation, contained a certain amount of a stimulating factor similar to that in the supernatant fraction.

The rate of incorporation of palmitic acid increased progressively with time, the reaction velocity after 1 hr. of incubation being about four times the initial reaction velocity (see Fig. 2b). This type of kinetic behaviour might be expected if there were a progressive removal or destruction of an inhibitor. Since between 12 and 32% of the palmitic acid added initially was esterified during the course of the reaction, its concentration changed significantly. If an excess of palmitic acid were inhibitory this might explain the kinetic behaviour mentioned above. However, when the concentration of palmitic acid in the system was varied, a concentration of 0.8 mM, which was the usual initial concentration, was not inhibitory. An alternative explanation may be that an autocatalytic process was involved and that a cofactor was being synthesized.

Some properties of the stimulating factor of the

Fig. 2. Effect of protein concentration and of time of incubation on glyceride biosynthesis. The standard incubation system was that described in Fig. 1. (a) The concentration of mitochondrial protein (●) or the concentration of a 0-45% saturated ammonium sulphate precipitate of the supernatant fraction in the presence of a constant amount of mitochondrial protein (4 mg.) (▲) was varied as indicated. Incubations were carried out for 40 min. at 37°. (b) The time of incubation was varied as indicated. ●, Mitochondrial protein (4 mg.); ▲, mitochondrial protein (4 mg.) + 0-45% saturated ammonium sulphate precipitate from the supernatant fraction (2 mg.).

supernatant fraction. Of the stimulating activity of the supernatant 85% was recovered in the fraction precipitated at 45% saturation with respect to ammonium sulphate, increases of specific activity (μ moles of palmitic acid incorporated/mg. of supernatant protein) of between three- and six-fold being obtained in different experiments. On a column of Sephadex G-200 the stimulating factor travelled as a relatively sharp band with the protein front, indicating molecular weight 200 000 or more (Fig. 3). The pooled middle fractions of the band had specific activity 1.15 μ moles of palmitic acid incorporated/hr./mg. of protein, giving an approximately twofold purification over the material applied.

Although these experiments suggested that the stimulating factor of the supernatant fraction was a protein, a more rigorous proof was sought by using a proteolytic enzyme. The stimulating activity of an ammonium sulphate precipitate (0-45% saturation) of the supernatant fraction was completely destroyed after incubation with papain (Table 2). Heat denaturation provided a further indication of the protein nature of the factor. Heating at various temperatures for 3 min. showed that almost all of the activity was retained at 50° but almost all of it was lost at 70° (Table 2).

Tzur & Shapiro (1964) reported on the stimulation of microsomal glyceride biosynthesis by

Fig. 3. Behaviour of the supernatant factor on Sephadex G-200. A 0-45%-saturated ammonium sulphate precipitate of the supernatant fraction was applied to a column of Sephadex G-200, 5 cm. $\times$ 18.5 cm., and 0.066M-Sörensen phosphate buffer, pH 7.4, was passed through the column at a flow rate of 13 ml./hr.; 8.3 ml. fractions were collected. O, Extinction at 280 m μ ; ●, enzymic activity (μ moles of palmitic acid incorporated/40 min./eluante fraction).

albumin and by various human lipoprotein fractions. Similar observations were obtained in the present investigation with mitochondrial preparations. However, although the stimulation of mitochondrial glyceride biosynthesis by the supernatant factor was constantly reproducible in at least 80 different preparations, the stimulation caused by bovine serum albumin was much smaller, varied a great deal with different mitochondrial preparations, and, in some of the experiments, was completely absent. To illustrate this point, the results in Table 3 may be quoted. In one experiment, bovine serum albumin brought about a sixfold stimulation of palmitic acid incorporation but in another experiment less than 1.5-fold stimulation was observed. Further, the effect of the stimulating factor in the liver supernatant was potentiated by bovine serum albumin in one experiment, whereas in another it was inhibited. No explanation can be given for these variations in the effect of bovine serum albumin. Interpretation is made difficult because bovine serum albumin is known to contain fatty acids and a radioisotope-dilution effect due to exchange between unlabelled fatty acids bound to albumin and labelled palmitic acid used as substrate may have taken place. This is suggested by the result of Expt. 2 (Table 3), where labelled palmitic acid and labelled L-3-glycerophosphate were used to determine the rate of formation of glycerides in parallel experiments. In the absence of albumin, the molar ratio of palmitic acid to glycerol incorporated was 2.5, whereas, in its presence, a value of 1.4 was obtained.

Table 2. *Effect of papain and of heating on the stimulation of glyceride biosynthesis by the supernatant fraction*

In Expt. 1, papain was incubated at 37° for 30 min. with 2.5-3.0 mg. of a 0-45%-saturated ammonium sulphate precipitate of the supernatant fraction (F-1) in the ratio 1 mg. of papain to 10 mg. of F-1 protein. Cysteine (4 mM) was also present. After incubation, the papain was inactivated with iodoacetamide (10 mM), the excess of iodoacetamide being removed by gel filtration with Sephadex G-25. In control I, 4 mg. of F-1 was incubated with cysteine alone. In control II, 4 mg. of F-1 was incubated as in control I and papain treated with iodoacetamide was added after incubation. In Expt. 2, 3.5 mg. of protein of the supernatant fraction (SF) was used. Palmitic acid incorporation was measured in the incubation system described in Fig. 1. Different mitochondrial and supernatant preparations were used in Expts. 1 and 2, but in each experiment between 4 and 5 mg. of mitochondrial protein was present. In Expts. 1 and 2, 100% refers to 560 and 158 μ moles respectively of palmitic acid incorporated/40 min.

Expt. no.	Additions	Relative amounts of palmitic acid incorporated (%)
1	F-1 not treated	100
	F-1 incubated with papain	3.5
	Control I	87.2
	Control II	72.6
	None	3.2
2	SF not treated	100
	SF heated for 3 min. at 50°	91.3
	SF heated for 3 min. at 70°	28.0
	SF heated for 3 min. at 100°	24.9
	None	24.7

Table 3. *Effect of bovine serum albumin and of the supernatant fraction on glyceride biosynthesis*

Palmitic acid or glycerol incorporation was measured in the incubation system described in Fig. 1. Mitochondrial protein (5mg.) was present and bovine serum albumin (30mg.), supernatant fraction (SF; 21mg. of protein) and the proteins of SF precipitated at 45% saturation with respect to ammonium sulphate (F-1; 2.7mg. of protein) were added as indicated.

Expt. no.	Additions	Glycerol incorporated (mμmoles/40 min.)	Palmitic acid incorporated (mμmoles/40 min.)
1	None	—	10.3
	SF	—	187.2
	Bovine serum albumin	—	67.0
	SF + bovine serum albumin	—	459.7
2	None	10.4	27.8
	F-1	103.2	257.4
	Bovine serum albumin	12.4	38.4
	F-1 + bovine serum albumin	115.2	162.0

Fig. 4. Effect of rat serum and of a lipoprotein-rich fraction from rat serum on glyceride biosynthesis. The incubation system was that described in Fig. 1. A 0-45%-saturated ammonium sulphate precipitate of the supernatant fraction (○), rat serum (●) or a lipoprotein-rich fraction from rat serum (▲) were added as indicated.

The effect of rat serum and of a lipoprotein-rich fraction from rat serum on glyceride biosynthesis is shown in Fig. 4. Although both preparations stimulated glyceride formation they were less effective than the supernatant factor. Low-density lipoprotein and high-density lipoprotein preparations from human serum also stimulated glyceride biosynthesis but were, at their respective optimum

concentrations, even less effective than the lipoprotein-rich fraction from rat serum. Only the high-density lipoprotein slightly accentuated the stimulation of palmitic acid incorporation obtained with the supernatant factor.

Possible function of the stimulating protein from the supernatant fraction. Though the individual enzymes participating in the biosynthesis of complex lipids are known to reside in particulate subcellular structures (Wilgram & Kennedy, 1963; Hübscher *et al.* 1963; Brindley & Hübscher, 1965), one or more of these enzymes could leak into the supernatant fraction during the preparation of the subcellular fractions. This (or these) enzyme(s) may then become the rate-limiting one(s) in glyceride biosynthesis by the mitochondrial multi-enzyme system. However, when acyl-CoA synthetase, phosphatidate phosphatase and diglyceride acyltransferase were each assayed in the absence and presence of the supernatant factor there was little difference in their activities (Table 4). The slight increase in activity observed in the assay of acyl-CoA synthetase and diglyceride acyltransferase in the presence of the supernatant factor could not account for the pronounced stimulation by this factor of glyceride biosynthesis from glycerophosphate and palmitic acid. Furthermore, the supernatant fraction contained only negligible amounts of these enzymes.

In view of the increased glyceride formation obtained with CTP (Table 1) it was thought possible that the stimulating protein might catalyse the formation of CDP-glycerol from CTP and glycerophosphate and that this might be a better precursor than glycerophosphate. From the results summarized in Table 5 it may be concluded that this is not so; in fact, glycerophosphate was a much better precursor than CDP-glycerol.

To be oxidized by mitochondria, long-chain fatty

Table 4. *Effect of the supernatant fraction on acyl-CoA-synthetase, phosphatidate-phosphatase and diglyceride-acyltransferase activities*

The assay systems for the enzymes are described in the Materials and Methods section. A 0-45%-saturated ammonium sulphate precipitate of the supernatant fraction (F-1) was added as indicated.

Enzyme tested	Expt. no.	Additions (mg. of protein)	Product formed (mμmoles/60 min.)
Acyl-CoA synthetase	1	None	1820
		F-1 (0.5)	2460
	2	None	1420
		F-1 (0.5)	1760
Phosphatidate phosphatase	3	None	320
		F-1 (1.8)	310
Diglyceride acyltransferase	4	None	25
		F-1 (3.5)	30

Table 5. *Effect of CDP-glycerol on glyceride biosynthesis*

The incubation system contained, in final volume 3.0 ml., 6.6 mm-ATP, 0.033 mm-CoA, 6.6 mm-MgCl₂, 6.6 mm-KF, 2 mm-reduced glutathione, 11 mm-Sørensen phosphate buffer, pH 7.4, 0.8 mm-[1-¹⁴C]palmitate (1 μC/6 μmoles) and 4 mg. of mitochondrial protein. Other additions were as indicated. F-1 is a precipitate of the supernatant fraction obtained after 45% saturation with ammonium sulphate.

Additions	Palmitic acid incorporated (mμmoles/40 min.)
None	0.7
CDP-glycerol (0.830 mm)	3.4
CDP-glycerol (4.15 mm)	5.1
CDP-glycerol (8.30 mm)	4.5
DL-3-Glycerophosphate (20 mm)	322.8
+ F-1 (4 mg.)	

acids are thought to be transported from the extra-mitochondrial to the intramitochondrial space by a carrier and acyl-carnitine has been proposed to fulfil such a carrier function (Bremer, 1962; Fritz & Yue, 1963). It seemed possible that fatty acids might also need to be carried into the mitochondrion before they could be esterified and this transport process might also involve acyl-carnitine. Although previous experiments (Hübscher *et al.* 1964) had suggested that the stimulating effect of the supernatant fraction could not be explained solely by assuming the presence in this fraction of carnitine or of enzymes catalysing the formation of acyl-carnitine, a more direct assessment of the role of carnitine in glyceride biosynthesis by mitochondria was obtained by comparing the formation of glycerides from palmitoylcarnitine with that from palmitoyl-CoA or free palmitic acid. The incor-

poration of palmitic acid from palmitoylcarnitine or palmitoyl-CoA was critically dependent on the concentrations used, high concentrations causing substrate inhibitions. These were less severe when bovine serum albumin was present and this protein was therefore included in the incubation system.

The results in Table 6 show that palmitoylcarnitine was not as effective a precursor as palmitoyl-CoA, which was, in turn, not as good a precursor as free palmitic acid in the presence of the supernatant fraction, ATP and CoA. However, in the absence of the supernatant factor, palmitoyl-CoA was a better precursor than palmitic acid.

Both the mitochondrial preparation and the supernatant fraction contained deacylases which reduced the effective concentrations of palmitoyl-CoA and palmitoylcarnitine by 50% and 16% respectively during the incubation period used for studying the incorporation of palmitic acid into glycerides from these precursors. When the amounts of palmitoyl-CoA and palmitoylcarnitine which were either deacylated or incorporated were allowed for, however, the effective concentrations of these substrates at the end of the incubation period were still higher than the maximum concentration of 0.05 mm-palmitoyl-CoA which could be formed when mitochondria were incubated with free palmitic acid, ATP, CoA and supernatant fraction (see discussion of results of Fig. 1).

DISCUSSION

Although the detailed function of the supernatant factor that stimulates glyceride biosynthesis by mitochondria is still not understood some of its properties can now be described and some possible functions can be excluded.

It has been shown that the factor is a protein molecule with molecular weight 200 000 or more, which is almost completely inactivated by heating

Table 6. *Palmitoylcarnitine, palmitoyl-CoA and palmitic acid as precursors of glycerides*

The incubation systems contained, in final volume 1.5 ml., 20 mM-DL-3-glycerophosphate, 6.6 mM-MgCl₂, 6.6 mM-KF, 2.5 mM-reduced glutathione and 11 mM-Sørensen phosphate buffer, pH 7.4. Palmitoylcarnitine (0.33 mM), palmitoyl-CoA (0.53 mM), palmitate (0.8 mM), CoA (0.033 mM), ATP (6.6 mM) and F-1 (3.1–8.6 mg. of protein) were present as indicated. F-1 is a precipitate of the supernatant fraction obtained after 45% saturation with ammonium sulphate. When palmitoylcarnitine or palmitoyl-CoA was used the assay system also contained 15 mg. of bovine serum albumin. n.d., Not determined.

Additions to basic system	Palmitic acid incorporated (μ moles/40 min.)		
	Expt. I	Expt. II	Expt. III
[¹⁴ C]Palmitoylcarnitine + CoA	32	18	n.d.
[¹⁴ C]Palmitoyl-CoA	185	205	212
[¹⁴ C]Palmitate + ATP + CoA	88	46	21
[¹⁴ C]Palmitate + ATP + CoA + F-1	367	256	296

at 70°. That it probably has a specific function is indicated by the observation that it cannot be replaced by serum albumin or by various lipoprotein preparations from human or rat serum. The requirement for ATP suggests that a lipase-catalysed exchange reaction between endogenous glycerides and labelled palmitic acid (Borgström, 1954) can be excluded. This view is supported by the finding that the incorporation of glycerophosphate and of palmitic acid are stimulated to similar extents (see Table 3).

The biosynthesis of glycerides by rat-liver mitochondria may be compared with their biosynthesis by the microsomal fraction from the mucosa of the small intestine. This subcellular fraction, too, requires the supernatant fraction for optimum synthesis of glycerides from glycerophosphate and palmitic acid, whereas synthesis from monoglycerides and palmitic acid does not show such a requirement (Hübscher *et al.* 1963, 1964). This suggests that the supernatant factor is likely to influence enzymic step(s) which are peculiar to the glycerophosphate pathway. Such a view would disfavour its possible function as a carrier of fatty acid, and indeed the experiments done with palmitoylcarnitine in the present study indicate that this type of carrier is of little, if any, importance in glyceride biosynthesis by liver mitochondria. There is thus the paradoxical situation that palmitoylcarnitine is an important intermediate in the series of reactions accompanying the uptake and oxidation of palmitic acid by mitochondria, whereas the uptake and esterification of palmitic acid does not seem to involve this intermediate. These contrasting observations could be reconciled if the oxidation of palmitic acid were to take place in the internal compartment of the mitochondrion and the esterification at its outer membrane.

The slight but significant stimulation of glyceride biosynthesis by CTP in the presence of optimum

amounts of ATP agrees with observations of Marinetti, Erbland & Brossard (1964) on homogenates of rat liver. Nevertheless, glycerophosphate was a better precursor of glyceride glycerol than CDP-glycerol.

When three individual enzymic steps of the glycerophosphate pathway for glyceride synthesis by mitochondria were tested none of them was sufficiently stimulated by the supernatant factor to explain its stimulating effect on the overall synthesis. Since the enzymes tested were acyl-CoA synthetase, phosphatidate phosphatase and diglyceride acyl-transferase, stimulation by the supernatant factor, if at all affecting an individual enzymic step, could only be concerned with the formation of phosphatidic acid from glycerophosphate and acyl-CoA. With the microsomal fraction of guinea-pig liver, Lands & Hart (1965) reported a stimulation of phosphatidic acid formation from lysophosphatidic acid and acyl-CoA by albumin and by a boiled microsomal fraction. The effect of the supernatant fraction was not tested.

The supernatant factor may, on the other hand, be concerned with the removal of triglycerides from the multi-enzyme complex where they are formed. Lipoproteins are carriers of glycerides and, although large amounts of free glycerides are found in the soluble compartment of the cell (Getz *et al.* 1961), the initial transport from their site of synthesis on intracellular membrane structures to the soluble cell compartment could be facilitated by a carrier lipoprotein.

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