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The Effect of Starvation on the Incorporation of Palmitate into Glycerides and Phospholipids of Rat Liver Homogenates

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1. Glyceride biosynthesis from glycerol phosphate and [1-^{14}C]palmitate was studied in liver homogenates of rats that were fed *ad libitum* or starved for 36–40hr. The changes in enzyme activity were related to total DNA content or total liver homogenate as these were found to be equivalent and to be the most meaningful parameters. 2. In liver homogenates from fed rats, labelled palmitate was incorporated mainly into phosphatidate (58% of the total incorporation into lipids), diglycerides (25%) and triglycerides (16%), whereas monoglycerides, cholesterol esters and phospholipids other than phosphatidate were labelled only to a small extent. Addition of particle-free supernatant to full homogenates increased the total incorporation of palmitate by 45% and the pattern of incorporation altered to 53% incorporated into triglycerides, 24% into diglycerides and 17% into phosphatidate. This result suggested that, in liver homogenates, phosphatidate phosphohydrolase (EC 3.1.3.4) may be rate-limiting in the biosynthesis of glycerides via the glycerol phosphate pathway. 3. Upon starvation, the amount of palmitate incorporated per liver into total phospholipids plus glycerides was decreased to between 68% and 75% of that observed with fed animals. In homogenates from fed animals 41–44% of the labelled phospholipids plus glycerides was in glycerides; this value increased to between 63% and 75% with starved rats. Of the palmitate incorporated into total phospholipids, between 85% and 86% was found in phosphatidate, independent of the nutritional state of the animal. The ratio of palmitate incorporated into triglycerides/diglycerides rose from 0·7, obtained with fed rats, to 1·0 with starved animals. 4. These results indicate that starvation caused a decrease in the activity (per total liver) of acyl-CoA–glycerol phosphate acyltransferase(s) (EC 2.3.1.15) and an increase in the activity of acyl-CoA–diglyceride acyltransferase (EC 2.3.1.20). The largest change, however, seemed to be related to the increased activity of the phosphatidate phosphohydrolase in the particle-free supernatant. 5. The latter enzyme was assayed in the particle-free supernatant with membrane-bound phosphatidate as substrate. In starvation, the activity per total liver was increased to between 130% and 190% and the specific activity to between 180% and 320% of the values for fed rats.

During starvation of rats there is an increased concentration of serum free fatty acids (Frederickson & Gordon, 1958) and a consequent increase in the uptake of free fatty acids by the liver (Fine & Williams, 1960; McElroy, Siefert & Spitzer, 1960; Aydin & Sokal, 1963). Further, there is an increase of free fatty acids in the liver (Foster, 1967), an elevated activity of hepatic acyl-CoA synthetase (EC 6.2.1.3) (Farstad, 1968) and a higher concentration of long-chain acyl-CoA in the liver (Bortz & Lynen, 1963; Tubbs & Garland, 1964). These changes might be expected to lead to increased biosynthesis of glycerides in the liver of rats and there are reports that the concentration of liver triglycerides rises during starvation (Harrison, 1953; Deuel, 1955). The extent of this increase depended on the duration of starvation (Harrison, 1953; Mayes, 1962) and on the sex of the animal (Harrison, 1953). However, there is also a report that starvation may lead to a lowered concentration of glycerides in rat liver (Altman, Hubbard & Gibson, 1965; Williams, Tamai & McIntosh, 1967).

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The concentration of liver triglycerides is not a direct indication of the rate of their biosynthesis, but kinetic studies *in vivo* gave some answers to this question. Within 5min. of intravenous injection into rats, labelled free palmitate disappeared almost completely from the blood (Göranson & Olivecrona, 1964) and was present in esterified form in tissue lipids, glycerides and phospholipids being almost equally labelled in the liver (Bragdon & Gordon, 1958; Laurell, 1959). It is therefore significant that 5min. after injection of labelled free palmitate, less of the label was incorporated into liver triglycerides and phospholipids of starved than of fed rats (Laurell, 1959; Göranson & Olivecrona, 1964; Schotz & Olivecrona, 1966). A decreased incorporation of fatty acids into liver lipids of starved rats was also observed with the perfused organ (Mayes & Felts, 1967) and slices (Vavrečka, Poledne & Petrašek, 1967).

These results point to a decreased rate of hepatic glyceride biosynthesis in starvation and it has been suggested (Tzur, Tal & Shapiro, 1964) that the lowered concentration of glycerol phosphate observed in the liver of starved rats (Rapoport, Leva & Guest, 1943; Bortz & Lynen, 1963; Tzur *et al.* 1964) is responsible for this. However, recent studies on the flux of carbon atoms from labelled glucose to glyceride glycerol (Denton & Randle, 1967; Denton & Halperin, 1968) throw doubt on this assumption.

The present study was undertaken to see if starved rats showed a change in the activity of the enzymes catalysing the biosynthesis of glycerides in the liver.

MATERIALS AND METHODS

Animals. Female rats of C.F.E. strain weighing between 180 and 240g. were used. They were purchased from Carworth Research Laboratories, Alconbury, Huntingdon. After arrival, they were kept for at least 2 weeks to adjust to partially reversed daylight conditions, in which the light period was from 10.30 to 00.30 hours. The rats were maintained on Spillers Autoclaved Laboratory Small Animals Diet (Spillers Ltd., Gainsborough, Lincs.) which was supplied *ad lib.*

For starvation, the rats were kept for 36–40hr. in metabolism cages and had free access to water. The starvation period was started between 18.00 and 19.00 hours.

Perfusion of rat liver. In some experiments, perfusion was performed to assess more accurately the changes in protein content of the liver. After killing the animal by a blow on the neck, a plastic cannula was inserted into the aorta just below the heart. Ice-cold saline (50–60ml.) was circulated from a syringe through the lower part of the animal's body and the right ventricle was cut open to allow the fluids to escape.

Preparation of phospholipids. Phosphatidate was prepared from egg phosphatidylcholine as described by Hübscher & Clark (1960). Total phospholipids were prepared from rat liver by chromatography on silicic acid according to Hanahan, Dittmer & Warashina (1957).

Preparation of homogenates and subcellular fractions. Liver homogenates (10%, w/v) were prepared in 0·3M-sucrose containing 15mM-tris–HCl, pH 7·4, and subcellular fractions were obtained as described by Smith & Hübscher (1966).

Incorporation of [^{14}C]palmitate into glycerides and phospholipids. The assay system was that described by Smith & Hübscher (1966), except that fluoride was omitted and the labelled palmitate was added as an albumin complex. Fluoride was omitted because it was shown (Smith, Sedgwick, Brindley & Hübscher, 1967) to inhibit the phosphatidate phosphohydrolase acting on membrane-bound phosphatidate, which is formed as an intermediate in the biosynthesis of glycerides. The albumin complex was prepared by the method of Hillyard, Cornelius & Chaikoff (1959) and contained (per ml.): 44mg. of bovine serum albumin (fraction V powder, fatty acid-poor, from Pentex Inc., Kankakee, Ill., U.S.A.), 4·8μmoles of [1-^{14}C]palmitate (1μc/6μmoles) and 0·48μmole of oleate. A portion (0·5ml.) of this complex was added to each 3ml. of assay system.

This assay system was originally devised for the study of mitochondrial glyceride biosynthesis (Smith & Hübscher, 1966), and in initial experiments with homogenates it was established that the same assay conditions were optimum. It was also established that the amount of palmitate incorporated was proportional to the amount of homogenate added (up to 7·5mg. of protein) and to the time of incubation (up to 40min. at 37°).

In routine assays, the incubation period was 20min. and 3–4mg. of protein was added. The energy-dependent incorporation of palmitate into glycerides and phospholipids was measured by subtracting the amount of palmitate incorporated in an assay system from which ATP and CoA were omitted.

The reaction was stopped by addition of 10ml. of methanol followed by 10ml. of chloroform. To the one-phase system was added 0·2ml. of chloroform containing 20mg. of olive oil, 1·5mg. of phosphatidate and 3·8mg. of total phospholipids from rat liver. The incubation tubes were kept for approx. 1hr. at room temperature and then 6ml. of 10mM-$CaCl_2$ soln. was added. After shaking and separation of the two phases, the lower phase was collected and evaporated to dryness under reduced pressure. The dry lipids were dissolved in 10ml. of chloroform.

Separation of reaction products was achieved by chromatography on silicic acid (Brindley, Smith, Sedgwick & Hübscher, 1967) and alumina (Clark & Hübscher, 1961). In some experiments, glycerides and phospholipids were further separated by t.l.c. For the separation of glycerides, the solvent system diethyl ether–*n*-hexane–acetic acid (20:78:2, by vol.) was used and for the separation of phospholipids oxalic acid containing silica gel G (Possmayer, Scherphof, Dubbelman, Van Golde & Van Deenen, 1969) with the solvent system recommended by Lands & Hart (1961).

Assay of soluble phosphatidate phosphohydrolase. The enzyme was assayed with mitochondrial membrane-bound phosphatidate as substrate essentially as described by Smith *et al.* (1967) but with the following modifications. The assay system contained, in a final vol. of 1ml., the following (final) concentrations: 10mM-sodium phosphate–potassium phosphate buffer, pH6·8; 5mg. of bovine serum albumin;

0·6–0·8mg. of particle-free supernatant; 1·6–2·5mg. of mitochondrial protein containing 250–360nmoles of phosphatidate. After incubation for 15min. at 37°, the reaction was stopped by addition of 3·75ml. of chloroform–methanol (1:2, v/v) followed by 1·25ml. of 10mM-$CaCl_2$ soln., 1·25ml. of chloroform and approximately 20mg. of olive oil. After separation and collection of the lower phase, the diglyceride formed was separated from unchanged phosphatidate by chromatography on alumina (Clark & Hübscher, 1961).

Other determinations. Protein and DNA were determined as described by Hübscher, West & Brindley (1965).

EXPERIMENTAL AND RESULTS

Incorporation of [^{14}C]*palmitate into lipids of liver homogenates of fed rats.* During initial experiments in which optimum conditions for the incorporation of palmitate into lipids were established, it was found that this incorporation depended almost completely on added ATP, whereas there was only a partial requirement for CoA and glycerol phosphate, presumably due to their endogenous presence. In the absence of ATP, the amount of palmitate incorporated into phospholipids and into glycerides was decreased to 8·5% and 6·5% respectively as compared with the complete assay system.

Table 1. *Distribution of labelled palmitate in individual classes of lipids*

Neutral lipids and total phospholipids were separated by t.l.c. (see the Materials and Methods section). Pooled portions of the reaction products from homogenates from four rats were analysed.

Compound	Palmitate incorporated (nmoles/20min.)	Palmitate incorporated into total lipids (%)
Triglyceride	77	15·9
Diglyceride	120	24·7
Monoglyceride	4	0·8
Cholesterol ester	2	0·4
Phosphatidate	242	49·9
Phosphatidylcholine	18	3·7
Other phospholipids	22	4·5

In the presence of optimum substrate and cofactor concentrations, palmitate was incorporated mainly into glycerides and phosphatidate (see Table 1). Phosphatidylcholine plus phospholipids other than phosphatidate were found to contain 8% of the palmitate incorporated into total lipids. This is of the same order as the energy-independent incorporation mentioned above. It is therefore possible that some of the palmitate found in phosphatidylcholine plus phospholipids other than phosphatidate was incorporated by a phospholipase-catalysed exchange reaction though an acylation of endogenous lysophospholipid or a new synthesis of some phosphatidylcholine owing to endogenous CDP-choline cannot be excluded.

The preponderance of labelled phosphatidate relative to labelled di- plus tri-glycerides suggested that phosphatidate phosphohydrolase may be rate-limiting in the formation of glycerides. This enzyme is present in the particle-free supernatant prepared from rat liver homogenates (Smith *et al.* 1967) and the effect of adding particle-free supernatant to full homogenates was therefore tested (see Table 2). It can be seen that addition of particle-free supernatant resulted in an increased incorporation of palmitate into higher glycerides and total lipids, whereas the amount of palmitate present in phosphatidate was decreased. This result would support the contention that, even in full homogenates, phosphatidate phosphohydrolase is rate-limiting although, in view of increased incorporation into total lipids, a further stimulating factor may have been added with the particle-free supernatant. It is likely that this factor affects acyl-CoA–diglyceride acyltransferase, as the addition of particle-free supernatant brought about an increase in the ratio of labelled palmitate recovered in triglyceride/diglyceride from 0·7 to almost 2, the incorporation into triglyceride being more than quadrupled.

Effect of starvation on the incorporation of [1-^{14}C]-*palmitate into lipids of liver homogenate.* In initial experiments the effect of starvation on body and liver weights, and on protein and DNA content of

Table 2. *Effect of particle-free supernatant on glyceride biosynthesis in liver homogenate*

Pooled portions of the reaction products from homogenates from four rats were analysed. The same preparation of particle-free supernatant was added to the four homogenates.

Additions (mg. of protein)	Palmitate incorporated (μmoles/liver/min.) Into monoglycerides	Into diglycerides	Into triglycerides	Into total phospholipids	Total
Full homogenate (3·5)	0·10	3·06	2·04	6·40	11·6
Full homogenate (3·5) + particle-free supernatant (9·5)	0·09	4·50	8·71	3·20	16·5

Table 3. *Effect of starvation for 36–40 hr. on body and liver weights and on protein and DNA content of perfused liver*

The homogenate was centrifuged at 104000*g* for 60 min. giving a total particulate fraction and a particle-free supernatant. The body weight of the starved group was (before starvation) 244 ± 6 g. The weight of the liver was taken from animals that were not perfused. All the results are expressed as mean ± S.D. of five rats.

	Fed rats (*a*)	Starved rats (*b*)	100 (*b*/*a*)
Body wt. (g.)	248 ± 6	215 ± 7	88·1
Wt. of liver (g.)	8·5 ± 0·6	5·8 ± 0·3	68·2
DNA (μmoles of DNA P/liver)	86·4 ± 4·6	89·7 ± 2·5	103·8
Protein (mg./liver):			
Full homogenate	1600 ± 154	1020 ± 51	63·8
Total particulate fraction	1060 ± 57	770 ± 73	72·6
Particle-free supernatant	540 ± 42	215 ± 16	39·8

Table 4. *Effect of starvation on the incorporation of palmitate into glycerides and phospholipids of liver homogenates*

Each group had four (Expt. 1) or five animals (Expt. 2). Results are quoted as means ± S.D.

		Palmitate incorporated (μmoles/min./total homogenate)	
Expt.		Fed rats	Starved rats
1	Glycerides	5·2 ± 0·6	6·5 ± 0·6
	Total phospholipids	6·4 ± 0·6	2·2 ± 0·5
	Glycerides plus phospholipids	11·6 ± 0·3	8·7 ± 1·0
2	Glycerides	4·4 ± 0·8	4·5 ± 0·2
	Total phospholipids	6·1 ± 0·8	2·7 ± 0·1
	Glycerides plus phospholipids	10·5 ± 0·6	7·2 ± 0·5

the liver, was measured (see Table 3). Only the DNA content of the liver remained unchanged during starvation. This result is in agreement with those of Harrison (1953) and of Herrera & Freinkel (1968). It was decided that the most meaningful parameter to which changes in enzyme activities should be related was the total DNA content of the liver and thus the total liver homogenate. These expressions would reflect enzyme changes per cell assuming that the DNA content per cell is constant (Leslie, 1965; Hotchkiss, 1965).

The results obtained on the incorporation of palmitate into the liver lipids of starved and fed rats are summarized in Table 4. There was a slight but significant decrease in the amount of palmitate incorporated into total lipids of homogenates from starved rats as compared with those from fed animals. Further, although the amount of palmitate incorporated into glycerides was unchanged or increased only slightly in starvation (103–126% as compared with fed rats), the amount of palmitate incorporated into phospholipids decreased significantly to between 34% and 44% of the values obtained with fed rats.

The distribution of label among the individual lipids was influenced only slightly by starvation. Over 80% of the palmitate incorporated into total phospholipids was in phosphatidate. The ratio of labelled palmitate in triglycerides/diglycerides increased in preparations from starved rats only slightly, from 0·7 to 1·0.

These results may be interpreted as indicating that in starvation there was a decrease in phosphatidate formation and an increased activity of phosphatidate phosphohydrolase and acyl-CoA–diglyceride acyltransferase. The first assumption was tested by studying the biosynthesis of phosphatidate in the presence of F^- ions. Phosphatidate phosphohydrolase activity and thus glyceride formation are known to be inhibited by F^- ions (Smith *et al.* 1967). In preliminary experiments with preparations from fed rats it was established that at 80 mM-sodium fluoride there was almost complete inhibition of phosphatidate phosphohydrolase activity, although the incorporation into total lipids remained unchanged (see also Table 5). In the presence of 80 mM-sodium fluoride, homogenates from starved rats showed, in comparison with homogenates from fed animals, a decreased incorporation into phospholipids, which represented

mainly labelled phosphatidate. This result supports the contention that one of the effects of starvation is to decrease the activity of acyl-CoA–glycerol phosphate acyltransferase(s).

The second assumption, that starvation resulted in an increased phosphatidate phosphohydrolase activity in the liver, was tested by assaying this enzyme, which is present in the particle-free supernatant, by using membrane-bound phosphatidate as substrate (see Table 6). After starvation, there was an increase in the activity of this enzyme per total liver and as the protein content of the particle-free supernatant decreased during starvation (see Table 3) the increase in specific activity of phosphatidate phosphohydrolase was even more pronounced. After refeeding (for 40hr.) of previously starved rats, the phosphatidate phosphohydrolase activity of the particle-free supernatant had returned to the value observed with fed animals.

DISCUSSION

The glycerol phosphate pathway represents the major pathway for the new synthesis of glycerides and phospholipids in the liver. However, it was recently shown that dihydroxyacetone phosphate may act as an alternative or competing acyl acceptor (Hajra, 1968). In the liver, the enzymes of the glycerol phosphate pathway are, with one exception, localized in particulate subcellular structures (Kornberg & Pricer, 1953; Wilgram & Kennedy, 1963; Smith & Hübscher, 1966; Shephard & Hübscher, 1969). Phosphatidate phosphohydrolase, when assayed with aqueous dispersions of phosphatidate as substrate, is localized in all subcellular fractions, the lysosome-rich fractions having the highest specific activity (Wilgram & Kennedy, 1963; Sedgwick & Hübscher, 1965). The occurrence of another phosphatidate phosphohydrolase was recently described. This enzyme is present in the particle-free supernatant and hydrolyses biosynthetically formed membrane-bound phosphatidate rather than aqueous dispersions of phosphatidate (Smith *et al.* 1967; Johnston, Rao, Lowe & Schwarz, 1967; Hübscher, Brindley, Smith & Sedgwick, 1967).

A change of enzyme activity with diet is regarded as one of the properties of a regulatory enzyme (Newsholme & Gevers, 1967). However, the activity of a non-regulatory enzyme may also be increased owing to an increase of overall flux of metabolites (Weber, Banerjee & Bronstein, 1961; Schimke, 1962; Newsholme & Gevers, 1967). As starvation and diabetes have many features in common as regards the metabolism of long-chain fatty acids, it is perhaps relevant that in the heart muscle of alloxan-diabetic rats, there is an increased flux of carbon atoms from glucose to glycerol phosphate and thence to glyceride glycerol, whereas the tissue concentration of glycerol phosphate is actually decreased (Denton & Randle, 1967). Furthermore, Denton & Halperin (1968), during an investigation into the control of glyceride biosynthesis in adipose tissue, came to the conclusion that the rate of biosynthesis could not be correlated with the tissue concentration of glycerol phosphate or of long-chain fatty acyl-CoA.

Table 5. *Effect of fluoride ions on the incorporation of palmitate into glycerides and phospholipids of liver homogenates from fed and starved rats*

Samples of pooled homogenates from two rats were assayed in each group.

	Concn. of NaF(mM)	Palmitate incorporated (μmoles/min./total homogenate): Glycerides	Phospholipids
Fed rats	0	3·79	5·44
	80	0·47	9·03
Starved rats	0	3·59	2·37
	80	0·48	3·90

Table 6. *Effect of starvation on the activity of phosphatidate phosphohydrolase in the particle-free supernatant*

Results are expressed as means ± S.D. of four rats.

Expt.		Phosphatidate hydrolysed: nmoles/min./mg. of protein	nmoles/min./total liver
1	Fed rats	2·20 ± 0·35	1180 ± 197
	Starved rats	7·08 ± 0·66	2490 ± 316
2	Fed rats	3·58 ± 0·48	2025 ± 350
	Starved rats	6·51 ± 0·91	2625 ± 515
	Starved and refed rats	3·75 ± 1·05	1960 ± 590

The present work was undertaken to investigate the possibility that the control of glyceride biosynthesis is mediated by changes of enzyme activity. Starvation of rats is reported to result in decreased glyceride biosynthesis in homogenates of the liver (Rubinstein & Rubinstein, 1966; Fallon & Kemp, 1968). In our experiments, optimum substrate and cofactor concentrations were employed so that the rate of incorporation of palmitate into lipids depended only on the activities of the enzymes involved. Under these conditions, glyceride biosynthesis was found to be unchanged or slightly increased after starvation for 36–40hr. and the difference between our results and those reported earlier (Rubinstein & Rubinstein, 1966; Fallon & Kemp, 1968) may well be explained by the different compositions of the assay systems used.

The decreased activity of acyl-CoA–glycerol phosphate acyltransferase(s) in the liver of starved rats that was observed in this investigation could explain why the formation of glycerides *in vivo* is not increased even though the amounts of acyl-CoA are raised in starvation. The rate of incorporation *in vitro* of palmitate into phosphatidate was almost ten times as high as the rate *in vivo* reported by Baker & Schotz (1967). The synthesis *in vitro* of acyl-CoA is usually greater than its subsequent utilization for esterification of glycerol phosphate (Brindley & Hübscher, 1966; Pande & Mead, 1968). A control of glyceride biosynthesis *in vivo* by the activity of acyl-CoA–glycerol phosphate acyltransferase could be expected only if this enzyme were rate-limiting. Whether this is so is not known, but experiments *in vitro* indicated that phosphatidate phosphohydrolase is more likely to be the rate-limiting enzyme (see Table 2). The increased activity of phosphatidate phosphohydrolase in the particle-free supernatant of the liver of starved rats is the most significant change observed in this investigation, but again it is not known if this enzyme is rate-limiting in the intact animal.

An unequivocal interpretation of the results obtained in this study with homogenates is difficult, as phosphatidate biosynthesis occurs in at least two subcellular compartments, i.e. the outer mitochondrial membrane and the microsomal fraction (Shephard & Hübscher, 1969), and possibly also in the particle-free supernatant (Rao, Wiegland & Reiser, 1969). The role of phosphatidate biosynthesis in each of the subcellular compartments in relation to the metabolism of the whole cell is probably not the same for all subcellular compartments. Consequently, the changes of enzyme activity observed after starvation should be further investigated by studying specific subcellular fractions.

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