Triacylglycerol Biosynthesis in Rat Adipose-Tissue Homogenates

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The optimum cofactor requirements for triacylglycerol biosynthesis in rat adipose-tissue homogenates containing mitochondrial, microsomal and cytosolic fractions were investigated. In general the optimum concentrations of cofactors for triacylglycerol biosynthesis were found to differ from those for total fatty acid esterification. The results provided further evidence for the key role of phosphatidate phosphohydrolase in the regulation of triacylglycerol biosynthesis. Albumin was included in the incubation medium to permit the use of concentrations of added fatty acids that would swamp the effects of endogenous fatty acids. The addition of albumin had little effect on the incorporation of palmitic acid and stearic acid into lipids including triacylglycerols. By contrast, a critical concentration of albumin (about 60 μ M) was required before incorporation of oleic acid or linoleic acid into triacylglycerols occurred. The system was used to study the incorporation of different 1- 14 C-labelled fatty acids from a mixture of unesterified fatty acids [palmitic acid 30%; stearic acid 10%; oleic acid 40%; linoleic acid 20% (molar percentages)] separately into the positions 1, 2 and 3 of triacyl-*sn*-glycerols. In general the stereospecific distribution of the labelled fatty acids incorporated into triacylglycerols paralleled the normal distribution of fatty acids within rat adipose-tissue triacylglycerols, suggesting that the specificities of the relevant acyltransferases have the major role in determining the positional distribution of fatty acids within triacylglycerols.

Separate acyltransferases are believed to control the esterification of the three distinct positions of the glycerol moiety during triacylglycerol biosynthesis by the *sn*-glycerol 3-phosphate pathway (Hübscher, 1970). The specificities of these enzymes probably play a major role in controlling the asymmetric distribution of fatty acids that is known to exist in rat adipose-tissue triacylglycerols (Brockerhoff *et al.*, 1966). Indeed, Stokes *et al.* (1975) have presented evidence that, with microsomal plus cytosolic preparations of adipose tissue from several species, the composition of position 2 of the triacyl-*sn*-glycerols may be controlled solely by the specificity of the appropriate acyltransferase. In pig adipose tissue, on the other hand, there appeared to be differences in the manner in which fatty acids synthesized *de novo* were esterified to the three positions of the triacylglycerol molecule in comparison with fatty acids from other sources (Christie & Moore, 1970). In lactating bovine mammary gland, the desaturation of stearic acid was coupled directly to the acylation of the primary positions of the *sn*-glycerols, possibly specifically to position 3 (Kinsella, 1972). In addition, Christie & Vernon (1975) used rat adipose-tissue slices to demonstrate that positional distributions of fatty acids in triacylglycerols were not controlled solely by the availability of fatty acids and the specificities of the acyltransferases. Other factors such as

the source of fatty acids were shown to be important, since oleic acid formed *de novo* in the tissue from stearic acid was esterified in a different manner from oleic acid added to the medium as such. In the present study, fat-free homogenates of rat adipose tissue, containing mitochondria, the microsomal fraction and cytosol, all of which are involved in triacylglycerol biosynthesis in this tissue (Roncari & Hollenberg, 1967), were used to study the incorporation of a number of different 1- 14 C-labelled fatty acids from mixtures of unesterified fatty acids of controlled composition separately into positions 1, 2 and 3 of rat adipose-tissue triacyl-*sn*-glycerols. In preliminary experiments, the cofactor requirements of fat-free homogenates of rat adipose tissue for triacylglycerol biosynthesis and the effects of albumin on the system were examined.

Materials and Methods

Animals and tissue

Parametrial adipose tissue from adult female Wistar rats, weighing 300–350 g, was used in all experiments.

Chemicals

The various cofactors used were from Sigma (London) Chemical Co. (Kingston-upon-Thames,

Surrey, U.K.) and all other chemicals were from BDH Chemicals (Poole, Dorset, U.K.). Labelled fatty acids were from The Radiochemical Centre (Amersham, Bucks, U.K.). Bovine serum albumin was defatted and then was dialysed by the procedure of Hanson & Ballard (1968).

Preparation of homogenates and incubation procedure

Parametrial adipose tissue from rats killed by cerebral fracture was homogenized in 0.15M-KCl (1:4, w/v) buffered with potassium phosphate buffer (pH 7.0, 46.5 mM) in a Potter-Elvehjem-type homogenizer with a Teflon pestle at 4°C. The cooled homogenate was centrifuged at 700g for 12 min, then the intermediate layer was aspirated for use as the enzyme source.

In the optimum system for triacylglycerol biosynthesis, the incubation medium contained the homogenate (1 ml), potassium phosphate buffer (pH 7.0, 93 μ mol in total), ATP (10 μ mol), Mg^{2+} (5 μ mol), CoA (0.12 μ mol), dithiothreitol (0.5 mg), *rac-sn*-glycerol 1-phosphate (25 μ mol), F^- (20 μ mol), KCl (0.25 mmol in total) and the labelled fatty acid, generally [1- ^{14}C]palmitic acid (0.1 μ Ci, 1.25 μ mol) bound to defatted bovine serum albumin (0.09 μ mol), in a final volume of 2 ml. Incubations were performed in duplicate with gentle agitation in a water bath at 37°C for 1 h.

In studies of the positional distribution of fatty acids esterified by the system, the fortified enzyme solution (2 ml final volume) was incubated with a mixture of free (i.e. unesterified) fatty acids (1.25 μ mol in total), consisting of palmitic acid (30 mol%), stearic acid (10 mol%), oleic acid (40 mol%) and linoleic acid (20 mol%) bound to bovine serum albumin (10 mg); the component carrying the 1- ^{14}C label (0.2 μ Ci) in each instance was varied. Control incubations with [1- ^{14}C]palmitic acid (1.25 μ mol, 0.2 μ Ci) alone were carried out simultaneously. Incubations were performed at 37°C in duplicate and the reaction was stopped by adding chloroform/methanol (2:1, v/v), and the lipid was extracted after 10, 20, 40 and 60 min intervals.

Analytical methods

Lipids were extracted by the method of Folch *et al.* (1957). A mixture of unlabelled dipalmitoylglycerol and tripalmitoylglycerol, palmitic acid and phosphatidylcholine (5 mg in total) was added to each sample during the extraction step to act as carriers. Lipids were separated into triacylglycerol, free fatty acid, diacylglycerol and phospholipid fractions by t.l.c. on silica gel G (0.5 mm thick); hexane/diethyl ether/formic acid (40:10:1, by vol.) was the developing solvent. Bands were detected under u.v. light after a light spraying with 0.1% 2',7'-dichlorofluorescein in 95% methanol. Fractions were counted for radioactivity after suspension of

the bands of adsorbent in Unisolve 1 (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) in a Tri-Carb 2425 liquid-scintillation spectrometer (Packard Instruments, Reading, Berks., U.K.). Recoveries from the plates were as a routine determined and were generally in the range 95–102%. The amounts of residual free fatty acids in the defatted and dialysed bovine serum albumin, and in the homogenate, after extraction as above, were determined by means of g.l.c. in the presence of heptadecanoic acid as the internal standard after separation by preparative t.l.c. (Christie, 1972). The protein content of the homogenate was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Stereospecific analysis of triacylglycerols

Triacylglycerols were isolated from the lipid extracts by preparative t.l.c. by using the procedure described above, with unlabelled rat adipose-tissue triacylglycerols added as carrier. The appropriate bands of adsorbent were placed in chromatography columns and were eluted with diethyl ether from which the triacylglycerols were recovered. The procedure for determining the relative proportions of 1- ^{14}C -labelled fatty acids incorporated into positions 1, 2 and 3 of triacyl-*sn*-glycerol has been described in detail (Christie & Hunter, 1973; Christie, 1975). The method was based on that devised by Brockerhoff (1965); tri[10,11- 3H]heptadecanoylglycerol of similar activity to the samples was added as internal standard. The triacylglycerols were partially hydrolysed by means of ethylmagnesium bromide, and the 1,2- and 2,3-diacyl-*sn*-glycerols formed were isolated and converted synthetically into phospholipids, which were in turn reacted with the stereospecific phospholipase A of snake venom. The *sn*-1-lysophosphatide and the fatty acids released from position *sn*-2 during the reaction, together with the unchanged *sn*-2,3-diacyl phosphatide, were isolated and the $^3H/^{14}C$ ratio in each was determined. As a control, 2-acyl-*sn*-glycerols, produced independently by pancreatic lipase hydrolysis, were isolated for analysis. The relative proportions of 1- ^{14}C -labelled fatty acids in the three positions were then calculated by relating the amount of ^{14}C in each position to the amount of 3H from the internal standard. With duplicate samples, results generally agreed within 5% (various checks on the analyses were possible).

Expression of results

Duplicate samples were obtained in all incubations and the mean result was used. Each experiment was performed twice with different rats and, when the results of separate experiments differed by more than 10%, it was performed a third time so that the s.e.m. of each result from three experiments was less than 5%. The molar proportions of free fatty acids endo-

genous to the enzyme solution and remaining in the albumin were determined. In calculating the amount of fatty acid incorporated, it was assumed that these were esterified at the same rate as the added labelled fatty acid, which was generally added to the medium in large excess.

Results

Cofactor requirements

In all experiments, the amounts of labelled fatty acid esterified to triacylglycerol, diacylglycerol and phospholipid, and any remaining labelled free fatty acid, were determined. No attempt was made to confirm that the phospholipid formed in each instance was indeed phosphatidic acid (1,2-diacyl-*sn*-glycerol 3-phosphate), as would be expected if triacylglycerol biosynthesis were by the *sn*-glycerol 3-phosphate pathway. In most studies with rat adipose-tissue homogenates (Steinberg *et al.*, 1961; Angel & Roncari, 1967; Daniel & Rubinstein, 1968; Jamdar & Fallon, 1973*a,b*; Stokes *et al.*, 1975), the incorporation of fatty acids into total lipid products (i.e. triacylglycerols plus diacylglycerols and phospholipids) was studied rather than into triacylglycerols specifically. In the present study, it was found that the optimum cofactor concentrations for triacylglycerol formation and for total esterification could differ appreciably; for example, the optimum concentrations of CoA or ATP for triacylglycerol biosynthesis were one-half those for total esterification (Fig. 1). On the other hand, the optimum concentrations of *sn*-glycerol 3-phosphate and Mg^{2+} were similar both for triacylglycerol biosynthesis and total esterification. Again the optimum fatty acid concentration for triacylglycerol biosynthesis was lower than that for total esterification. The initial free

fatty acid concentration used in most experiments (0.625 mM) was in fact slightly greater than the optimum concentration for triacylglycerol biosynthesis, but this was chosen to minimize the effect of residual free fatty acid in the homogenate and in association with the albumin (approx. 0.075 mM in total).

Effect of albumin

The effect of added albumin on the rate of esterification of [$1-^{14}C$]palmitic acid, [^{14}C]stearic acid, [^{14}C]oleic acid and [^{14}C]linoleic acid and on the nature of the products formed are shown in Fig. 2. When palmitic acid and stearic acid were the substrates, the rate of esterification was high in the absence of albumin. Indeed, addition of albumin caused increasing inhibition of palmitic acid incorporation into lipid in general and into triacylglycerol in particular. When stearic acid was the substrate, the effect of albumin was less marked, but there appeared to be optimum esterification with between 0.05 and 1.0 μ mol of albumin (25–50 μ M) in the medium. Phospholipids, rather than triacylglycerols, were the main product in this instance.

A very different effect was observed when unsaturated fatty acids were used as substrates. With oleic acid, some limited synthesis of phospholipid and diacylglycerol occurred in the absence or with low concentrations of albumin. No measurable triacylglycerol formation occurred until about 0.1 μ mol of albumin had been added to the system, i.e. 1 mol of albumin for 14 mol of fatty acids, when there was a very rapid onset of triacylglycerol production. The optimum rate of triacylglycerol biosynthesis occurred when the molar ratio of oleic acid to albumin was about 10:1, after which esterification tended to decrease. A similar effect was seen when linoleic acid was the substrate, since appreciable,

Fig. 1. Rate of incorporation of [$1-^{14}C$]palmitic acid (nmol esterified/min per mg of homogenate protein) into triacyl- and diacyl-glycerols, and phospholipids by rat adipose-tissue homogenates

Effect of: ATP concentration (a); CoA concentration (b); substrate free fatty acid concentration (c). Full incubation conditions and analytical methods are described in the Materials and Methods section. ●, Total lipid; ▲, triacylglycerols; ○, diacylglycerols; ■, phospholipids.

Fig. 2. Effect of varying the amount of albumin on the medium on the incorporation of 1-¹⁴C-labelled fatty acids (nmol esterified/min per mg of enzyme protein) into lipids

(a) Palmitic acid; (b) stearic acid; (c) oleic acid; (d) linoleic acid. ●, Total lipid; ▲, triacylglycerol; ○, diacylglycerol; ■, phospholipids (■ was used to represent both diacylglycerols and phospholipids when the points were too close together to be distinguished). Full incubation conditions and analytical methods are given in the Materials and Methods section.

if somewhat variable, amounts of diacylglycerols and phospholipids were formed in the absence of albumin. No detectable triacylglycerol synthesis occurred until the amount of albumin in the medium reached a critical value of 0.12 μmol (0.06 mM), when a rapid onset of triacylglycerol biosynthesis again took place. In fact the absolute concentration of albumin in the medium rather than the molar ratio of albumin to free fatty acid appeared to be relevant. When the experiments were repeated with one-half the initial concentration of each of the unsaturated fatty acids, analogous proportions of the various products were synthesized and the initiation of triacylglycerol synthesis occurred at exactly the same concentration of albumin in the medium as before. When equimolar amounts of [1-¹⁴C]palmitic acid and unlabelled oleic acid (1.25 μmol in total) were incubated with various concentrations of albumin, no synthesis of triacylglycerol occurred again until the critical concentration of albumin required when oleic acid alone was the substrate was reached.

Esterification of fatty acids from mixtures of controlled composition

The fatty acid mixture used as the substrate was chosen as representative in composition of the free fatty acids normally found in rat plasma or of the triacylglycerol of rat adipose tissue and was therefore similar to those fatty acids normally available to the tissue. Higher concentrations of free fatty acid were used than have been used by other workers to minimize the contributions of fatty acids from the homogenate itself and from the albumin and to ensure that some control could be exercised over the composition of the substrate.

The amounts of each fatty acid incorporated into triacyl- and diacyl-glycerols and phospholipids (presumably phosphatidic acid) when each was incubated as part of the fatty acid mixture relative to palmitic acid incubated on its own are illustrated in Fig. 3. Palmitic acid incubated as part of the fatty acid mixture was incorporated much more rapidly into

Fig. 3. Amount of incorporation of $1\text{-}^{14}\text{C}$ -labelled fatty acids ($\mu\text{mol esterified/mg of homogenate protein}$) into triacylglycerols (a), diacylglycerols (b) and phospholipids (c) by rat adipose-tissue homogenates as the time of incubation was varied

○, Palmitic acid only (control). As constituents of the fatty acid mixture: ■, palmitic acid; □, stearic acid; ●, oleic acid, Δ, linoleic acid. Full incubation conditions and analytical methods are described in the Materials and Methods section.

and phospholipids when it was incubated as part of the mixture, so that total esterification was only marginally higher. Oleic acid and linoleic acid in the mixture were also esterified much more rapidly than the palmitic acid control, and indeed greater amounts appeared to be incorporated into triacylglycerols in 60 min incubations than were found when the fatty acids were incubated on their own. Stearic acid was incorporated into lipids more slowly than the palmitic acid control. Triacylglycerols were again the main product, although increasing incorporation into phospholipids was observed as the reaction progressed. When stearic acid was incubated on its own with the enzyme preparation, phospholipid rather than triacylglycerol was the main product (see above).

Positional distribution of fatty acids

The relative proportions of each of the labelled fatty acids that were incorporated from the mixed substrate separately into positions 1, 2 and 3 of the *sn*-glycerol at various time-intervals were determined. A stereospecific analysis procedure was used for comparison with the natural distribution of the fatty acids in rat adipose-tissue triacylglycerols (Christie & Vernon, 1975). The results are in Table 1. Despite the high degree of disruption of the tissue and the fact that a complex range of enzymes was required for triacylglycerol biosynthesis, such differences as were found between the distributions obtained here and those that occurred naturally were comparatively minor. The distributions obtained after 10 min incubations were slightly anomalous, but analogous samples incubated for longer periods gave very similar results, which are therefore given as means. In triacylglycerols obtained from the longer (20–60 min) incubations, the relative proportions (not the absolute amounts, which would be dependent on the amount of substrate) of the two saturated fatty acids that were incorporated into each of the three positions were similar to each other. The same was true of the distributions of the two unsaturated fatty acids, although there were some differences in the natural distributions of these. Somewhat less stearic acid was incorporated into position *sn*-2 by the homogenate *in vitro*. Correspondingly more was found in position *sn*-3 than occurred naturally, and a higher proportion of linoleic acid was esterified in position *sn*-1 with correspondingly less in position *sn*-2 than was expected. In the triacylglycerols formed during the brief (10 min) incubations, higher proportions of the esterified palmitic acid and stearic acid were found in position *sn*-3, and of oleic acid and linoleic acid in position *sn*-1, than might have been anticipated from the natural distributions. Esterification of small amounts of unlabelled endogenous partially acylated intermediates may have influenced these results.

triacylglycerols than when it was incubated alone. At the same time, there were considerably lower accumulations of the intermediate diacylglycerols

Table 1. *Relative incorporation of ^{14}C -labelled fatty acids into positions 1, 2 and 3 of triacyl-sn-glycerols by rat adipose tissue homogenates*

Incubation conditions and analytical methods are described in the Materials and Methods section.

Fatty acid	Proportional distribution in each position (%)								
	Normal distribution*			Distribution after 10 min incubation			Distribution after 20, 40 and 60 min incubation†		
	sn-1	sn-2	sn-3	sn-1	sn-2	sn-3	sn-1	sn-2	sn-3
16:0	54.2±1.5	11.8±0.3	34.0±1.5	43.3	13.8	42.9	50.7±2.1	14.8±0.6	34.5±2.4
18:0	56.9±4.3	21.3±1.6	21.8±2.9	52.0	15.2	32.8	53.5±3.4	9.5±0.6	37.0±3.0
18:1	24.3±0.8	40.0±0.8	35.7±1.4	35.7	45.6	18.7	25.3±3.0	45.7±0.7	29.0±2.4
18:2	16.0±0.8	56.4±0.9	27.6±0.4	31.4	48.8	19.8	26.7±3.1	49.4±1.8	23.9±2.1

* Mean ± s.d. of three analyses of rat adipose-tissue triacylglycerols.

† Mean ± s.d. of duplicate samples from 20, 40 and 60 min incubations.

Discussion

The results of this study confirmed those of a number of other workers that fat-free homogenates of adipose tissue contained an active system for the synthesis of triacylglycerols by the *sn*-glycerol 3-phosphate pathway in which phosphatidic acid and diacylglycerol are the isolatable intermediates (Steinberg *et al.*, 1961; Roncari & Hollenberg, 1967; Angel & Roncari, 1967; Daniel & Rubinstein, 1968; Jamdar & Fallon, 1973*a,b*; Stokes *et al.*, 1975; Raju & Six, 1975). As cofactor concentrations were varied and triacylglycerol formation departed from the optimum value, increasing amounts of phosphatidic acid tended to accumulate, frequently in such quantities that there was relatively constant total esterification of palmitic acid. On the other hand, the amount of diacylglycerol formed never varied appreciably. It would thus appear that the activity of phosphatidate phosphohydrolase (EC 3.1.3.4) may have been rate-limiting for triacylglycerol biosynthesis in rat adipose-tissue homogenates. A similar conclusion was reached for triacylglycerol biosynthesis in rat liver cells by Lamb & Fallon (1974).

The observed effects of albumin on esterification of the various fatty acids by adipose-tissue homogenates were complex, and any explanation must be speculative. Bovine serum albumin can bind 6 mol of fatty acids at strong binding sites and over 60 mol at comparatively weak binding sites (Spector *et al.*, 1969). This lowers the effective concentration of free fatty acids in the medium and diminishes the potential inhibitory effects of unesterified fatty acids on any of the relevant enzymes. Similarly, it can decrease the concentration of CoA esters in the medium by decreasing the concentration of substrate fatty acids or by direct binding (Tubbs & Garland, 1964). The effects of different fatty acids and their acyl-CoA esters on rat adipose-tissue phosphatidate

phosphohydrolase do not appear to have been reported, but oleate and linoleate were found to be more effective inhibitors of the rat liver enzyme than palmitate and stearate (Sedgwick & Hübscher, 1967). If similar inhibition had occurred in adipose tissue, it might have been expected that greater amounts of phosphatidic acid would have accumulated in the incubations when oleic acid or linoleic acid was used as substrate unless there was inhibition of acyl-CoA synthetase (EC 6.2.1.3) as well. Palmitate did not inhibit rat adipose-tissue acyl-CoA synthetase at concentrations up to 4 mM (Daniel & Rubinstein, 1968), but the effects of other fatty acids were not studied. On the other hand, acyl-CoA synthetase of guinea-pig intestinal mucosal cells was inhibited by oleate and linoleate at concentrations greater than 0.25 mM, whereas palmitate and stearate had no inhibitory effects (Brindley & Hübscher, 1966), although the rat liver enzyme was inhibited by palmitoyl-CoA (Pande, 1973). These effects were very different in magnitude from those reported in the present paper, however.

It was known that unsaturated fatty acids stimulated the incorporation of palmitic acid into glycerols by rat liver mitochondria and cat intestinal mucosal microsomal fraction *in vitro* (Brindley *et al.*, 1967), and in the present study it was shown to be true of triacylglycerol biosynthesis in rat adipose-tissue homogenates. In this instance, also, the amount of phosphatidic acid that accumulated relative to triacylglycerol was diminished particularly when stearic acid was the substrate. It is possible that the physical properties, particularly the high melting points of tripalmitoylglycerol and tristearoylglycerol, which would be formed if palmitic acid and stearic acid respectively were the sole substrates for the reaction, were such that the formation of these compounds was not favoured. On the other hand, in the presence of unsaturated fatty acids, the products

would be expected to be the complex range of molecular species of triacylglycerols that are found normally in rat adipose tissue (Bugaut & Bezard, 1973).

When the degree of disruption of the tissue is considered, the proportions of the individual ^{14}C -labelled fatty acids that entered each of the three distinct positions of the glycerol molecules were remarkably similar to those distributions that were found naturally in rat adipose tissue. An indication that this might be so was obtained by Stokes *et al.* (1975), who demonstrated that microsomal plus cytosol preparations of adipose tissue from several species appeared to incorporate palmitic acid and oleic acid into position 2 of the triacyl-*sn*-glycerols in similar proportions to those pertaining naturally. No attempt was made in this instance to control or define the composition of the substrate unesterified fatty acids. On the other hand, Christie & Vernon (1975) showed that rat adipose-tissue slices did not always incorporate fatty acids *in vitro* into triacylglycerols in the proportions found naturally. For example, oleic acid formed *de novo* in the tissue by desaturation of stearic acid was esterified in a different manner from exogenous oleic acid. Stearic acid desaturation did not occur at a sufficient rate in the rat adipose-tissue homogenates, supplemented with NADH in addition to the other cofactors, for the phenomenon to be examined in this study, possibly because the high concentrations of unsaturated fatty acids in the medium were inhibitory (Ullman & Sprecher, 1971). It would also be worth while examining whether those fatty acids formed *de novo* in adipose tissue from acetate were incorporated into the three positions of the triacylglycerols in similar proportions to those fatty acids added preformed to the medium. Subject to this being determined, it appeared from this study that, in homogenates of rat adipose tissue, the positional distribution of the fatty acids was governed very largely by the specificities of the relevant acyltransferases.

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