Metabolism of the diacetyl derivatives of stereoisomeric monoacyl-*sn*-glycerols by rat adipocytes *in vitro*

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Diacetyl long-chain 1(3)- and 2-acyl-*sn*-glycerols containing either [9,10-³H]oleic acid or [1-¹⁴C]palmitic acid were synthesized by partial hydrolysis of the corresponding labelled triacylglycerols and acetylation. They were obtained in a high degree of stereochemical purity by preparative h.p.l.c. on a column containing a diol bonded phase. Each compound was rapidly metabolized by adipocyte preparations *in vitro*, and a high proportion of the label was recovered in the unesterified fatty acid and triacylglycerol fractions. Negligible amounts of intermediate products of hydrolysis were detected. Triacylglycerols were formed from [9,10-³H]oleic acid and from diacetyl-1(3)-[9,10-³H]oleoyl glycerol precursors at about the same rate, but the 2-isomer was metabolized rather more slowly. The results were consistent with the hypothesis that essentially complete hydrolysis occurred in the medium or at the plasma membrane, through the actions of lipoprotein lipase and monoacylglycerol lipase, and that subsequent esterification took place within the cell. To confirm that no putative intermediate monoacylglycerols were utilized for triacylglycerol biosynthesis via the monoacylglycerol pathway, the positional distributions of fatty acids in triacylglycerols from each substrate were determined. No positional selectivity was observed. It was concluded that monoacylglycerols, of an origin exogenous to the tissue, e.g. those derived from plasma triacylglycerols, were not utilized to a significant degree for triacylglycerol biosynthesis in adipose tissue. The diacetyl derivatives of monoacylglycerols may serve as useful stereochemical probes in studies of triacylglycerol biosynthesis via the monoacylglycerol pathway in other tissues.

INTRODUCTION

Triacylglycerols are rapidly hydrolysed by adipocyte preparations *in vitro*, because of the presence of the enzyme lipoprotein lipase at the plasma membrane (Nilsson-Ehle *et al.*, 1976; Spencer *et al.*, 1978), and the unesterified fatty acids released are utilized within the cell for triacylglycerol biosynthesis. Indeed, there is evidence that these fatty acids do not equilibrate with the extracellular pool prior to esterification (in mammary tissue) (Clegg, 1981), possibly because the lipoprotein lipase acts as a transacylase (Benoit & Boyer, 1983). The fate of any intermediate monoacylglycerols, which may be produced, is less clear. A monoacylglycerol lipase is known to be present in adipose tissue at the plasma membrane (Arnaud *et al.*, 1979), and this could release the fatty acid moieties for re-esterification in the cell. On the other hand, it appears to have been demonstrated unequivocally that the enzymes of the monoacylglycerol pathway are indeed present in adipose tissue (Schultz *et al.*, 1971; Schultz & Johnston, 1971; Dodds *et al.*, 1976a,b).

At present, there is no method that is free of major flaws for studying the monoacylglycerol pathway for the biosynthesis of triacylglycerols *in vitro*. For example, isotopically labelled monoacylglycerols cannot readily be used, as acyl migration occurs very rapidly (Thomas *et al.*, 1965). In addition, as monoacylglycerols are powerful detergents, they can cause disruption of cell membranes at concentrations approaching 0.25 mM (R. A. Clegg, personal communication). The analogous monoalkylglycerols have been used as substrates, and while their use can confirm the presence of the relevant enzymes, it cannot give any estimate of the relative

importance of the monoacylglycerol pathway, since ethers are not affected by the monoacylglycerol lipase at the plasma membrane. The ideal substrate for studying the monoacylglycerol pathway *in vitro* would be triacylglycerols with labelled fatty acids in specific positions of the glycerol moiety. However, it is not possible technically to distinguish between triacylglycerols used as substrates from those formed following hydrolysis and resynthesis in the tissue, because triacylglycerols and adipocytes have virtually identical flotation characteristics. For this reason, diacetyl derivatives of monoacylglycerols, having distinctive chromatographic properties, were synthesized and their value as substrates for hydrolysis and resynthesis in adipocytes *in vitro* has been examined.

MATERIALS AND METHODS

Animals and tissue

Parametrial adipose tissue from female Wistar rats, weighing from 200 to 220 g, was used in all experiments.

Chemicals

Insulin (25.3 i.u./mg), bovine serum albumin (defatted fraction V), all lipid standards, and phospholipase A (*O. hannah*) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) and all other chemicals were from Fisons (Loughborough, Leics., U.K.). All isotopically labelled lipids were obtained from Amersham International (Amersham, Bucks., U.K.). Bovine serum albumin was dialysed by the method of Hanson & Ballard (1968) before use.

Preparation of diacetyl derivatives of monoacylglycerols

Glycerol tri-[9,10-³H]oleate (200 mg, 1.85 MBq) was hydrolysed by means of pancreatic lipase for sufficient time for a high proportion of monoacylglycerols to be formed (Luddy *et al.*, 1964). To minimize acyl migration, the lipids were extracted immediately with cold diethyl ether/acetone (9:1, v/v) and the 2-monoacyl-*sn*-glycerols were isolated by preparative t.l.c. on silica gel (Kieselgel G; E. Merck, Darmstadt, Germany) layers impregnated with 10% (w/w) boric acid (four 20 cm × 20 cm plates with a layer 0.5 cm thick) (Thomas *et al.*, 1965); the developing solvent was hexane/diethyl ether (1:1, v/v). The required band was detected by spraying with 2',7'-dichlorofluorescein solution (Christie, 1982); it was extracted from the adsorbent with diethyl ether/acetone (9:1, v/v), and was acetylated immediately with acetic anhydride/pyridine (2 ml; 5:1, v/v) (Christie, 1982). The product was freed from major contaminants by column chromatography on Florisil (5 g), with diethyl ether as eluting solvent.

A crude 1(3)-[9,10-³H]oleoylglycerol preparation was obtained by heating the 2-monoacylglycerol, prepared by pancreatic lipase hydrolysis, at 80 °C for 2 h in toluene (5 ml) to cause acyl migration; the relatively pure compound was then isolated by boric acid t.l.c. as above and was acetylated immediately.

The diacetyl derivatives of 1- and 2-[1-¹⁴C]palmitoylglycerol and the corresponding unlabelled materials were prepared similarly.

Each of the products contained 5–10% (w/w) of the other isomer, and they were purified by means of h.p.l.c. A model 8770 (Spectra-Physics, St. Albans, Herts., U.K.) isocratic solvent delivery system was used together with a Knauer differential refractometer (Dr. H. Knauer, Oberursel/Taunus, Germany). The column (25 cm × 0.5 cm) was packed with Nucleosil 7-OH (HPLC Technology, Macclesfield, Cheshire, U.K.), and it was eluted with iso-octane/tetrahydrofuran (19:1, v/v) at a flow rate of 1.0 ml/min. Samples were applied to the column in portions of about 5 mg.

Preparation of adipocytes and incubation conditions

Adipocytes were prepared from parametrial adipose tissue, from rats killed by cervical dislocation, by the method of Rodbell (1964) as described by Fain (1975). They were suspended in Krebs–Ringer bicarbonate buffer (pH 7.4) (Krebs & Henseleit, 1932) with half the usual calcium concentration but containing 4% (w/v) albumin and maintained at 37 °C. All incubations were carried out under an atmosphere of O₂/CO₂ (19:1) in polythene bottles (30 ml capacity) sealed with Suba-Seal rubber stoppers (Gallenkamp, London, U.K.) at 37 °C in a thermostatically controlled room and were shaken continuously (2 Hz). The basic incubation medium contained 1 ml of adipocyte suspension (40–60 mg wet weight of adipocytes), Krebs–Ringer bicarbonate buffer, glucose (5 mM), insulin (20 munits/ml), albumin (40 mg/ml), bovine serum (0.5 ml) and the isotopically labelled lipid substrate (1 mM) in a total volume of 3 ml. The normal incubation time was 2 h. In some experiments, the volume of adipocytes or the substrate concentration or the time of incubation was varied, but the concentrations of all the other materials in the incubation medium were as specified above. Fatty acids (1 mM) were added in the form of a complex with albumin and, to

mimic the plasma unesterified fatty acid fraction, had the composition: palmitic acid, 30%; stearic acid, 10%; oleic acid, 40%; linoleic acid, 20%; only one of these fatty acids carried the label. The diacetyl derivatives of the monoacylglycerols were added to the medium in the form of an emulsion with glycerol, lecithin, and albumin, formulated and prepared by the method of Nilsson-Ehle & Schotz (1976). In some experiments, triacylglycerols (1 mM), isolated by preparative t.l.c. from rat adipose tissue lipids, were added with the labelled substrate in the form of an emulsion.

Analytical methods

Lipids were extracted from the tissues and separated by t.l.c. for analysis as described previously (Christie *et al.*, 1976); in incubations in which diacetyl derivatives of monoacylglycerols were included, it was necessary to use a more polar solvent to develop the t.l.c. plates, i.e. hexane/diethyl ether/formic acid (70:30:1, by vol.). Bands of lipids labelled with ¹⁴C were scraped into liquid-scintillation vials and were counted for radioactivity after suspension in scintillator 299 (Packard Instruments, Reading, Berks., U.K.). Lipids labelled with ³H were extracted from the adsorbent, simple lipids with diethyl ether and phospholipids with methanol, prior to counting in a conventional toluene scintillator (Packard Instruments). Unlabelled carrier lipids were added to facilitate detection and to ensure quantitative recoveries. The liquid-scintillation counter was a Packard Tricarb model 4530.

Stereospecific analysis of triacylglycerols

The procedure for the stereospecific analysis of triacylglycerols was as described earlier (Christie, 1982; Christie & Hunter, 1980), except that h.p.l.c. was used to separate the phospholipid products in the final stage of the analysis (Christie & Hunter, 1984), and to isolate monoacylglycerols formed by means of hydrolysis with pancreatic lipase (Christie, 1986). In experiments with ³H-labelled substrates, tri-[1-¹⁴C]palmitin was added as an internal standard, and in experiments with ¹⁴C-labelled substrates, tri-[9,10-³H]olein was used for the purpose (Christie & Hunter, 1973).

RESULTS

Substrates

As labelled diacetylmonoacylglycerols of defined stereochemistry were not available commercially, they were synthesized from triacylglycerols, which appeared to be the simplest precursors. 2-Monoacylglycerols were prepared relatively easily by means of pancreatic lipase hydrolysis, and they were acetylated by a standard procedure. Removal of a small amount of the 1(3)-isomer proved impossible by t.l.c., or by h.p.l.c. on a column containing silicic acid as an absorbent, or with one containing an octadecylsilyl bonded phase in the reversed-phase mode. However, it was found that the two isomers could be readily separated with a column packing consisting of diol groups bonded to an inert matrix, on a sufficient scale to be of preparative value, as shown in Fig. 1. 1(3)-Monoacylglycerols were prepared by isomerization of 2-monoacylglycerols, and the diacetates were also purified by preparative h.p.l.c.

Following incubation of labelled diacetylmonoacyl-

Fig. 1. Separation of the 1(3)- and 2-isomers of diacetylmonoacylglycerol by means of h.p.l.c. on a column of Nucleosil 70H with refractive index detection

See the Materials and methods section for further details.

glycerols with adipocytes, the lipids were extracted and the various lipid classes separated by means of t.l.c., so that the amount of incorporation of the label into each could be determined. The substrates migrated well behind the main products of hydrolysis (fatty acids) and resynthesis (triacylglycerols), and ahead of partial hydrolysis products or biosynthetic intermediates as shown in Fig. 2. In fact, no significant accumulation of putative monoacylmonoacylglycerol (which migrated between diacylglycerols and monoacylglycerols on t.l.c.) and monoacylglycerol intermediates was ever observed.

Effects of adipocyte and substrate concentrations

The results of an experiment in which diacetyl 1(3)- and 2-[9,10-³H]oleoylglycerols and [9,10-³H]oleic acid were each incubated with increasing amounts of the rat adipocyte preparation are shown in Fig. 3. Substrate unesterified fatty acids were rapidly taken up and esterified, with triacylglycerols as the main product, as expected. The diacetylmonoacylglycerols were also metabolized rapidly by the adipocyte preparations. With each isomer, an appreciable proportion of the label was recovered in the triacylglycerols and as fatty acids, with a small amount only as diacylglycerols and as phospholipids. The remaining label was in the unchanged

Fig. 2. Separation of the products of hydrolysis and resynthesis of diacetylmonoacylglycerol by means of t.l.c.

See the Materials and methods section for further details.

substrate. It seems improbable that any of these esterified substrates were taken up in an intact form into the adipocyte, but it was not possible to determine this experimentally, i.e. to distinguish between esterified lipid in the medium and that in the adipocytes. In contrast, it must be assumed that hydrolysis occurs largely at the plasma membrane of the adipocyte but also to some extent in the medium, because of release of the enzyme from the cell, and that most of the products of the reaction are present within the cell. Triacylglycerols were formed in similar amounts from fatty acids and diacetyl-1-oleoylglycerol, in a rectilinear manner until the medium contained 1 ml of adipocyte suspension in 3 ml total volume. Any monoacylglycerols formed transiently as intermediates could not have had any marked detergent effects on the cell membranes. A smaller proportion of the diacetyl-2-oleoylglycerols, relative to the other substrates, was metabolized, although the relationship between triacylglycerol formation and adipocyte volume was also rectilinear over the initial part of the range. Acetic acid would be released by the action of the lipases, but is of course water-soluble and would not be expected to interfere with the metabolism of long-chain fatty acyl derivatives.

The relationships between substrate concentration and product formation were determined, and they are illustrated in Fig. 4. With diacetyl-1(3)-[9,10-³H]oleoylglycerol as substrate, the amount of incorporation of the label into total products was related to substrate concentrations in a manner that was almost rectilinear over much of the range studied. A higher proportion of triacylglycerols were formed with unesterified fatty acids

Fig. 3. Effect of varying the volume of the adipocyte preparation on the metabolism of [9,10-³H]oleic acid and of the diacetyl 1(3)- and 2-[9,10-³H]oleoylglycerols

(a) Total metabolic products, i.e. free fatty acids (except when oleic acid was the substrate), triacylglycerols, diacylglycerols and phospholipids. (b) Free fatty acids formed. (c) Triacylglycerols formed. Substrates: □, oleic acid; ○, diacetyl-1(3)- and △, diacetyl-2-oleoylglycerols. The Materials and methods section contains details of the incubation conditions and analytical methods.

Fig. 4. Effect of varying the substrate concentration on the metabolism of [9,10-³H]oleic acid and of the diacetyl 1(3)- and 2-[9,10-³H]oleoylglycerols

(a) Total metabolic products, i.e. free fatty acids (except when oleic acid was the substrate), triacylglycerols, diacylglycerols and phospholipids. (b) Free fatty acids formed. (c) Triacylglycerols formed. Substrates: □, oleic acid; ○, diacetyl-1(3)- and △, diacetyl-2-oleoylglycerols. The Materials and methods section contains details of the incubation conditions and analytical methods.

as substrate than were formed from diacetyl-1-oleoylglycerols, while the diacetyl-2-oleoylglycerols were the poorest substrate for triacylglycerol formation. Similarly,

a higher proportion of the label was recovered in the fatty acid fraction in relation to precursor concentration with the diacetyl 1- than with the 2-oleoylglycerol substrate.

Fig. 5. Variation in the metabolism of [9,10-³H]oleic acid and of diacetyl 1(3)- and 2-[9,10-³H]oleoylglycerols with respect to time

(a) Total metabolic products, i.e. free fatty acids (except when oleic acid was the substrate), triacylglycerols, diacylglycerols and phospholipids. (b) Free fatty acids formed. (c) Triacylglycerols formed. Substrates: $\square$, oleic acid; $\circ$, diacetyl-1(3)- and $\triangle$, diacetyl-2-oleoylglycerols. The Materials and methods section contains details of the incubation conditions and analytical methods.

Time course of the reaction

The amounts of incorporation of labelled fatty acids, derived from [9,10-³H]oleic acid and diacetyl 1(3)- and 2-[9,10-³H]oleoylglycerols, into each of the products of hydrolysis/resynthesis by adipocytes *in vitro* with respect to time are shown in Fig. 5. Label was incorporated rapidly and at about the same rate into triacylglycerols from the free acid and the diacetyl-1(3)-oleoylglycerol substrates. On the other hand, the 2-oleoylglycerol derivatives were utilized for triacylglycerol biosynthesis at only about half this rate. Label accumulated rapidly in the free fatty acid fraction with the 1-oleoylglycerol derivative as substrate, but then fell with time, while there was a slower but steady accumulation from the 2-isomer.

It seems probable that the 1(3)-oleoylglycerol derivative is hydrolysed rapidly by lipoprotein lipase, and that uptake, into the cell, of the fatty acid released or the subsequent esterification is rate-limiting. The results are consistent with the hypothesis that the fatty acid of the 2-oleoylglycerol derivative must be released by the sequential action of lipoprotein lipase and monoacylglycerol lipase, and that the latter is the rate-limiting step. On the other hand, no appreciable accumulation of label in a monoacylglycerol intermediate was ever observed.

Positional distributions of fatty acids in triacyl-*sn*-glycerols

It appeared probable from the above results that the diacetyl-oleoylglycerols were largely hydrolysed, and that the oleic acid moiety was incorporated into triacylglycerols via its coenzyme A ester. The possibility that some could have been incorporated via a transient monoacylglycerol intermediate by the monoacylglycerol pathway for triacylglycerol biosynthesis could not be ruled out, however. Accordingly, the distributions of fatty acids in each of positions 1, 2 and 3 of the triacyl-*sn*-glycerols formed from [1-¹⁴C]palmitic acid, [9,10-³H]oleic acid, and the diacetyl 1(3)- and 2-monoacylglycerol derivatives

prepared from these compounds were determined. The results are shown in Table 1.

The free acid substrates each gave positional distributions similar to those found naturally or obtained in similar experiments (Henderson *et al.*, 1979). The results obtained for each position from incubations of the two isomeric diacetylacylglycerols (corresponding fatty acid derivatives) were indistinguishable from each other, and differed only a little from the free fatty acid incubations. The substrates were added to the medium on their own or in an emulsion together with purified rat adipose tissue triacylglycerols to ensure that a sufficiency of other fatty acids were present to permit normal esterification to occur, i.e. with formation of a wide range of molecular species. The results were not significantly different, presumably because there were enough fatty acids present of endogenous or exogenous origin to produce representative triacylglycerols, so these data are not listed. Results for triacylglycerols synthesized from the precursors containing [9,10-³H]oleic acid after incubations of 1 h and 3 h duration (results also not shown) were indistinguishable from those in Table 1. It is particularly noteworthy that the results for the distributions of fatty acids in position *sn*-2 were in remarkably good agreement, no matter whether the fatty acid originated in a primary position or in position *sn*-2 of the substrate. No significant proportion of the triacylglycerols (more than about 3%) could then have been synthesized via the monoacylglycerol pathway.

DISCUSSION

It has now been demonstrated that the diacetyl derivatives of stereoisomeric monoacyl-*sn*-glycerols are hydrolysed rapidly by adipocytes *in vitro*, presumably by lipoprotein lipase in the medium and in the plasma membrane, and by monoacylglycerol lipase in the plasma membrane. The fatty acids released are utilized for triacylglycerol biosynthesis within the cell.

Table 1. Relative incorporation of labelled fatty acids derived from diacetyl 1- and 2-acylglycerols and unesterified fatty acids into positions 1, 2 and 3 of triacyl-*sn*-glycerols by rat adipocytes

Incubation conditions and analytical methods are described in the Materials and methods section. Each labelled substrate was present in the medium at a concentration of 1 mM.

Substrate	Proportional distribution in each position (%)		
	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3
[1- ¹⁴ C]Palmitic acid*	50.2±5.2	21.5±3.1	28.3±6.2
Diacetyl-2-[1- ¹⁴ C]palmitoylglycerol*	44.2±3.3	26.8±2.7	29.0±3.3
Diacetyl-1(3)-[1- ¹⁴ C]palmitoylglycerol*	43.3±4.8	26.3±0.5	30.4±4.9
[9,10- ³ H]Oleic acid†	24.1±1.9	38.0±1.4	37.9±1.9
Diacetyl-2-[9,10- ³ H]oleoylglycerol†	28.5±3.1	37.2±0.3	34.3±2.8
Diacetyl-1(3)-[9,10- ³ H]oleoylglycerol†	31.1±2.7	37.3±2.6	31.6±2.6

* Mean±S.D. for four experiments.

† Mean±S.D. for three experiments.

It appears certain from this study that monoacylglycerols derived from plasma triacylglycerols by the action of lipoprotein lipase are not utilized as such by the adipocyte, in spite of the presence of the enzymes of the monoglyceride pathway in the tissue. This was perhaps also indicated from experiments in which triacylglycerols labelled in the glycerol moiety were utilized as substrates for adipocytes *in vitro*; recovery of glycerol from the medium was virtually quantitative, suggesting that little could have passed through the plasma membrane in any form (Scow *et al.*, 1977; Verine *et al.*, 1982). Similar conclusions were reached recently for rat mammary gland from structural comparisons of triacylglycerols in milk and plasma (Christie, 1985). A question remains, therefore, as to the physiological significance of the presence of the enzymes of the monoacylglycerol pathway to adipose tissue and other organs, assuming these are distinct from the glycerol phosphate acyltransferases. It is possible that they are involved in the recycling of monoacylglycerols, derived from partial hydrolysis of the triacylglycerols of the fat droplet within the tissue; there is, for example, some evidence that monoacylglycerols are produced in the brown adipose tissue of the hamster under certain conditions (Linck *et al.*, 1975). Dodds *et al.* (1976a) put forward a hypothesis of this kind to explain parallel changes in the activities of monoacylglycerol acyltransferase and glycerol phosphate acyltransferase in rat adipose tissue under altered physiological conditions.

Diacetyl 1(3)- and 2-acyl-*sn*-glycerols make excellent stereochemical probes to assess the physiological significance of the monoacylglycerol pathway in tissues, and may be of value in determining the structural specificities of the various enzymes involved, especially in studies of triacylglycerol biosynthesis in mammary gland and intestinal tissue.

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