Stereospecific distribution of palmitic acid in the triacylglycerols of rat adipocytes

Effects of varying the composition of the substrate fatty acid *in vitro*

William W. CHRISTIE and Margaret L. HUNTER
The Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

(Received 6 May 1980/Accepted 8 July 1980)

The effects of inclusion of different fatty acids in the medium on the rate of esterification of palmitic acid and its stereospecific distribution among the three positions of the triacyl-*sn*-glycerols by preparations of rat adipocytes *in vitro* have been determined. Myristic acid, stearic acid, oleic acid and linoleic acid were used as diluents and the concentration of the combined unesterified fatty acids in the medium was held constant; only the proportion of palmitic acid was varied. The amount of palmitic acid esterified was always linearly related to its relative concentration in the medium and was not significantly affected by the nature of the diluent fatty acid chosen. Constant relative proportions were recovered in triacylglycerols and in intermediates in each instance. The amount of palmitic acid esterified to each of the positions of the triacyl-*sn*-glycerols was linearly dependent on the relative proportion in the medium but the nature of the relationship was markedly influenced by which fatty acid was present. When stearic acid was present, simple relationships were found over the whole range tested. When either myristic acid, oleic acid or linoleic acid was present, abrupt changes in the manner of esterification of palmitic acid were observed in position *sn*-1 when the relative concentrations of palmitic acid and the diluent reached critical values, which differed with each fatty acid. In position *sn*-2 when oleic acid or linoleic acid was present, a similar change was observed, and in position *sn*-3 it was obtained with myristic acid as diluent. The results are discussed in terms of changes in the relative affinities of the acyltransferases for palmitic acid. Palmitic acid was esterified into various molecular species in proportions that indicated acylation with non-correlative specificity at higher relative concentrations but not at lower.

Separate acyltransferases are known to control the esterification of the three distinct positions of the glycerol moiety during triacyl-*sn*-glycerol 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 known to exist in most natural triacyl-*sn*-glycerols (Breckenridge, 1978). This specificity can be expressed by the enzymes in two ways: by selection of particular fatty acids as substrates for esterification, or by selection of partially-acylated intermediates with particular esterified acyl moieties as acceptors for the substrate fatty acids. In intact cells, some apparent specificity can also be introduced as a consequence of compartmentation of the relevant enzymes within particular organelles. For example, Henderson *et al.* (1979*a,b*) demonstrated that fatty acids synthesized

de novo within the cell were esterified in a different manner from those fatty acids of exogenous origin by rat adipocytes *in vitro*. These observations were interpreted in terms of selective esterification either at the plasma membrane or at the endoplasmic reticulum.

The manner of esterification of a given fatty acid to the various positions of triacyl-*sn*-glycerols must depend to some extent on its concentration relative to that of the other fatty acid components in the medium. For example, when [1-¹⁴C]palmitic acid alone was incubated with rat adipocytes *in vitro*, it was recovered in each of the positions in approximately equal proportions (Henderson *et al.*, 1979*b*). In similar circumstances, a very high proportion of the label (approx. 60%) was found in the tri-saturated molecular species (Christie *et al.*, 1979), although this normally amounts to only 2% of the

triacylglycerols (Bezard & Bugaut, 1972; Bugaut & Bezard, 1973). On the other hand, when palmitic acid was incubated as part of a mixture, which resembled the unesterified fatty acid fraction of rat plasma in composition, its distribution in the triacyl-*sn*-glycerols (*sn*-1/*sn*-2/*sn*-3 ratio, 53:14:33) resembled the natural distributions (Henderson *et al.*, 1979b). The acyltransferases involved in the biosynthesis of triacylglycerols in adipose tissue can, therefore, make use of fatty acids, varying widely in composition, as substrates. In experiments in which the composition of the triacylglycerols in the adipose tissue of pigs and of rabbits was varied by dietary means in a limited way, the relative distributions of the fatty acid among the three positions appeared to bear simple relationships to their overall concentrations in the tissue (Christie & Moore, 1970; Christie *et al.*, 1974). In the present study, the effects of systematically varying the nature and the composition of the combined substrate fatty acids on the manner of esterification of palmitic acid specifically into triacyl-*sn*-glycerols by rat adipocytes *in vitro* have been examined.

Materials and methods

Animals and tissue

Parametrial adipose tissue from female Wistar rats, weighing 180–220 g, was used in all experiments.

Chemicals

Insulin (25.3 i.u./mg), bovine serum albumin (defatted fraction V) and phospholipase A (*O. hannah*) were purchased from Sigma (London) Chemical Co. (Poole, Dorset, U.K.) and all other chemicals were from BDH Chemicals (Poole, Dorset, U.K.). [$1\text{-}^{14}\text{C}$]Palmitic acid was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Bovine serum albumin was dialysed by the method of Hanson & Ballard (1968) before use.

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 shaking water bath (2 cycles/s). The basic incubation system contained 1 ml of

adipocyte suspension (40–60 mg wet wt. of adipocytes), Krebs–Ringer bicarbonate buffer, glucose (5 mM), insulin (20 m-units/ml), albumin (40 mg/ml) and the fatty acid substrate (1 mM) in a total volume of 3 ml. The isotopically labelled substrate was [$1\text{-}^{14}\text{C}$]palmitic acid (0.3 μCi) and various unlabelled fatty acids were added to this as indicated in the text; they were added to the incubation medium as the potassium salts bound to albumin. All incubations were of 60 min duration.

Analytical methods

Procedures for the extraction of lipids, the separation of lipid classes for liquid-scintillation counting, and for determining the amounts of residual unesterified fatty acids in the defatted albumin and adipocytes have been described previously (Christie *et al.*, 1976).

Stereospecific analysis of triacylglycerols

Triacylglycerols were isolated from the lipid extracts by preparative t.l.c. (Christie *et al.*, 1976). The procedure for determining the relative proportions of [$1\text{-}^{14}\text{C}$]palmitic acid incorporated into positions 1, 2 and 3 of the triacyl-*sn*-glycerols is based on that devised by Brockerhoff (1965), but tri[10,11- ^3H]heptadecanoylglycerol of similar specific radioactivity to the samples was added as internal standard (Christie & Hunter, 1973; Christie, 1975). The triacylglycerols were partially hydrolysed by means of ethylmagnesium bromide (Christie & Moore, 1969) and the 1,2- and 2,3-diacyl-*sn*-glycerols formed were isolated and converted synthetically into phosphatidylcholines (Myher & Kuksis, 1979), which were in turn reacted with the stereospecific phospholipase A of snake venom.

The *sn*-1-lysophosphatidylcholine and the fatty acids released from position *sn*-2 during the reaction, together with unchanged *sn*-2,3-diacylphosphatidylcholine, were isolated by column chromatography on carboxymethylcellulose as adsorbent as suggested by Comfurius & Zwaal (1977). Pre-swollen CM-cellulose (CM52, Na⁺ form, Whatman) was converted into the acetate form as described by Rouser *et al.* (1969) for DEAE-cellulose and was stored in chloroform until required. The adsorbent (about 1 g) was packed in chloroform into a disposable Pasteur pipette, which served as the chromatography column. The products of the stereospecific analysis reaction were applied in chloroform; unesterified fatty acids were eluted with chloroform (10 ml), unchanged 2,3-diacylphosphatidylcholine was eluted with chloroform/methanol (97.5:2.5, v/v; 10 ml) and lysophosphatidylcholine with methanol (10 ml). The separation was effected in about 30 min, so was more rapid, used less polar

solvents and gave better recoveries than conventional preparative t.l.c. methods.

The phospholipid products of the reaction were methylated and the $^{14}\text{C}/^3\text{H}$ ratio in each was determined so that the proportion of the $[1-^{14}\text{C}]$ palmitic acid in each position could be calculated (Christie, 1975; Christie & Hunter, 1973; Christie *et al.*, 1976).

Silver nitrate chromatography

The amount of substrate incorporated into molecular species of triacylglycerols, separated according to degree of unsaturation, was determined after t.l.c. on silver nitrate-impregnated layers (Christie *et al.*, 1979).

Results

To assess the effects of different fatty acids on the esterification of palmitic acid, adipocytes were incubated *in vitro* with $[1-^{14}\text{C}]$ palmitic acid alone or diluted with various amounts (10–90 mol% in 10 mol% steps) of either myristic acid, stearic acid, oleic acid or linoleic acid (i.e. those most abundant in rat adipose tissue); the initial overall unesterified fatty acid content of the medium or the potential substrate concentration of the medium remained constant. The concentration chosen (1 mM) was within the normal physiological range for the unesterified fatty acid fraction of rat plasma (0.3–1.7 mM) (Scow & Chernick, 1970). The defatted bovine serum albumin and the adipocytes contained residual unesterified fatty acids (approx. 0.2 mM) and the amount and compositions of these were determined so that the true palmitic acid concentration could be used in calculations.

Effect on rate of esterification

The amount of the substrate $[1-^{14}\text{C}]$ palmitic acid esterified was proportionally related to its initial concentration in the medium, or to its relative proportion in the total unesterified fatty acid fraction, with each of the fatty acid diluents tested. For example, the amount esterified as the proportion was varied when myristic acid was the diluent is illustrated in Fig. 1. Corresponding points with different fatty acid diluents were not significantly different statistically ($P < 0.05$). With each of the fatty acids at each concentration, between 60 and 75% of the label was recovered in the triacylglycerol fraction, 10–12% of the label was in the diacylglycerol fraction and approx. 1% of the label was in the phospholipid fraction in each instance. When stearic acid and myristic acid were the diluents, there appeared to be a slight decrease in the proportion of the label recovered in the triacylglycerol fraction as the proportion of palmitic acid in the medium was increased, whereas the

Fig. 1. Incorporation of $[1-^{14}\text{C}]$ palmitic acid ($\mu\text{mol}/100 \text{ mg of lipid per h}$) into lipids by rat adipocytes *in vitro* as the proportion of palmitic acid relative to myristic acid in the substrate was varied

Results are means $\pm$ S.D. for three separate experiments.

opposite was indicated when oleic acid and linoleic acid were the diluents, but the trends were not statistically significant.

Effect on the positional distributions of palmitic acid in the triacylglycerols

The triacyl-*sn*-glycerols were isolated and subjected to stereospecific analysis to determine the relative proportion of the $[1-^{14}\text{C}]$ palmitic acid esterified to positions 1, 2 and 3 in each instance. It has previously been shown that the proportionate distributions did not vary with the time of incubation *in vitro* for between 50 and 130 min incubations (Henderson *et al.*, 1979b). From these results, the molar proportion of palmitic acid esterified to each of the positions could be calculated as the proportion in the medium initially was varied relative to that of each of the diluent fatty acids. Each experiment was carried out at least three times and standard deviations were generally within 5% of the mean values. Although only the palmitic acid distribution was measured directly, it can be assumed that much of the remaining fatty acid in each position is made up of that chosen as the diluent, though some of the endogenous unesterified fatty acid fraction will also be esterified.

The results obtained with myristic acid, stearic acid, oleic acid or linoleic acid as the diluent are illustrated in Fig. 2. These acids affected the esterification of palmitic acid within the triacylglycerols in very different ways. In the presence of

Fig. 2. Incorporation of $[1-^{14}\text{C}]$ palmitic acid (proportion in mol %) into each position of the triacyl-*sn*-glycerols by rat adipocytes *in vitro* as the proportion of palmitic acid relative to myristic acid (a), stearic acid (b), oleic acid (c) or linoleic acid (d) in the substrate was varied

Results are means for three separate experiments. Symbols: ●, position *sn*-1; ■, position *sn*-2; ▲, position *sn*-3.

myristic acid over much of the range of palmitic acid concentrations tested, the highest proportion of palmitic acid was found in position *sn*-1, with somewhat less in position *sn*-2 and least in position *sn*-3, except at the higher palmitic acid concentrations, when the proportion in this position was greater than in the other two and indeed approached 100% of the total fatty acids in the position. As the relative proportion of palmitic acid in the medium was increased, the proportion of palmitate of the total fatty acids entering position *sn*-1 increased linearly until palmitic acid comprised 66% of the substrate when the slope of the line changed abruptly. The slopes of the lines must be a function of the relative affinities of *sn*-glycerol 3-phosphate 1-acyl-transferase for palmitic acid and for myristic acid. When the proportion of palmitic acid in the medium

reached values twice that of myristic acid or greater, the affinity of the enzyme for palmitic acid relative to myristic acid was decreased to one-fifth of the original value (using the values for the slopes obtained from the regression equations). In position *sn*-2, the proportion of palmitic acid of the total fatty acids esterified to this position was linearly related to the substrate concentration over the range tested, i.e. the affinity of 1-acyl-*sn*-glycerol 3-phosphate acyl-transferase for palmitic acid relative to myristic acid did not change. On the other hand, with position *sn*-3, the amount of palmitic acid esterified was again proportionally related to the substrate concentration until the ratio of palmitic acid to myristic acid was two to one when the slope of the line changed abruptly once more. In this instance, the relative affinity of the relevant enzyme, i.e. 1,2-diacyl-

sn-glycerol acyltransferase, for palmitic acid relative to myristic acid, calculated from the slopes of the lines, increased by a factor of two.

When stearic acid was the diluent fatty acid, the highest proportion of the palmitic acid esterified was again found in position *sn*-1, though only slightly less was found in each of positions *sn*-2 and *sn*-3, the relative importance varying with the substrate concentration. In each position, the relative proportion of palmitic acid of the total fatty acids esterified was linearly related to its concentration in the unesterified fatty acids in the medium at all concentrations tested. It appeared that in this instance, the affinities of each of the three enzymes involved in acylation for palmitic acid did not change with relative concentration.

With both oleic acid and linoleic acid as diluents, position *sn*-1 tended to contain the highest proportion of esterified palmitic acid, with appreciable amounts in position *sn*-3 but least in position *sn*-2. When the proportion of palmitic acid was 30–40% of the substrate, the relative proportion found in each position was close to the natural distribution in the triacylglycerols of rat adipose tissue (Christie & Vernon, 1975; Henderson *et al.*, 1979b). In the presence of oleic acid, the proportions of palmitic acid of the total fatty acids esterified to positions *sn*-1 and *sn*-2 were linearly related to the proportions in the medium again, until this reached approx. 40% of the total. There was then a sharp decrease in the rate of esterification of position *sn*-1 with palmitic acid and a corresponding increase in that of position *sn*-2; in position *sn*-3, the amount of palmitic acid esterified was linearly related to that in medium over most of the range tested. When palmitic acid comprised 80% of the fatty acids present, position *sn*-1 was fully occupied with this component and further additions were esterified almost entirely to position *sn*-2. When linoleic acid was present in the medium, palmitic acid was esterified to positions *sn*-1, *sn*-2 and *sn*-3 in similar proportions to those obtained when oleic acid was the diluent. Sharp changes in the manner of esterification of palmitic acid in positions *sn*-1 and *sn*-2 occurred when linoleic acid comprised 50% of the substrate; the amount esterified was still linearly related to the concentration in the medium but the slopes of the lines differed considerably from those obtained when oleic acid was present. The proportion of palmitic acid of the total fatty acids esterified to position *sn*-3 was directly proportional to the amount in the medium over the entire range tested.

It is also noteworthy that in position *sn*-1 of the triacylglycerols, at substrate concentrations of palmitic acid below the critical concentrations with each of the diluent fatty acids tested, the straight lines obtained on plotting the proportion esterified versus that in the medium were parallel to each

Table 1. Proportions of $[1-^{14}\text{C}]$ palmitic acid found in the more saturated molecular species of the triacylglycerols after incubation of the labelled substrate, diluted with various proportions of unlabelled oleic acid, with rat adipocytes *in vitro*

See the Materials and methods section for experimental details. The results are means $\pm$ s.d. of three separate experiments.

Molecular species	Proportion of $[1-^{14}\text{C}]$ palmitate in triacylglycerol species (%)									
	Proportion (mol%) of palmitic acid in the medium ...									
Trisaturated	19.4	20.7	34.7	42.3	50.0	57.7	65.3	72.9	80.6	88.3
Found	2.1 $\pm$ 0.3	2.8 $\pm$ 0.4	4.3 $\pm$ 0.7	5.9 $\pm$ 0.1	9.1 $\pm$ 0.5	13.0 $\pm$ 2.3	19.9 $\pm$ 1.7	36.3 $\pm$ 4.6	51.1 $\pm$ 5.9	67.3 $\pm$ 3.8
Calc.*	0.4	1.0	2.2	4.3	8.8	15.4	22.3	35.5	48.4	67.9
Disaturated-monoenoic	18.5 $\pm$ 4.3	24.1 $\pm$ 3.6	31.9 $\pm$ 5.3	39.8 $\pm$ 3.5	45.8 $\pm$ 1.9	50.4 $\pm$ 2.2	50.9 $\pm$ 1.2	45.1 $\pm$ 3.3	34.1 $\pm$ 4.9	22.5 $\pm$ 3.1
Found	8.0	19.5	23.4	31.8	39.3	44.0	48.3	45.0	37.3	20.8
Calc.*										

* Calculated assuming acylation with non-correlative specificity (Slakey & Lands, 1968), i.e. a 1-random, 2-random, 3-random distribution.

other. This suggests that there were minor changes only in the relative rates of esterifying this position with palmitic acid. Above the critical concentration of palmitic acid or in positions *sn*-2 and *sn*-3, this was not so. There is no doubt that the observations of abrupt changes in relative affinities of the various enzymes for substrates at particular concentrations were real ones as the regression coefficients for each part of the relationship were 0.98 or higher.

Effect on the proportions of molecular species of triacylglycerols formed

Triacylglycerols were isolated from adipocytes after incubation with $[1-^{14}\text{C}]$ palmitic acid and various amounts of unlabelled oleic acid, as above. The proportions of the label in the more saturated molecular species were determined after separation by silver nitrate chromatography. The results were compared with those calculated from the positional distributions of the fatty acids in the molecules, assuming that acylation had occurred with non-correlative specificity (Slakey & Lands, 1968), i.e. with random selection of the intermediate acyl acceptors. Table 1 contains the results. With the trisaturated molecular species, the calculated and actual results corresponded very well (within one standard deviation) at higher palmitic acid concentrations, but tended to break down at lower (less than 50%) ones. With the disaturated-monounsaturated molecular species, agreement was also excellent at higher palmitic acid concentrations, but tended to worsen much sooner (below 66% palmitate in the medium).

Discussion

Variation of the nature and the composition of the fatty acids as substrate for triacylglycerol biosynthesis by rat adipocytes had very little effect on the rate of esterification of palmitic acid, which was proportionally dependent on its relative concentration in the medium. Similarly, Henderson *et al.* (1979a) found no difference in the relative rate of esterification of palmitic acid by adipocytes whether it was supplied on its own to the tissue or as part of a fatty-acid mixture.

The presence of different fatty acids in the medium affected the stereospecific distribution of palmitic acid within the triacyl-*sn*-glycerols in markedly different ways, however. Stearic acid had least effect and over the range of concentrations tested, palmitic acid was distributed among the three positions in roughly similar proportions; the concentration of palmitic acid in each position was linearly related to that in the medium. With myristic acid in the medium, the proportions of palmitic acid found in each position tended to diverge more from each other and showed a simple linear relationship to the amount in the medium only in position *sn*-2. In

positions *sn*-1 and *sn*-3, abrupt changes in the affinity of the enzymes for the substrate occurred at a particular concentration of palmitic acid relative to that of myristic acid. Myristic acid and stearic acid are similar in structure to palmitic acid, have comparatively high melting points ($>58^\circ\text{C}$) and would be expected to adopt similar conformations. The specificity of 1-acyl-*sn*-glycerol 3-phosphate acyltransferase for palmitic acid was not markedly affected by the chain-length of competing substrates but those of the other enzymes involved in triacylglycerol biosynthesis certainly were.

The presence of oleic acid or linoleic acid in the medium had a much more profound but similar effect on the esterification of palmitic acid when the latter was present in lower relative proportions (akin to those under normal physiological conditions) but the effects were somewhat different at higher concentrations. At the lower palmitic acid concentrations, the specificities of the acyltransferases for palmitic acid appeared to be affected by the change in conformation, brought about probably by the presence of a *cis*-double bond between C-9 and C-10, in the competing fatty acids. Sharp changes in the manner of esterification of palmitic acid occurred when it reached a critical concentration in the medium but the nature of the effect differed somewhat according to whether oleic acid or linoleic acid was present.

The phenomenon of an abrupt change in the manner of esterification of palmitic acid, as its concentration relative to that of the competing fatty acid was increased, was observed in all three positions of the triacyl-*sn*-glycerols. It appeared to represent a change in the relative affinity of each enzyme for the substrate, corresponding to an increase or decrease of up to 5-fold. In position *sn*-1, it was observed when myristic acid, oleic acid and linoleic acid were the competing substrates, i.e. components that are markedly different in structure, conformation and physical properties, but at points that were characteristic for the particular fatty acids. It was observed in position *sn*-2 when oleic acid and linoleic acid were present and in position *sn*-3 when myristic acid was present. The reason for the effect is not clear. Some acyltransferases are known to accept fatty acids in monomeric form, whereas others accept fatty acids in micellar form (Hübscher, 1970). It is possible that micelles of palmitic acid have some special property when particular concentrations of other fatty acids are present. Alternatively, above certain concentrations of palmitic acid, it is possible that the nature of the competition for esterification changes. Acyltransferases involved in triacylglycerol biosynthesis in rat adipocytes are known to be located in both the endoplasmic reticulum and the plasma membrane (Giacobino &

Chmelar, 1977) and this can lead to compartmental effects in the esterification of fatty acids (Henderson *et al.*, 1979a,b). Possibly, the acyltransferases at one of these sites can be saturated with respect to palmitic acid so that a change in the manner of esterification is seen. Why this should occur at the observed ratios of palmitic to the competing acid remains obscure.

A clue to the nature of the change may be provided by examination of the proportions of the various molecular species of triacylglycerols formed. At high palmitic acid relative to oleic acid concentration, the substrate was esterified with non-correlative specificity (Slakey & Lands, 1968). This did not appear to be true at lower palmitic acid concentrations, i.e. in the former circumstance, the acyltransferases esterifying positions *sn*-2 and *sn*-3 did not differentiate between partially acylated intermediates according to the nature of the bound acyl groups, but did exhibit selectivity at lower palmitic acid concentrations. It is not known how the change is mediated. Acylation of position *sn*-3 of triacylglycerols with non-correlative specificity was found to occur when preparations of rat mammary tissue were incubated with various diacylglycerol intermediates and single fatty acid substrates (Lin *et al.*, 1976); it would be intriguing to see whether similar results can be obtained with mixed fatty-acid substrates.

The experiments described above should be repeated with a wider range of fatty-acid substrates and at a range of combined substrate concentrations with adipocytes and other tissue preparations, e.g. mammary acini, but it may require the isolation of purified acyltransferases before definitive explanations of the observed phenomenon are obtained. It has been established, however, that during triacylglycerol biosynthesis in rat adipocytes, the proportional distributions of a fatty acid among the three positions are dependent on its concentration in the medium and on the nature and the concentrations of any other fatty acids present.

Miss J. V. Wilson gave skilled technical assistance.

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