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Stereospecific Biosynthesis of Triacylglycerols in Mammary Glands from Lactating Rats

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Microsomal plus cytosol preparations from the mammary gland of lactating rats are capable of incorporating palmitic acid and oleic acid into triacylglycerols. These triacylglycerols are similar in structure to those found in rat milk, where palmitic acid tends to be confined to the *sn*-2-position of the glycerol. Both glycerol 3-phosphate and dihydroxyacetone phosphate function as acyl acceptors. The enzymic synthesis of triacylglycerols appears in late pregnancy, increases rapidly during early lactation, but disappears within 3 days of weaning.

The triacylglycerols from rat milk contain an unusual intramolecular distribution of fatty acids in that palmitic acid is concentrated at the *sn*-2-position of the glycerol (Lin *et al.*, 1976; Grigor, 1977). This structure is similar to that found in human milk (Breckenridge *et al.*, 1969) and certain tissues of the pig (Mattson *et al.*, 1964; Christie & Moore, 1970), and contrasts with that found for the triacylglycerols of most animal tissues, where palmitic acid tends to be confined to the *sn*-1-position and where the *sn*-2-position is occupied mainly by unsaturated fatty acids (Brockerhoff *et al.*, 1966; Kuksis, 1972).

Cell-free preparations from the mammary tissue of lactating rats have been used to study the synthesis of the usual triacylglycerol structure found in rat milk. This synthetic activity has been measured in mammary tissue taken from animals at various stages of pregnancy and lactation, and has been compared with other known markers of mammary-gland function.

Experimental

Animals

Pregnant and virgin female rats of the Wistar strain were obtained from the University of Otago Animal Breeding Station. The animals were housed individually and given food and water *ad libitum*. Litters were decreased to ten pups at least 2 days before the lactating females were killed.

Materials

Substrates, cofactors and enzymes were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Radioactive fatty acids were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was obtained from Ciba–Geigy (N.Z.) Ltd., Wellington, New Zealand.

Methods

Preparation of microsomal plus cytosol fractions. Rats were anaesthetized with diethyl ether and killed by cervical dislocation. The right abdominal mammary gland was excised and immediately placed in ice-cold homogenizing buffer [5mM-Tris/maleate (pH7.3)/0.25M-sucrose/1mM-EDTA/1mM-dithiothreitol]. The tissue was homogenized in 5vol. of freshly prepared ice-cold buffer by using a Sorvall Omni-Mixer at half-speed for 30s. This homogenate was centrifuged at $15000g_{av.}$ at 4°C for 15min and the supernatant was stored in small portions at −15°C. The enzyme activities assayed were stable for several weeks. Adipose tissue from virgin female rats was treated identically, except that the tissue was homogenized by hand in a ground-glass homogenizer. A purified microsomal preparation from mammary gland was prepared by centrifuging the microsomal plus cytosol fraction at $100000g_{av.}$ at 4°C for 60min and resuspending the pellet in 2.5ml of the homogenizing buffer. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme assays. sn-Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) was assayed spectrophotometrically in a system containing 0.3mM-NADH and 0.4mM-dihydroxyacetone phosphate in 1ml of 0.19M-Tris/HCl buffer, pH7.4. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the method of Cohen & Rosemeyer (1975).

Assay of triacylglycerol synthesis. This activity was assayed by measuring the rate of incorporation of radioactively labelled palmitic acid and oleic

acid into triacylglycerols. Each incubation contained 0.05mM-bovine serum albumin (fatty acid-poor), 10mM-ATP, 4mM-$MgCl_2$, 0.075mM-CoA, 0.1mM-palmitic acid, 0.1mM-oleic acid, 0.75μCi each of [1-^{14}C]palmitic acid (59mCi/μmol) and [9,10(n)-^{3}H]oleic acid (2.2Ci/μmol) and either 3mM-*rac*-glycerol 3-phosphate or 1mM-dihydroxyacetone phosphate plus 0.2mM-NADPH in 1ml of 0.1M-Tris/HCl buffer, pH7.4. Enzyme protein (0.2–0.5mg) was added and the mixture incubated at 37°C with gentle shaking for 40min when glycerol 3-phosphate was the acyl acceptor or for 60min when dihydroxyacetone phosphate was the acyl acceptor.

The reaction was stopped by adding 2ml of chloroform/methanol/acetic acid (1:1:0.01, by vol.). The lower (chloroform) phase was then removed, evaporated to dryness under N_2 at 40°C and redissolved in 0.2ml of chloroform. The lipids from a portion of this solution were separated by t.l.c. with hexane/diethyl ether/acetic acid (50:50:1, by vol.) as solvent. Fractions corresponding to lipid standards were scraped into scintillation vials, and 5ml of scintillant [0.55% (w/v) butyl-PBD in toluene/Triton X-100/water (20:10:3, by vol.)] was added to each. No quench corrections were necessary for the presence of the silica gel. Standards of [^{3}H]- and [^{14}C]-hexadecane were prepared in the same scintillant and their radioactivities counted. The d.p.m. of ^{3}H and ^{14}C for each fraction was measured to calculate the rate of incorporation of each acid into the triacylglycerols.

In preliminary experiments a microsomal preparation minus the cytosol fraction was used as the enzyme source and, to separate the phospholipid products by t.l.c., a solvent system of chloroform/methanol/aq. NH_3 (sp.gr. 0.9) (4:14:1, by vol.) was used.

Analysis of triacylglycerol structure. The triacylglycerols from the remaining lipid were purified by preparative t.l.c. Corn-oil triacylglycerols (10mg) were added as carriers. The digestion system, comprising 0.14M-$CaCl_2$, 0.05M-NaCl, 0.06mM-cholic acid and 0.5–0.75mg of pancreatic lipase in 2ml of 0.1M-Tris/HCl buffer, pH8.5, was added to the triacylglycerols and the mixture shaken vigorously for 20min at 37°C. The reaction was stopped by adding 4ml of chloroform/methanol/acetic acid (1:1:0.01, by vol.), and the chloroform layer removed, evaporated under N_2 and redissolved in 0.1ml of chloroform. The lipids were separated by t.l.c. as described above into fractions containing monoacylglycerols, diacylglycerols, non-esterified fatty acids and triacylglycerols. The recoveries of ^{3}H and ^{14}C in each fraction were determined and, from them, the molar ratios of palmitic acid to oleic acid in the 2-monoacylglycerols, non-esterified fatty acids (released from positions 1 and 3) and triacylglycerols calculated.

Results and Discussion

The assay for triacylglycerol synthesis is similar to that used by Stokes *et al.* (1975) to study the stereospecific synthesis of triacylglycerols by preparations from the adipose tissue of several species including the pig. We found that, when microsomal plus cytosol preparations from lactating mammary tissue were used as the enzyme source, ATP, CoA, Mg^{2+} and an acyl acceptor (either glycerol 3-phosphate or dihydroxyacetone phosphate plus NADPH) were necessary for incorporation of fatty acids into triacylglycerols. Triacylglycerols accounted for over 90% of the radioactivity in the esterified products if glycerol 3-phosphate was used as the acyl acceptor and over 70% if dihydroxyacetone phosphate plus NADPH was used. In the latter case between 15 and 25% of the radioactivity in the esterified fatty acids was recovered in phosphatidic acid. When a microsomal preparation lacking the cytosol fraction was used in the presence of 20mM-NaF, the major product (50–80%) with either acyl acceptor was phosphatidic acid. When dihydroxyacetone phosphate minus NADPH was used, a more polar product (thought to be acyldihydroxyacetone phosphate) was formed.

When the microsomal plus cytosol fraction from rat adipose tissue was used as the enzyme source the major product was again triacylglycerols, which comprised 75% of the radioactivity in the esterified products if glycerol 3-phosphate was used as the acyl acceptor and 40% if dihydroxyacetone phosphate plus NADPH was used. Here 20 and 35% respectively of the radioactivity in the esterified fatty acids were recovered in phosphatidic acid. These observations are consistent with triacylglycerol synthesis in the cell-free preparations of these tissues being the result of synthesis *de novo*.

The molar ratio of palmitic acid to oleic acid incorporated into triacylglycerols was independent of the concentration of the acyl acceptors, glycerol 3-phosphate and dihydroxyacetone phosphate, but was dependent on the concentration of fatty acids. High fatty acid concentrations partially inhibited triacylglycerol synthesis and favoured the incorporation of oleic acid. When the optimum fatty acid concentration was used the rates of incorporation of the two fatty acids were approximately equal.

Table 1 shows that mammary-gland preparations from rats on about the fourteenth day of lactation synthesized triacylglycerols with the characteristic structure of rat milk triacylglycerols with either glycerol 3-phosphate or dihydroxyacetone phosphate as the acyl acceptor. The molar ratio of palmitate to oleate for 2-monoacylglycerols was higher and that for non-esterified fatty acids lower than the ratio for the triacylglycerols. This shows that more palmitate was esterified on the 2-position of the triacylglycerols

Table 1. *Stereospecificity of triacylglycerol synthesis in microsomal plus cytosol preparations from 14-day lactating mammary gland and adipose tissue*

Incubations and digestions were carried out as described in the text. Molar ratios of palmitic acid to oleic acid in the triacylglycerols synthesized and in the *sn*-2-monoacylglycerols and non-esterified fatty acids released by the action of pancreatic lipase on the triacylglycerols were calculated. The number of rats in each experiment is given in parenthesis. Values are means±S.D. of estimates obtained with individual rats.

		Molar ratio of palmitic acid to oleic acid		
Tissue	Acyl acceptor	Triacyl-glycerol	*sn*-2-Monoacyl-glycerol	Non-esterified fatty acids
Mammary (7)	Glycerol 3-phosphate	0.82±0.03	3.90± 0.90	0.43±0.04
Mammary (5)	Dihydroxyacetone phosphate	1.31±0.22	15.20±11	0.84±0.07
Adipose (4)	Glycerol 3-phosphate	0.80±0.13	0.27± 0.07	1.54±0.18
Adipose (4)	Dihydroxyacetone phosphate	0.56±0.02	0.08± 0.02	0.81±0.24

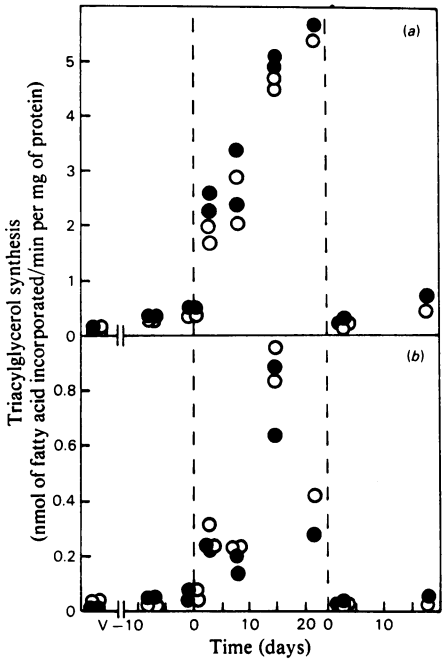

Fig. 1. *Synthesis of triacylglycerols by microsomal plus cytosol preparations of mammary gland at different stages of pregnancy and lactation*

The acyl acceptor was glycerol 3-phosphate (*a*) or dihydroxyacetone phosphate (*b*). Incubations containing [1-^{14}C]palmitic acid (●) and [9,10(n)-^{3}H]oleic acid (○) were carried out as described in the text. Each pair of points represents a single animal taken at the day indicated. Broken lines represent parturition and weaning. V, virgin animals.

than on the 1- and 3-positions. Adipose-tissue preparations, however, synthesized triacylglycerols with the structure characteristic of those found in adipose tissue.

A study was then carried out to determine the ability of mammary-gland preparations to synthesize the characteristic milk triacylglycerols at different stages of pregnancy and lactation. As markers of gland function, tissue weight and the protein content of the microsomal plus cytosol preparation were measured, and the activities of glucose 6-phosphate dehydrogenase and *sn*-glycerol 3-phosphate dehydrogenase were measured. The changes in these functions were similar to those reported previously (McLean, 1958; Baldwin & Milligan, 1966; Bartley *et al.*, 1966; Kuhn & Lowenstein, 1967). Fig. 1 shows that the rate of incorporation of fatty acids into triacylglycerols when glycerol 3-phosphate was the acyl acceptor increases 6-fold during pregnancy, rises markedly after parturition to reach a peak in late lactation 70 times the activity for glands from virgin rats, and falls rapidly to pre-lactation levels after weaning. A similar pattern was obtained when dihydroxyacetone phosphate was the acyl acceptor, except the highest activity was only one-seventh of that when *sn*-glycerol 3-phosphate was the acyl acceptor.

Analysis of the digestion products of the triacylglycerols synthesized when glycerol 3-phosphate was the acyl acceptor gave the values shown in Fig. 2. The products of preparations from virgin animals or animals at mid-pregnancy (14 days) resembled those of the adipose tissue rather than the lactating mammary gland (Table 1). Mammary tissue from virgin

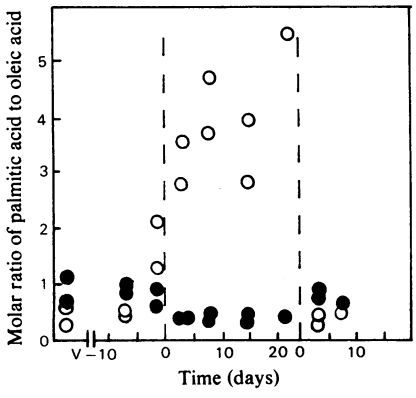

Fig. 2. *Stereospecificity of triacylglycerol synthesis in microsomal plus cytosol preparations from mammary glands at different stages of pregnancy and lactation*
Molar ratios of palmitic acid to oleic acid in the *sn*-2-monoacylglycerols (○) and non-esterified fatty acids (●) released by the action of pancreatic lipase on the triacylglycerols are shown. Incubations and digestions were carried out as described in the text. Each point represents a single animal taken at the day indicated. Broken lines represent parturition and weaning. V, virgin animals.

rats is mainly a mixture of connective and adipose tissue (Rees & Eversol, 1964). The glands taken at day 21 of pregnancy produced a mixture of adipose-tissue- and milk-type triacylglycerols. However, by the third day of lactation the synthesis of the milk-type structure predominated. The product of the glands taken 3 days after weaning had reverted to that typical of adipose tissue. Similar observations were obtained when the triacylglycerols synthesized with dihydroxyacetone phosphate as the acyl acceptor were analysed.

It is not known whether milk-type triacylglycerols are synthesized by a set of enzymes specific to the mammary gland or if some factor is present in mammary gland that directs the stereochemistry of the triacylglycerol synthesis. Stokes & Tove (1975) have described the presence of such a factor in microsomal preparations from pig adipose tissue that is capable of causing the synthesis of the pig-type triacylglycerols when added to preparations from other species. Two factors have already been shown to be present in lactating mammary gland that modify other enzyme activities to make products found only in milk. These are α-lactalbumin, which is required for lactose synthesis (Brew *et al.*, 1968), and a medium-chain acyl-thioesterase from rabbit mammary gland responsible for the premature chain termination during fatty acid synthesis to produce the short-chain fatty acids found in the milk lipids (Knudsen *et al.*, 1976; Chivers *et al.*, 1977). Alternatively Caffrey & Kinsella (1977) have shown that there is an isoenzyme of monoacylglycerol phosphate acyltransferase that is specifically induced in rabbit mammary gland during lactation. This enzyme may represent one of a set of enzymes for triacylglycerol synthesis specific to the lactating mammary gland.

References

Baldwin, R. L. & Milligan, L. P. (1966) *J. Biol. Chem.* **241**, 2058–2066
Bartley, J. C., Abraham, S. & Chaikoff, I. L. (1966) *Proc. Soc. Exp. Biol. Med.* **123**, 670–675
Breckenridge, W. C., Marai, L. & Kuksis, A. (1969) *Can. J. Biochem.* **47**, 761–769
Brew, K., Vanamam, T. C. & Hill, R. L. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 491–497
Brockerhoff, H., Hoyle, R. J. & Wolmark, N. (1966) *Biochim. Biophys. Acta* **116**, 67–72
Caffrey, M. & Kinsella, J. E. (1977) *J. Lipid Res.* **18**, 44–52
Chivers, L., Knudsen, J. & Dills, R. (1977) *Biochim. Biophys. Acta* **481**, 361–367
Christie, W. W. & Moore, J. H. (1970) *Biochim. Biophys. Acta* **210**, 46–56
Cohen, P. & Rosemeyer, M. A. (1975) *Methods Enzymol.* **41**, 208–214
Grigor, M. R. (1977) *Proc. Univ. Otago Med. Sch.* **55**, 23–24
Knudsen, J., Clark, S. & Dils, R. (1976) *Biochem. J.* **160**, 683–691
Kuhn, N. J. & Lowenstein, J. M. (1967) *Biochem. J.* **105**, 995–1002
Kuksis, A. (1972) *Prog. Chem. Fats Other Lipids* **12**, 1–163
Lin, C. Y., Smith, S. & Abraham, S. (1976) *J. Lipid Res.* **17**, 647–656
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mattson, F. H., Volpenheim, R. A. & Lutton, E. S. (1964) *J. Lipid Res.* **5**, 363–365
McLean, P. (1958) *Biochim. Biophys. Acta* **30**, 303–315
Rees, E. D. & Eversol, A. (1964) *Am. J. Physiol.* **207**, 595–600
Stokes, G. B. & Tove, S. B. (1975) *J. Biol. Chem.* **250**, 6315–6319
Stokes, G. B., Poteat, L. W. & Tove, S. B. (1975) *Biochim. Biophys. Acta* **380**, 245–256