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A New Assay Procedure for Monoglyceride Acyltransferase

By VERENA J. SHORT, DAVID N. BRINDLEY and RAYMOND DILS
Department of Biochemistry, The Medical School, University of Nottingham, Nottingham NG7 2RD, U.K.

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1. A new assay system is described for monoglyceride acyltransferase (acylglycerol palmitoyltransferase, EC 2.3.1.22) in which palmitoyl-CoA is generated from palmitoyl-(−)-carnitine. 2. With the microsomal fraction from homogenates of guinea-pig intestinal mucosa, the $V_{max.}$ of this enzyme decreased with different acyl acceptors in the order 2-monopalmitoylglycerol > 2-hexadecylglycerol > *rac*-1-monopalmitoylglycerol. 3. There were highly significant correlations between the monoglyceride acyltransferase activity as measured with these three substrates. This demonstrates that each of these substrates can be used to measure the same enzyme activity. 4. The advantages of using generated palmitoyl-CoA with 2-hexadecylglycerol and *rac*-1-monopalmitoylglycerol as model substrates for this enzyme are discussed.

The synthesis of triacylglycerols in intestinal epithelium occurs predominantly by the stepwise acylation of 2-monoacylglycerol [i.e. the monoglyceride pathway; see Hübscher (1970) and Brindley (1974) for reviews]. However, this pathway has proved difficult to estimate *in vitro* for a number of reasons. 2-Monoacylglycerols, which are the physiological acyl acceptors, are sparingly soluble in aqueous media and readily undergo both acyl migration and hydrolysis. This has led to the use of *rac*-1-monoacylglycerols and 2-monoalkylglycerols as model substrates, since they are more stable (see Brindley, 1974).

Though long-chain acyl-CoA esters have been used as acyl donors in these assays, they present difficulties which are well documented (see Kuhn, 1967; Zahler & Cleland, 1969). They have strong detergent properties, and Michaelis kinetics are only obtained when they are used below their critical micellar concentrations. To circumvent this, low concentrations of acyl-CoA esters have been generated during the incubation from the corresponding fatty acid in the presence of ATP, CoA and Mg^{2+} ions. This method assumes that the acyl-CoA synthetase (EC 6.2.1.3) activity of the preparation is not rate-limiting. An alternative way of generating palmitoyl-CoA is to use palmitoyl-(−)-carnitine in the presence of CoA and excess of carnitine palmitoyltransferase (EC 2.3.1.21). This method was originally used by Daae & Bremer (1970) to assay the activity of glycerol phosphate acyltransferase (EC 2.3.1.15) in rat liver and has been found to give more favourable kinetics than with added acyl-CoA esters (Brindley, 1973).

In the present paper we have compared the rates of acylation of the acyl-acceptors 2-monopalmitoylglycerol, *rac*-1-monopalmitoylglycerol and 2-hexadecylglycerol by the microsomal fraction of homogenates from guinea-pig intestinal epithelium. We have also measured the rates obtained by using added palmitoyl-CoA and have compared the results with those obtained with palmitoyl-CoA generated from palmitoyl-(−)-carnitine. In each case, the composition of the products formed has been determined.

Materials

Animals

Male guinea pigs (600–800g) were supplied by the Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K., and were allowed free access to food and water.

Chemicals and enzymes

Unless otherwise stated, these were purchased or prepared as described previously (Sánchez *et al.*, 1973). 2-Hexadecylglycerol was prepared by the method of Baumann & Mangold (1964). The product had a m.p. (uncorrected) of 64–65°C (the published value is 62.5–63.3°C; Wood & Snyder, 1967). It was characterized by its i.r. spectrum, and by its behaviour on t.l.c. on silica gel G impregnated with 5% (w/w) boric acid and with chloroform–methanol (49:1, v/v) as a developing solvent (Thomas *et al.*, 1965). 2-Monopalmitoylglycerol was purchased from Supelco Inc., Bellefonte, Pa., U.S.A.

Experimental and Results

Protein determination

Protein was determined by a biuret method (Brindley & Hübscher, 1965).

Determination of radioactivity

Radioactivity was measured by liquid scintillation as described by Sánchez *et al.* (1973).

Preparation of microsomal fractions

Microsomal fractions were prepared from homogenates of the mucosal scrapings obtained from the small intestinal tract of guinea pigs (Hübscher *et al.*, 1965).

Preparation of glyceride emulsions

Aqueous emulsions of 2-monopalmitoylglycerol were prepared by sonication for four periods each of 30s at 4°C and 22kHz and were used within 30min. Aqueous emulsions of *rac*-1-monopalmitoylglycerol and 2-hexadecylglycerol were prepared and used as described above, but the emulsions were sometimes stored at −20°C and resonicated before use.

Surfactants were not added, since agents such as Lubrol AL18 and Tween 20 inhibit the enzymes of the monoglyceride pathway (Brindley & Ferrier, 1972). The addition of hexane improves the emulsion characteristics of diacylglycerols (Mangiapane *et al.*, 1973). When aqueous emulsions of 2-hexadecylglycerol were prepared by sonication in water–hexane (99:1, v/v) there was a 10-fold decrease in the apparent K_m value for the acylation of 2-hexadecylglycerol by added palmitoyl-CoA compared with that when hexane was not used. However, since there was a 2-fold decrease in the $V_{max.}$ value of the reaction, the emulsions used in the present work were prepared without added hexane.

Separation of neutral lipids

Neutral lipids were separated by t.l.c. on silica gel G with hexane–diethyl ether–acetic acid (70:30:1, by vol.) as developing solvent. They were detected by exposure to iodine vapour. Areas of gel corresponding to authentic lipid standards were scraped into vials and the radioactivity present was determined by liquid scintillation (Sánchez *et al.*, 1973).

Measurement of rates of esterification

Unless stated otherwise, the rates of esterification by acyl donors with acyl acceptors were measured by using optimum concentrations of substrates and cofactors. Reaction rates were constant during the incubation period and were measured at several protein concentrations. Incubations without microsomal protein were always included as controls.

Rates of esterification of rac-1-monopalmitoylglycerol and 2-hexadecylglycerol with exogenous palmitoyl-CoA

Rodgers (1969) has assayed spectrophotometrically the monoglyceride acyltransferase activity of rat small intestine by treating the liberated CoA with 5,5′-dithiobis-(2-nitrobenzoic acid). However, when Rao & Johnston (1966) had used this method previously to assay the enzyme in hamster small intestine they found little correlation with results obtained by measuring the incorporation of radioactive palmitoyl-CoA into acylglycerols. They concluded that the liberated CoA became tightly bound to the microsomal protein and was not available to react with the 5,5′-dithiobis-(2-nitrobenzoic acid). We have therefore used the incorporation of [9,10-^{3}H]palmitoyl-CoA into glycerides to measure its rate of esterification with monoacylglycerols and 2-hexadecylglycerol. Rodgers (1969) also reported that bovine serum albumin can partly overcome the inhibition by palmitoyl-CoA of the acylglycerol palmitoyltransferase activity in preparations of rat small intestine when assayed spectrophotometrically. We therefore tested the effect of albumin on the assay system described below. With *rac*-1-monopalmitoylglycerol as the acyl acceptor, the same maximum rates of incorporation were observed with 40μM-[9,10-^{3}H]palmitoyl-CoA plus 1.5mg of albumin/ml, 100μM-[9,10-^{3}H]palmitoyl-CoA plus 3.0mg of albumin/ml, or 200μM-[9,10-^{3}H]-palmitoyl-CoA plus 6.0mg of albumin/ml. These rates were approximately three times greater than that observed with 15μM-[9,10-^{3}H]palmitoyl-CoA in the absence of albumin. In all subsequent assays, 40μM-[9,10-^{3}H]palmitoyl-CoA plus 1.5mg of albumin/ml were used.

The assay conditions for optimum incorporation of [9,10-^{3}H]palmitoyl-CoA into monoglycerides were found to be 25mM-Tris buffer adjusted to pH7.4 with HCl, 5mM-dithiothreitol, 2.0mM-*rac*-1-monopalmitoylglycerol or 1.5mM-2-hexadecylglycerol, 40μM-[9,10-^{3}H]palmitoyl-CoA (48nCi) and 1.5mg of bovine serum albumin (fatty-acid-poor)/ml. The incubation volume was 0.25ml. The reaction was started by the addition of microsomal protein and stopped after 3min at 37°C with 1.88ml of chloroform–methanol (1:2, v/v). Chloroform (0.625ml) containing 0.4% (v/v) of olive oil as carrier was added followed by 0.875ml of water. After gentle mixing, the system was separated by centrifugation. Unused [9,10-^{3}H]palmitoyl-CoA was removed from the bottom phase by washing twice with 50mM-Tris buffer adjusted to pH8.0 with HCl. A portion (0.5ml) of the bottom phase was applied to a column (1g) of alumina in chloroform, and neutral lipids were eluted with 10ml of chloroform. Recoveries of neutral lipid from the columns were greater than 96%. The incorporation of [9,10-^{3}H]palmitoyl-CoA into total neutral lipids and into individual neutral lipid classes was determined by t.l.c.

With this assay system the activity of the monoglyceride acyltransferase was only proportional to protein concentration below 40μg of protein even when the incubation time was decreased to 2min. It was therefore difficult to measure the activity with

Table 1. *Specific activity of monoglyceride transacylase with different acyl acceptors, and the composition of the reaction products*

Optimum assay conditions were used to measure the rate of esterification of [9,10-^{3}H]palmitoyl-CoA into neutral lipids with the acyl-acceptors shown. The [9,10-^{3}H]palmitoyl-CoA was exogenous, or was generated from [9,10-^{3}H]palmitoyl-(−)-carnitine. Freshly prepared homogenates of the mucosa of guinea-pig small intestine were used to prepare the microsomal fractions. The numbers of animals used are given in parentheses and the results are means ± S.E.M. The monoglyceride acyltransferase activity was calculated from the rate of incorporation of palmitoyl-CoA into total neutral lipids and from the appropriate product composition.

		Specific activity (nmol of product formed/min per mg of protein)		Product composition (mol %)	
Acyl acceptor	Acyl donor	Incorporation of palmitoyl-CoA into total neutral lipids	Monoglyceride acyltransferase	Triacylglycerol	Diacylglycerol
rac-1-Monopalmitoyl-glycerol	Palmitoyl-CoA (exogenous)	16.8±0.3 (2)	12.8±0.4 (2)	22.1±2.2 (4)	77.9±2.2 (4)
2-Hexadecylglycerol	Palmitoyl-CoA (exogenous)	25.0±5.0 (2)	19.9±4.1 (3)	22.1±5.3 (4)	77.9±5.3 (4)
rac-1-Monopalmitoyl-glycerol	Palmotoyl-CoA (generated)	13.7±1.2 (13)	8.4±0.8 (13)	38.9±0.8 (13)	61.1±0.8 (13)
2-Monopalmitoylglycerol	Palmitoyl-CoA (generated)	42.2±4.5 (12)	32.9±3.1 (12)	18.2±1.3 (11)	81.8±1.3 (11)
2-Hexadecylglycerol	Palmitoyl-CoA (generated)	17.6±1.5 (13)	15.7±1.5 (13)	10.8±0.8 (13)	89.2±0.8 (13)

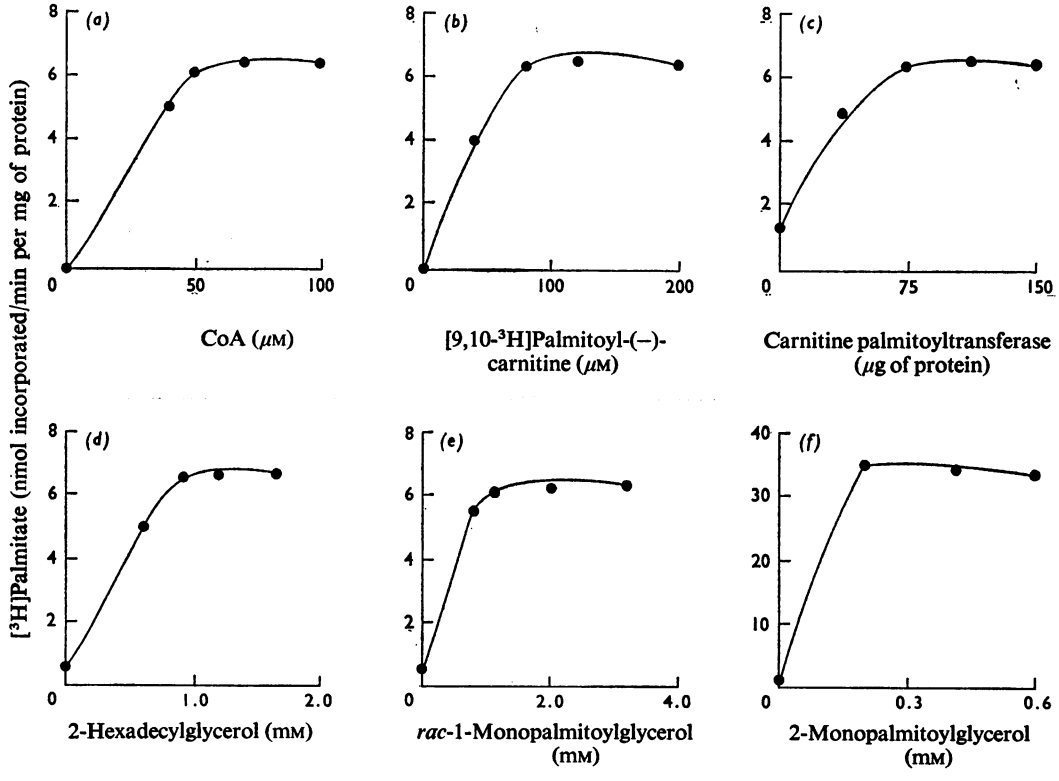

Fig. 1. *Conditions for the assay of monoglyceride acyltransferase by using palmitoyl-CoA generated from palmitoyl-(−)-carnitine*

Details of the assay system are given in the Experimental and Results section. The concentrations of cofactors and substrate were optimum except for the one under consideration, which was varied as shown.

accuracy, and the results quoted in Table 1 are only approximate.

Rates of esterification of rac-1-monopalmitoylglycerol, 2-monopalmitoylglycerol and 2-hexadecylglycerol with palmitoyl-CoA generated from palmitoyl-(−)-carnitine

These rates were measured by a method based on that of Daae & Bremer (1970) for determining the activity of glycerol phosphate acyltransferase. The optimum assay conditions were found to be 25mM-Tris buffer adjusted to pH7.4 with HCl, 5mM-dithiothreitol, 70μM-CoA, excess of bovine carnitine palmitoyltransferase (approx. 100μg of protein), 1.5mg of bovine serum albumin (fatty-acid-poor), 100μM-[9,10-^{3}H]palmitoyl-(−)-carnitine (20nCi) and 2.0mM-*rac*-1-monopalmitoylglycerol, 0.3mM-2-monopalmitoylglycerol or 1.44mM-2-hexadecylglycerol. These concentrations of acyl acceptors gave optimum rates of esterification. The cofactor and substrate requirements are shown in Fig. 1. In the absence of added acyl acceptors, the rates of esterification were negligible (see Fig. 1). The incubation volume was 0.25ml. The reaction was started by the addition of the microsomal fraction without prior preincubation of the palmitoyl-(−)-carnitine-generating system. The reaction was stopped after 6min at 37°C with 1.88ml of chloroform–methanol (1:2, v/v). Chloroform (0.625ml) containing 0.4% (v/v) of olive oil as carrier was added followed by 0.875ml of water. After gentle mixing, the system was separated by centrifugation. The bottom phase was washed with 2.0ml of a synthetic top phase of the mixture chloroform–methanol–1.2M-HCl (10:10:9, by vol.). A portion of the bottom phase was applied to a column (1g) of silicic acid in chloroform. Neutral lipids and unesterified fatty acids were eluted with 10ml of chloroform. The eluate was applied to a column (1g) of alumina in chloroform and neutral lipids were eluted with 5ml of chloroform. The incorporation of radioactivity from [9,10-^{3}H]palmitoyl-(−)-carnitine into total neutral lipids and into individual lipid classes was measured by liquid scintillation. The use of these two columns effectively removed residual palmitoyl-(−)-carnitine, palmitoyl-CoA and any palmitic acid formed by hydrolysis. Control incubations without added enzyme contained approx. 1–2% of the original radioactivity added.

With this assay system, the activity of the monoglyceride acyltransferase could be measured more accurately than when added palmitoyl-CoA was used. With all three acyl acceptors, the reaction rate was proportional to protein concentration up to about 60μg of microsomal protein and was proportional to the time of incubation for at least 6min.

Products of the reaction

Irrespective of the acyl acceptor or acyl donor used, the major products of the esterification reactions were diacylglycerol or its acyl-alkylglycerol analogue (Table 1). Similar results were obtained by Brindley & Ferrier (1972), who used palmitoyl-CoA generated from palmitic acid. The assays used are therefore primarily measuring the esterification of monoglycerides to diglycerides. The highest proportion of triglyceride was formed by intestinal preparations from guinea pig, when *rac*-1-monopalmitoylglycerol was acylated by generating palmitoyl-CoA from palmitoyl-(−)-carnitine (Table 1). However, the relative proportions of diglyceride and triglyceride synthesized by intestinal preparations is known to vary from species to species (Paltauf & Johnston, 1971) and the results shown in Table 1 may only apply to the guinea pig.

Discussion

The kinetic difficulties encountered when assaying monoglyceride acyltransferase activity with exogenous palmitoyl-CoA as acyl donor can be largely overcome by using palmitoyl-CoA generated from palmitoyl-(−)-carnitine. The specific activity of the enzyme was essentially similar with both acyl donor systems (Table 1).

With generated palmitoyl-CoA as acyl donor, paired *t* tests showed that the specific activity of monoglyceride acyltransferase measured with 2-monopalmitoylglycerol was 3.9 times ($P<0.001$) that with *rac*-1-monopalmitoylglycerol, and 2.1 times ($P<0.001$) that with 2-hexadecylglycerol. When the results were subjected to linear regression analysis, there were highly significant correlations ($P<0.001$, 13 animals) between the activities of monoglyceride acyltransferase measured with 2-hexadecylglycerol and *rac*-1-monopalmitoylglycerol as acyl acceptors. However, because the activities obtained with 2-palmitoylglycerol varied much more with different emulsions than did the activities obtained with the other two substrates, correlations involving 2-palmitoylglycerol were only significant within each experiment. For the largest experiment (six animals) the significance levels were $P<0.01$ for 2-palmitoylglycerol and *rac*-1-monopalmitoylglycerol, and $P<0.001$ for 2-monopalmitoylglycerol and 2-hexadecylglycerol. Schultz & Johnston (1971) have reported on the difficulties of interpreting results obtained with 2-monoacylglycerols which probably results from the rapid hydrolysis and isomerization of this substrate.

In conclusion, *rac*-1-monoglycerides and 2-alkylglycerols have the advantages over 2-monoglycerides in this assay in that they are less subject to isomerization and hydrolysis and also that they are more readily available. In addition, monoglyceride acyltransferase activity can be determined more accurately by generating palmitoyl-CoA from palmitoyl-(−)-carnitine than by adding palmitoyl-CoA directly to the assay system. With generated palmitoyl-CoA

the reaction rate is constant for longer time-periods and is also proportional to enzyme concentration over a wider range.

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