Intermolecular Specificity of Pancreatic Lipase in the Lipolysis of Triacylglycerols Containing Phytanic Acid

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Treatment of mixtures of glycerol tripalmitate and *rac*-glycerol 1,2-dipalmitate 3-phytate with pancreatic lipase demonstrated that phytanic acid retarded the release of palmitic acid esterified in the same triacylglycerol.

In the course of demonstrating that phytanate esters of glycerol are less susceptible to the action of lipases than are palmitate esters, Ellingboe & Steinberg (1972) found that an approximately equimolar mixture of *rac*-glycerol 1-palmitate 3-phytate and *rac*-glycerol 1-oleate 3-palmitate gave approximately equal amounts of palmitic acid and oleic acid on treatment with pancreatic lipase. This suggested to us that the palmitic acid that was esterified in the same diacylglycerol as phytanic acid may have been released much more slowly than that esterified in the same diacylglycerol as oleic acid. In order to test this possibility we have compared the rates of release of palmitic acid from *rac*-glycerol 1,2-dipalmitate 3-phytate with that from glycerol tripalmitate when mixtures of the two were treated with pancreatic lipase.

Materials and Methods

rac-Glycerol 1,2-dipalmitate 3-phytate was obtained from Analabs, North Haven, CT, U.S.A.; *rac*-glycerol 1,2-dipalmitate and phytol were from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; pig pancreatic lipase (28 units/mg) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; glycerol tripalmitate was from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.; behenic acid was from Fluka A. G., Buchs, Switzerland; methyl arachidate was from Applied Science, State College, PA, U.S.A.; other chemicals were from BDH, Poole, Dorset, U.K. Glycerol tri[1-¹⁴C]palmitate and glycerol tri[9,10(n)-³H]-palmitate were supplied by The Radiochemical Centre, Amersham, Bucks., U.K., and purified by column chromatography on Florisil (Bishop *et al.*, 1969) and by t.l.c. (Stead & Welch, 1975). Phytanic acid was prepared from phytol (Patton & Benson, 1966) and converted into the acid chloride by the method of Youngs *et al.* (1957). *rac*-Glycerol 1,2-di[1-¹⁴C]palmitate was prepared by partial lipolysis of glycerol tri[1-¹⁴C]palmitate with pancrea-

tic lipase. The products were separated by t.l.c. on silicic acid impregnated with boric acid (19:1, w/w) with the solvent system chloroform/acetone (24:1, v/v) (Thomas *et al.*, 1965). The ¹⁴C-labelled dipalmitate was eluted from the silicic acid with diethyl ether (Stead & Welch, 1975) and the diethyl ether extract washed with ice-cold water to remove boric acid and dried over anhydrous Na₂SO₄ (Christie, 1973). *rac*-Glycerol 1,2-di[¹⁴C]palmitate 3-phytate was prepared from phytanoyl chloride and glycerol di[¹⁴C]palmitate to which non-radioactive dipalmitate had been added (Mattson & Volpenhein, 1962) and purified by column chromatography on Florisil and by t.l.c. as described previously (Bishop *et al.*, 1969), but with the solvent system diethyl ether/n-hexane/formic acid (50:50:1, by vol.). The solutions used for lipolysis were extracted with diethyl ether, and the residual diethyl ether was removed with a stream of N₂; pancreatic lipase was extracted with acetone and diethyl ether.

A mixture of pancreatic lipase (125 µg/µmol of triacylglycerol), 1M-Tris buffer, pH 8 (1 ml), 0.2M-CaCl₂ (0.1 ml) and 1 mM-sodium taurocholate (0.2 ml) was emulsified with mixtures of *rac*-glycerol 1,2-dipalmitate and radioactive glycerol tripalmitate (1–6 µmol) at 22.5 or 40°C (Luddy *et al.*, 1964). Paraffin (12 mg) (Ellingboe & Steinberg, 1972) and/or n-hexane (0.5 ml) (Christie & Moore, 1969) were added to the mixture to assist emulsification. Behenic acid was added as a recovery indicator, and the lipolysis products were separated by t.l.c. on silicic acid as described above. The band corresponding to non-esterified fatty acids was eluted and methylated (Rogozinski, 1964), and the methyl esters were dissolved in n-hexane containing a known amount of methyl arachidate as internal standard. The methyl esters were assayed for radioactivity (Bishop *et al.*, 1969) and their fatty acid compositions determined by g.l.c. (Stead & Welch, 1975). In control determinations the enzyme was either omitted or acidified before it was added to the substrate.

When both triacylglycerols were radioactive,

Fig. 1. Specific radioactivity of palmitic acid released by treatment of mixtures of *rac*-glycerol dipalmitate phytanate and radioactive glycerol tripalmitate with pancreatic lipase

(a) Variation with time of specific radioactivity of palmitic acid released from an equimolar mixture at 40°C. S_T is specific radioactivity of palmitic acid in glycerol tri[^3H]palmitate and S_p is calculated specific radioactivity when all of the palmitic acid on the primary positions of the triacylglycerols has been released. (b) Variation with degree of lipolysis of glycerol tripalmitate ($(S - S_p)/(S_T - S_p)$), where S is specific radioactivity of released palmitic acid. Δ , Mixture of glycerol tri[^3H]palmitate and *rac*-glycerol dipalmitate phytanate (molar ratio 1:1) at 40°C (as in a); $\circ$, mixture of glycerol tri[^{14}C]palmitate and *rac*-glycerol dipalmitate phytanate (molar ratio 1:2) at 22.5°C; $\blacktriangle$, mixture of glycerol tri[^{14}C]palmitate and *rac*-glycerol dipalmitate phytanate (molar ratio 1:2) at 22.5°C; $\bullet$, mixture of glycerol tri[^{14}C]palmitate and *rac*-glycerol dipalmitate phytanate (molar ratio 1:2) at 24°C.

glycerol tri[^3H]palmitate (about 0.06 μmol and 0.7 μCi), *rac*-glycerol di[^{14}C]palmitate phytanate (about 3.5 μmol and 0.14 μCi) and glycerol trioleate (about 45 μmol) were dissolved in *n*-hexane (10 ml) and 0.12–1.5 ml samples were taken for lipolysis at 40°C, the smaller samples being lipolysed for longer times to conserve materials. The procedure was as before, except that fatty acids were separated by column chromatography on Florisil and the $^{14}\text{C}/^3\text{H}$ ratios were determined as described by Bishop *et al.* (1971).

Results and Discussion

When we treated mixtures of *rac*-glycerol dipalmitate phytanate and radioactive glycerol tripalmitate with pancreatic lipase, the specific radioactivity of the released palmitic acid decreased as the time of incubation increased and eventually reached a constant value. This we calculated to be the value expected when all the palmitic acid on the primary positions of the triacylglycerol mixture had been released. The specific radioactivity of the glycerol tripalmitate and the relative proportions of glycerol tripalmitate and *rac*-glycerol dipalmitate phytanate were different for each of the four experiments and so only one of the plots of specific radioactivity against time is shown in Fig. 1(a). In order to repre-

Fig. 2. Variation with time of $^{14}\text{C}/^3\text{H}$ ratio of palmitic acid released by treatment of mixtures of glycerol tri[^3H]palmitate and *rac*-glycerol di[^{14}C]palmitate phytanate with pancreatic lipase at 40°C

$\bullet$, $\circ$, First preparation of *rac*-glycerol di[^{14}C]palmitate phytanate; $\blacktriangle$, second preparation of *rac*-glycerol di[^{14}C]palmitate phytanate.

sent the data from the four experiments on one Figure, we have plotted the values for the expression $(S - S_p)/(S_T - S_p)$ against the degree of lipolysis of glycerol tripalmitate (Fig. 1b), where S represents the specific radioactivity of released palmitic acid, S_T that of palmitic acid in glycerol tripalmitate, and S_p that calculated for palmitic acid released from all the primary positions of the mixture of *rac*-glycerol dipalmitate phytanate and glycerol tripalmitate. The expression represents the difference between the observed and final specific radioactivities of palmitic acid released, divided by the difference between the maximum and minimum specific radioactivities. The specific radioactivity of released palmitic acid decreased as the degree of lipolysis increased and we concluded that the proportion of palmitic acid that is derived from *rac*-glycerol dipalmitate phytanate increases as the lipolysis proceeds.

In similar experiments with glycerol tri[^3H]-palmitate and *rac*-glycerol di[^{14}C]palmitate phytanate, which thus avoided the measurement of small amounts of palmitic acid, we found that the $^{14}\text{C}/^3\text{H}$ ratio increased with time of incubation (Fig. 2), thereby confirming our previous conclusion.

A low rate of lipolysis is sometimes observed with high-melting-point triacylglycerols because of difficulties with emulsification (Luddy *et al.*, 1964), but the addition of *n*-hexane (Brockhoff, 1965) or paraffin (Ellingboe & Steinberg, 1972) should overcome this. However, since *rac*-glycerol dipalmitate phytanate has a lower melting point than glycerol tripalmitate, the difference in melting point cannot be the explanation for our observations.

The intermolecular specificity of pancreatic lipase has been observed previously. Sampugna *et al.* (1967) demonstrated that triacylglycerols containing both palmitate and butyrate were lipolysed faster than was glycerol trioleate, and they found no evidence of fatty acid specificity.

Very little phytanic acid was released from *rac*-glycerol dipalmitate phytanate during our experiments, in agreement with earlier reports of the resistance of phytanate esters to lipolysis (Brockhoff, 1970; Ellingboe & Steinberg, 1972). In the lipolysis of *rac*-glycerol dipalmitate phytanate, therefore, pancreatic lipase exhibits both intramolecular and intermolecular specificity.

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