Degradation of Monogalactosyl Diglyceride and Digalactosyl Diglyceride by Sheep Pancreatic Enzymes

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1. Saline extract of sheep pancreas acetone-dried powder was shown to catalyse acyl ester hydrolysis of spinach leaf galactosyl diglycerides and also galactosylglucosyl diglyceride of *Lactobacillus casei*. 2. Sodium deoxycholate stimulated the enzyme activity. Ca^{2+} had no effect on the hydrolysis of monogalactosyl diglyceride, but it enhanced that of digalactosyl diglyceride. When added together, there was considerably less activity with both the substrates. 3. Optimal hydrolysis was observed at pH 7.2. 4. The initial point of hydrolysis was at position-1, leading to the formation of monogalactosyl monoglyceride and digalactosyl monoglyceride. Further hydrolysis to the corresponding galactosylglycerols and later to galactose and glycerol was also observed, indicating the presence of α - and β -galactosidases in the enzyme preparation. 5. Formation of monogalactosyl diglyceride from digalactosyl diglyceride by the action of α -galactosidase was noted. 6. Monogalactosyl diglyceride was also hydrolysed by β -galactosidase to a limited extent, giving rise to diacylglycerol and galactose. 7. Attempts at purification of monogalactosyl diglyceride acyl hydrolase by using protamine sulphate treatment, Sephadex G-100 filtration and DEAE-cellulose chromatography gave a partially purified enzyme which showed 9- and 81-fold higher specific activity towards monogalactosyl diglyceride and digalactosyl diglyceride respectively. This still showed acyl ester hydrolysis activity towards methyl oleate, phosphatidylcholine and triacylglycerol. 8. When sheep, rat and guinea-pig tissues were compared, guinea-pig tissues showed the highest activity towards both monogalactosyl diglyceride and digalactosyl diglyceride. In all the species pancreas showed higher activity than intestine.

The occurrence of glycosyl diglycerides in plants, animals and bacteria is now well established, although the nature and the number of glycosyl residues associated with these lipids vary among the organisms. In glycosyl diglycerides from plants and animals, galactose is the only sugar found so far, whereas a variety of carbohydrate moieties are encountered in bacteria (Sastry, 1974). Monogalactosyl diglyceride and digalactosyl diglyceride are major lipid components in the plant kingdom, and occur at particularly high concentrations in the photosynthetic parts and account for about 40% by weight of the total lipids (Kates, 1970). Further, in leaves and forage crops these lipids contain mainly trienoic acids, especially α -linolenic acid (Sastry & Kates, 1964a; Weenink, 1964). It is therefore possible that glycosyl diglycerides of plant origin may serve as good sources of carbohydrate and fatty acids for herbivores, provided that these lipids are digested.

The enzymic deacylation of galactosyl diglycerides by plant tissues is well documented (Sastry & Kates, 1964b; Helmsing, 1967, 1969; Galliard, 1971). Galactosidases that hydrolyse monogalactosyl diglyceride or the deacylation products of monogalactosyl diglyceride and digalactosyl diglyceride have also been observed in leaves (Gatt & Baker, 1970; Sastry & Kates, 1964b). This shows that leaf

tissues possess the necessary enzymes for the complete hydrolysis of galactosyl diglycerides. Evidence exists for similar enzymic breakdown of glycosyl glycerides in animals. The ability of commercial pancreatin to deacylate both monogalactosyl diglyceride and digalactosyl diglyceride as well as sulphoquinovosyl diglyceride has been reported (Sastry & Kates, 1964b; Noda & Fujiwara, 1967; Safford & Nichols, 1970; Auling *et al.*, 1971; Yagi & Benson, 1962). Enzymes metabolizing glycosyl glycerides were also found in brain tissue (Subba Rao *et al.*, 1970; Subba Rao & Pieringer, 1970). In ruminants, the rumen micro-organisms can hydrolyse galactosyl glycerides (Viviani, 1970). However, as pancreas is the most likely tissue to play a general role in the digestion of these lipids in animals, the metabolic degradation of glycosyl glycerides by pancreatic enzymes and their characterization was investigated.

Materials and Methods

Materials

Fresh sheep pancreas and intestine were obtained from the local slaughterhouse. The rats and guinea pigs used in these experiments were obtained from the Central Animal Facility of this Institute.

Sodium deoxycholate and DEAE-cellulose were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-100 was obtained from Pharmacia, Uppsala, Sweden. Silicic acid (100–200 mesh) for column chromatography was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A., and silica gel C (13% $\text{CaSO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$) for t.l.c. was bought from ACME Chemicals, Poona, India. Solvents of A.R. grade were obtained from BDH Chemicals Ltd., Bombay, India and were redistilled before use.

Preparation of substrates

Monogalactosyl diglyceride and digalactosyl diglyceride were prepared from New Zealand spinach (*Tetragonia expansa*) obtained from the Horticulture Institute, Bangalore, India. Leaves (300g wet wt.) were extracted by the procedure of Sastry & Kates (1964a). The total lipids were chromatographed on a 40g column of silicic acid–Celite (4:1, w/w) and successively eluted with 600ml of chloroform (fraction I), 300ml of chloroform–methanol (5:1, v/v) (fraction II) and 300ml of methanol (fraction III). Fraction II, which contained glycolipids, was subjected to precipitation with cold acetone. The lipids in the supernatant fraction were rechromatographed on a 20g silicic acid column and eluted successively with 150ml of chloroform (fraction A), 2% (v/v) methanol in chloroform (fraction B), 5% (v/v) methanol in chloroform (fraction C) and 10% (v/v) methanol in chloroform (fraction D). Fractions B and D contained monogalactosyl diglyceride and digalactosyl diglyceride respectively. These gave single spots on t.l.c. with chloroform–methanol–acetic acid–water (170:25:25:3, by vol.) as the solvent system and also on silicic acid paper chromatograms (Marinetti, 1962). The galactose/ester molar ratios of monogalactosyl diglyceride and digalactosyl diglyceride were 1:1.94 and 1:1.12 respectively. The fatty acid composition of the monogalactosyl diglyceride was: $\text{C}_{16:0}$ acid, 3.7%; $\text{C}_{16:3}$ acid, 8.5%; $\text{C}_{18:0}$ acid, 9.6%; $\text{C}_{18:1}$ acid, 9.3%; $\text{C}_{18:2}$ acid, 6.3%; $\text{C}_{18:3}$ acid, 62.4%, by weight. The fatty acid composition of digalactosyl diglyceride was: $\text{C}_{16:0}$ acid, 19.2%; $\text{C}_{16:3}$ acid, 1.1%; $\text{C}_{18:0}$ acid, 2.7%; $\text{C}_{18:1}$ acid, 4.4%; $\text{C}_{18:2}$ acid, 6.8%; $\text{C}_{18:3}$ acid, 65.8%, by weight.

Galactosylglucosyl diglyceride was prepared from *Lactobacillus casei* 7469 (supplied by the National Chemical Laboratory, Poona, India) grown at 37°C on a medium containing glucose (500mg), lactose (500mg), liver extract (600mg wet wt.), sodium acetate (600mg), yeast extract (500mg wet wt.), 0.5ml of salt A [KH_2PO_4 (25g) and K_2HPO_4 (25g) in 250ml of water] and 0.5ml of salt B [$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (10g), NaCl (500mg), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (500mg) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (500mg) in 250ml of water] in 100ml of water and the pH was adjusted to 7.6. The cells were

harvested after 24h of growth, and the lipids were extracted by the method of Shaw *et al.* (1968). The lipid extract (0.36g) that was obtained from 43g of wet cells was chromatographed on a 20g silicic acid–Celite column (4:1, w/w) and eluted successively with 100ml of chloroform (fraction I), 350ml of chloroform–methanol (5:1, v/v) fraction (II) and 100ml of methanol (fraction III). Fraction II, which contained glycolipids, was rechromatographed on a 10g silicic acid column and eluted successively with 100ml each of chloroform, 2% (v/v) methanol in chloroform, 5% (v/v) methanol in chloroform, 10% (v/v) methanol in chloroform, 15% (v/v) methanol in chloroform and finally with methanol. Galactosylglucosyl diglyceride was found in the fraction eluted with 15% (v/v) methanol in chloroform. It gave a single spot on t.l.c. with chloroform–methanol–acetic acid–water (170:25:25:3, by vol.) as the solvent system and on silicic acid paper chromatogram (Marinetti, 1962). The sugar/ester molar ratio was 1.18:1.00. It was not further characterized and was assumed to be 1,2-di-*O*-acyl-3-*O*-[α -D-galactopyranosyl-(1→2)-*O*- α -D-glucopyranosyl]-sn-glycerol (Shaw *et al.*, 1968). Galactosylglycerols were prepared by alkaline hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride (Sastry & Kates, 1964a). Phosphatidylcholine was isolated from spinach leaves (Bajwa & Sastry, 1972), and triacylglycerol was prepared from linseed oil (Hirsch & Ahrens, 1958).

Preparation of enzyme extracts

An acetone-dried powder of sheep pancreas was prepared by homogenizing the tissue in a Waring Blendor with 10vol. of cold acetone at –10°C and filtered immediately. The residue was homogenized with acetone at –10°C and filtered twice more. It was finally dried over CaCl_2 *in vacuo* and stored at –10°C. Sheep intestines were washed with ice-cold 0.9% NaCl (saline), the mucosa was then scraped and an acetone-dried powder of the scrapings was prepared as described above. For the extraction of enzymes, 1g of acetone-dried powder was suspended in 20ml of 0.9% NaCl, kept overnight at 1–5°C and centrifuged at 1000g_{av.} for 2min. The supernatant was used as the source of enzyme.

Enzyme assay

Substrate (usually about 1 μmol in chloroform) was taken in a test tube and the solvent was evaporated off under N_2 . The lipid was dissolved in 0.05ml of methanol and dispersed in 0.2ml of 0.1M-Tris-maleate buffer, pH7.2, and 0.2ml of sodium deoxycholate in the same buffer, with the aid of a Vortex mixer. Preliminary experiments had shown that substrate concentrations up to 2.5mM were not inhibitory and in all the experiments re-

ported the concentration of glycosyl diglycerides was kept between 0.8 and 2.0 mM. Enzyme solution (0.4 ml) was added to each tube in a final volume of 0.8 ml. After incubation at 37°C the reaction was terminated by the addition of 2.0 ml of methanol, and the lipids were extracted by the method of Bligh & Dyer (1959). Zero-time controls were run simultaneously. The ester content of the lipid extracts was determined. Although methanol (6–7%, by volume) was used in the assay mixture, the formation of fatty acid methyl esters during the incubation was not detected by t.l.c.; therefore the decrease in acyl ester content was taken as a valid measure of enzyme activity and expressed as nequiv. of ester hydrolysed/h per mg of protein.

Analytical methods

G.l.c. of fatty acid methyl esters was carried out on 6% (w/w) diethylene glycol succinate as described by Bajwa & Sastry (1972). Unless otherwise stated silica-gel plates were prepared by spreading 40 g of silica gel C in 80 ml of water by the procedure of Stahl (1965). The solvent systems used for the separation of lipid components on t.l.c. are indicated in the figure legends. Silicic acid paper chromatography was by the procedure of Marinetti (1962). Sugars and deacylation products of monogalactosyl diglyceride and digalactosyl diglyceride were separated by descending chromatography on Whatman no. 1 filter paper with ethyl acetate–pyridine–water (8:2:1, by vol.) as the solvent system for 72 h; spots were detected by spraying the paper with a freshly prepared mixture of 2% (w/v)

NaIO₄–1% (w/v) KMnO₄ (2:1, v/v) (Sastry & Kates, 1964a). The paper was washed with water after 15 min. Sugars in the lipid samples were measured by the phenol–H₂SO₄ method of Dubois *et al.* (1956) as described by Roughan & Batt (1968). The total sugars in the aqueous phase of the enzyme digest were determined by the procedure of Morris (1948), and reducing sugars were determined by the method of Nelson (1944). The acyl ester concentration was determined by the procedure of Snyder & Stephens (1959). Protein was measured by the method of Lowry *et al.* (1951). In the experiments on purification of the enzyme, protein was determined by measuring the *E*₂₈₀ with a Beckman spectrophotometer with 1 cm-light-path cells.

Results

Hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

In initial experiments carried out with rat tissues, fresh pancreatic homogenates hydrolysed 36 and 11% of the acyl ester bonds in monogalactosyl diglyceride and digalactosyl diglyceride respectively within 1 h. By contrast, homogenates of intestinal mucosa hydrolysed only 5% of the monogalactosyl diglyceride and no hydrolysis of digalactosyl diglyceride could be detected. However, the assay of enzymic activity with homogenates was complicated by the large amounts of endogenous lipids present, and therefore further experiments were conducted with extracts of acetone-dried powders.

Table 1. *Hydrolysis of glycosyl diglycerides by saline extracts of sheep pancreas acetone-dried powder*

The reaction mixture consisted of the substrate dissolved in 0.05 ml of methanol and dispersed in 0.2 ml of 0.1 M-Tris-maleate buffer, pH 7.2, and 0.4 ml of saline extract of sheep pancreas acetone-dried powder (containing 8.1 mg of protein in Expt. 1 and 6.1 mg of protein in Expt. 2). Where indicated, 0.1 ml of CaCl₂ (5 μmol in 0.1 M-Tris-maleate buffer, pH 7.2) and/or 0.1 ml of sodium deoxycholate (1.2 μmol in 0.1 M-Tris-maleate buffer, pH 7.2) was added. The total volume was adjusted to 0.8 ml with 0.1 M-Tris-maleate buffer, pH 7.2, and incubated at 37°C for the times shown.

Expt. no.	Substrate	Additions	Time (min)	Specific activity (nequiv. of ester hydrolysed/h per mg of protein)
1	Monogalactosyl diglyceride (0.85 μmol)	None	60	52.1
		+ CaCl ₂	60	52.1
		+ Sodium deoxycholate	60	139.7
		+ CaCl ₂ + sodium deoxycholate	60	83.5
	Digalactosyl diglyceride (1.03 μmol)	None	60	40.1
		+ CaCl ₂	60	72.0
		+ Sodium deoxycholate	60	175.0
		+ CaCl ₂ + sodium deoxycholate	60	68.0
2	Digalactosyl diglyceride (1.03 μmol)	+ Sodium deoxycholate	30	213.8
			60	207.0
	Galactosyl diglyceride (from <i>L. casei</i>) (0.63 μmol) + Sodium deoxycholate		30	42.6
			60	49.2

Saline extracts of sheep pancreas acetone-dried powder actively hydrolysed the acyl ester bonds in both monogalactosyl diglyceride and digalactosyl diglyceride (Table 1). The addition of Ca^{2+} showed essentially no effect on the hydrolysis of monogalactosyl diglyceride, but it enhanced the hydrolysis of digalactosyl diglyceride considerably. On the other hand, sodium deoxycholate enhanced the enzyme activity 2.5-fold with monogalactosyl diglyceride and more than fourfold with digalactosyl diglyceride. When Ca^{2+} and sodium deoxycholate were added together, the rate of hydrolysis of both substrates was much lower than that found with sodium deoxycholate alone. Under the latter conditions, hydrolysis of digalactosyl diglyceride was 1.25 times that of monogalactosyl diglyceride. Inhibition of the galactolipase by sodium deoxycholate and MgCl_2 when added together, but not separately, has also been observed with rat brain microsomal fraction (Subba Rao *et al.*, 1970). The pancreatic enzyme extract also showed activity towards galactosylglucosyl diglyceride isolated from *L. casei*, but the rate of hydrolysis was only one-fourth of that observed with digalactosyl diglyceride.

pH optimum of the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

The hydrolysis of both monogalactosyl diglyceride and digalactosyl diglyceride was optimal at pH 7.2 (Fig. 1) when Tris-maleate buffer was used and sodium deoxycholate (1.5mm) was present in the medium. This pH optimum agrees with that reported by Subba Rao *et al.* (1970) for the rat brain microsomal enzyme determined in the absence of sodium deoxycholate. But, this optimum is in contrast with the situation with the plant enzymes, where monogalactosyl diglyceride and digalactosyl diglyceride were hydrolysed at different pH values: usually monogalactosyl diglyceride hydrolysis showed a pH optimum between pH 6.0 and 7.0, whereas digalactosyl diglyceride hydrolysis occurred optimally between pH 4.5 and 5.6 (Sastry & Kates, 1964b; Helmsing, 1969; Galliard, 1971); however, these determinations were also made in the absence of sodium deoxycholate.

Identification of the products of the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

The chloroform-soluble products of hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride were analysed by t.l.c. with a solvent system for polar lipids (Fig. 2). During the hydrolysis of monogalactosyl diglyceride, monogalactosyl mono-

glyceride and unesterified fatty acids were formed. Similarly, with digalactosyl diglyceride, digalactosyl monoglyceride and unesterified fatty acids were formed. In addition, the formation of monogalactosyl diglyceride from digalactosyl diglyceride was also observed indicating the action of an α -galactosidase on this substrate. These results were confirmed by the silicic acid paper-chromatographic procedure of Marinetti (1962). The chloroform-soluble products of the hydrolysis of monogalactosyl diglyceride were also analysed by t.l.c. with a solvent system for non-polar lipids (Fig. 3). The formation of 2-monoacylglycerol and 1,2-diacylglycerol was observed, indicating the action of β -galactosidase on monogalactosyl diglyceride. A small amount of 1,3-diacylglycerol was also formed, probably owing to isomerization of the 1,2-diacylglycerol. However, monoacyl- and diacyl-glycerols were observed as hydrolytic products only when larger amounts of monogalactosyl diglyceride (2.0 μmol) were used as substrate.

Monogalactosyl monoglyceride was isolated by preparative t.l.c. of the lipid extract from enzyme digests of monogalactosyl diglyceride. It was hydrolysed with 2% (v/v) H_2SO_4 in a sealed tube at 100°C for 2h. The hydrolysate was extracted thrice with light petroleum (b.p. 40–60°C) and the extract was chromatographed on silica-gel t.l.c. plates prepared with 2% (w/v) boric acid in water by

Fig. 1. Effect of pH on the galactosyl diglyceride acyl hydrolase activity of saline extracts of sheep pancreas acetone-dried powder

The incubation mixture contained 1.09 μmol of monogalactosyl diglyceride (○) or 1.15 μmol of digalactosyl diglyceride (△) dissolved in 0.05 ml of methanol, 40 μmol of Tris-maleate buffer at different pH values, 1.2 μmol of sodium deoxycholate and 8.0 mg of protein in a final volume of 0.8 ml. The rest of the procedure was as described in the Materials and Methods section.

Fig. 2. Thin-layer chromatogram of chloroform-soluble products of the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

The reaction mixture contained $0.85\mu\text{mol}$ of monogalactosyl diglyceride or $1.03\mu\text{mol}$ of digalactosyl diglyceride dissolved in 0.05ml of methanol, $40\mu\text{mol}$ Tris-maleate buffer, pH 7.2, $1.2\mu\text{mol}$ of sodium deoxycholate and 8.1mg of protein in a final volume of 0.8ml . After incubation for the required time the reaction was stopped and the lipids extracted as described in the Materials and Methods section. The chloroform layer was chromatographed on t.l.c. with chloroform-methanol-acetic acid-water (170:25:25:3, by vol.) as solvent. Spots were located by exposure to I_2 vapour. (A)–(E) Hydrolysis products of monogalactosyl diglyceride: (A) authentic monogalactosyl diglyceride; (B) 30 min control; (C) 60 min control; (D) 30 min experimental; (E) 60 min experimental. (F)–(J) Hydrolysis products of digalactosyl diglyceride: (F) authentic digalactosyl diglyceride; (G) 30 min control; (H) 60 min control; (I) 30 min experimental; (J) 60 min experimental. Key: 1, fatty acids; 2, monogalactosyl diglyceride; 3, monogalactosyl monoglyceride; 4, digalactosyl diglyceride; 5, digalactosyl monoglyceride.

using hexane-diethyl ether-acetic acid (50:50:1, by vol.) as the solvent system. A major spot corresponding to 2-monoacylglycerol was observed on the chromatogram; this indicates that the pancreatic monogalactosyl diglyceride acyl hydrolase acts initially on the 1-ester linkage of glycosyl glycerides. This observation confirms the findings obtained by Noda & Fujiwara (1967).

The water-soluble products were analysed by paper chromatography after treatment of the aqueous phase with a mixture of Dowex 50 X8 (H^+ form) and Dowex 1 X8 (Cl^- form) to remove salts. Formation of galactosylglycerol and galactose from monogalactosyl diglyceride was observed. With

Fig. 3. Thin-layer chromatogram of chloroform-soluble products of hydrolysis of monogalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

(a) The reaction mixture was as described in Fig. 2, except that $1.7\mu\text{mol}$ of monogalactosyl diglyceride was used as substrate. The silica-gel plate was prepared with 2% (w/v) boric acid solution in water, spotted and developed in hexane-diethyl ether-acetic acid (50:50:1, by vol.). (b) The chromatogram was developed in hexane-diethyl ether-acetic acid (90:10:1, by vol.). Spots were located by exposure to I_2 vapour. Key: 1, triacylglycerol; 2, 1,3-diacylglycerol; 3, 1,2-diacylglycerol + unesterified fatty acids; 4, 2-monoacylglycerol; 5, 1-monoacylglycerol; 6, triacylglycerol; 7, unesterified fatty acids; 8, 1,3-diacylglycerol; 9, 1,2-diacylglycerol.

digalactosyl diglyceride, the formation of digalactosylglycerol as a major component and small amounts of galactosylglycerol and galactose were also observed.

Time-course studies on the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

The above studies showed that extracts of pancreas acetone-dried powder contained acyl hydrolases as well as galactosidases acting on glycosyl glycerides. The metabolic degradation of monogalactosyl diglyceride and digalactosyl diglyceride by these pancreatic extracts was therefore followed by determining the decrease in the acyl ester content as well as by the measurement of total and reducing sugars formed with time. The results (Fig. 4) show that 53 and 74% of the acyl ester bonds in monogalactosyl diglyceride were hydrolysed in 1 h and 2 h respectively. During this 2 h period of incubation the sugar released

Fig. 4. Time-course of the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride by saline extracts of sheep pancreas acetone-dried powder

The incubation mixture contained (a) $1.015\mu\text{mol}$ of monogalactosyl diglyceride or (b) $1.225\mu\text{mol}$ of digalactosyl diglyceride dissolved in 0.05ml of methanol, $40\mu\text{mol}$ of Tris-maleate buffer, pH 7.2, $1.2\mu\text{mol}$ of sodium deoxycholate and enzyme extract (8.0mg of protein) in a final reaction volume of 0.8ml for each time-interval. The rest of the procedure was as described in the Materials and Methods section. ○, Ester hydrolysed; △, total sugar liberated; ●, reducing sugar liberated.

was less than stoichiometric amounts, thus confirming that a part of the substrate was hydrolysed to the monogalactosyl monoglyceride stage only. It can be calculated from the data in Fig. 4, if it is assumed that the main pathway involves the initial hydrolysis of ester bonds, that 5.2% and 0.6% of the monogalactosyl diglyceride hydrolysed accumulated as monogalactosyl monoglyceride at 1 h and 2 h. Measurement of reducing sugars showed that 22.2 and 27% of monogalactosyl diglyceride were totally converted

into galactose and glycerol respectively. With digalactosyl diglyceride, the hydrolysis as determined by the decrease in the acyl ester content was 42% after 1 h and 52% after 2 h. Here again the release of total sugar was not stoichiometric. It can be calculated from the amount of total sugar released, without account being taken of the observed conversion of digalactosyl diglyceride into monogalactosyl diglyceride, that 22 and 18.5% of the substrate hydrolysed were present as digalactosyl monoglyceride at 1 h and 2 h respectively. Of the digalactosyl diglyceride hydrolysed 3.1 and 3.2% of the sugar components were found as reducing sugars after 1 h and 2 h respectively.

Attempts at the purification of sheep pancreatic acyl hydrolase

The various steps used for purification are described below and all operations were performed at $1-5^\circ\text{C}$.

Step 1. Sheep pancreas acetone-dried powder (0.5g) was mixed with 8.0ml of 0.05M -potassium phosphate buffer, pH 7.0, and kept at $0-5^\circ\text{C}$ overnight. It was then centrifuged at $1000g_{av}$ for 2 min and the supernatant was retained. It has been observed that phosphate-buffer extract showed much higher activity towards monogalactosyl diglyceride and a considerably lower activity towards digalactosyl diglyceride when compared with saline extract.

Step 2. To the supernatant (4.0ml) was added 0.8ml of 1% (w/v) protamine sulphate solution. This mixture was stirred, left to stand for 15 min and then centrifuged at $12000g_{av}$ for 10 min. The supernatant was retained.

Step 3. A column ($60\text{cm}\times 1.5\text{cm}$) of Sephadex G-100 was prepared and equilibrated with 0.05M -potassium phosphate buffer (pH 7.0). The supernatant from step 2 (4.5ml) was applied to the column and eluted with the same buffer at a flow rate of 30ml/h . Fractions (8.0ml) were collected and analysed for protein and enzyme activity. Three protein peaks were observed (Fig. 5). The acyl hydrolase activity closely followed the first protein peak when monogalactosyl diglyceride was used as substrate for the assay. Fractions containing monogalactosyl diglyceride acyl hydrolase activity were pooled.

Step 4. DEAE-cellulose was equilibrated with 0.05M -potassium phosphate buffer, pH 7.0, and packed in a column ($45\text{cm}\times 1.2\text{cm}$). The pooled fractions from step 3 were applied to the column and eluted with 50ml of the same buffer followed by a NaCl gradient. The gradient was achieved by using a mixing chamber containing 200ml of the buffer connected to a reservoir containing 200ml of 0.5M -NaCl in the same buffer. Fractions (8.0ml) were

Fig. 5. Gel-filtration pattern on a Sephadex G-100 column of sheep pancreatic acyl hydrolase in step 3 of the purification procedure

The supernatant after protamine sulphate treatment was applied to a column (60cm×1.5cm) of Sephadex G-100 prepared and equilibrated with 0.05M-potassium phosphate buffer, pH 7.0, and eluted with the same buffer. Fractions (8.0ml) were collected and analysed for their protein content (○) and enzyme activity with monogalactosyl diglyceride as substrate (△).

Fig. 6. Elution pattern of sheep pancreatic monogalactosyl diglyceride acyl hydrolase on a DEAE-cellulose column in step 4 of the purification procedure

Fractions 6–25 in step 3 of the purification procedure were pooled, applied to a DEAE-cellulose column (45cm×1.2cm) equilibrated with 0.05M-potassium phosphate buffer, pH 7.0, and eluted with 50ml of the same buffer and then with a linear NaCl gradient in the same buffer. Fractions (8.0ml) were collected and analysed for their protein content (○) and enzyme activity with monogalactosyl diglyceride as substrate (△). ----, NaCl gradient.

collected and assayed for protein and enzyme activity. The results obtained (Fig. 6), with monogalactosyl diglyceride as substrate, indicated that enzyme activity was present in fractions 28–38, although a

distinct protein peak coinciding with this activity was not observed. These fractions were pooled. The enzyme preparation at various steps of purification was tested for its activity to hydrolyse acyl

Table 2. Purification of sheep pancreatic monogalactosyl diglyceride acyl hydrolase

The reaction mixture contained substrate dissolved in 0.05 ml of methanol, 70 μ mol of potassium phosphate buffer, pH 7.0, 2.4 μ mol of sodium deoxycholate and enzyme in a reaction volume of 1.8 ml. The quantity of substrate used was 1.0 μ mol of monogalactosyl diglyceride, 1.0 μ mol of phosphatidylcholine, 0.6 μ mol of triacylglycerol or 3.8 μ mol of methyl oleate. The amount of enzyme used was 0.66 mg of protein in step 1, 0.55 mg of protein in step 2, 0.40 mg of protein in step 3, and 0.08 mg of protein in step 4. The boiled saline extract (1.28 mg of protein) was used as the source of co-lipase. One unit of activity is equal to 1 μ equiv. of ester hydrolysed/h and the specific activity is expressed as units/mg of protein.

Step	Total protein (mg)	Total activity (units)	Recovery (%)	Methyl oleate		Monogalactosyl diglyceride		Digalactosyl diglyceride		Phosphatidylcholine		Triacylglycerol	
				Specific activity	Purification	Specific activity	Purification	Specific activity	Purification	Specific activity	Purification	Specific activity	Purification
1. Buffer extract	57.5	63.3	100	1.3	1.0	1.1	1.0	0.03	1.0	0.2	1.0	0.3	1.0
2. Protamine sulphate	34.2	57.0	90	1.7	1.3	1.7	1.5	0.04	1.3	0.6	4.0	0.3	1.0
3. Sephadex G-100													
(a) Without co-lipase	14.4	38.4	60	3.1	2.5	2.6	2.3	0.05	1.6	0.9	5.6	0.5	1.6
(b) With co-lipase	14.4	56.2	89	3.9	3.0	3.1	3.5	0.26	8.5	2.6	16.5	3.4	10.8
4. DEAE-cellulose													
(a) Without co-lipase	5.4	46.8	72	6.2	4.1	8.7	7.9	1.23	40.6	4.9	31.2	8.7	27.6
(b) With co-lipase	5.4	53.2	84	12.4	9.8	9.9	9.0	2.46	81.2	7.5	47.7	15.0	47.6

Table 3. Distribution of galactosyl diglyceride acyl hydrolase activity in the pancreas and the intestine of sheep, rat and guinea pig

The incubation mixture consisted of 1.05 μ mol of monogalactosyl diglyceride or 1.3 μ mol of digalactosyl diglyceride dissolved in 0.05 ml of methanol, 40 μ mol of Tris-maleate buffer, pH 7.2, 1.2 μ mol of sodium deoxycholate and enzyme in a final volume of 0.8 ml. Saline extracts of acetone-dried powders of the tissues were used as enzyme sources and the amounts of enzyme added were: sheep intestine, 6.44 mg of protein; sheep pancreas, 8.0 mg of protein; rat intestine, 5.0 mg of protein; rat pancreas, 6.0 mg of protein; guinea-pig intestine, 4.4 mg of protein; guinea-pig pancreas, 3.3 mg of protein. The rest of the procedure was as described in the text.

Tissue source of enzyme	Specific activity (nequiv. of ester hydrolysed/h per mg of protein)	
	Monogalactosyl diglyceride	Digalactosyl diglyceride
Sheep pancreas	145.0	127.0
Sheep intestine	46.6	109.0
Rat pancreas	116.6	200.0
Rat intestine	40.0	180.0
Guinea-pig pancreas	273.0	515.1
Guinea-pig intestine	204.0	204.0

ester bonds in monogalactosyl diglyceride, digalactosyl diglyceride, methyl oleate, phosphatidylcholine and triacylglycerol (Table 2). In these experiments the enzyme concentration was chosen such that the activity was linear with time for the period of incubation.

It has been reported that for optimum activity pancreatic lipase requires a cofactor, described as 'co-lipase' (Morgan *et al.*, 1969; Julien *et al.*, 1972), which separated from the lipase during gel filtration, and that full activity can be restored by the addition of co-lipase (Maylié *et al.*, 1971). In the present study, the enzyme preparation after gel filtration and DEAE-cellulose column fractionation was therefore assayed in the presence and in the absence of co-lipase. Boiled extract of pancreatic acetone-dried powder (0.5 g in 10 ml of 0.9% NaCl boiled for 10 min and centrifuged at 1000 g_{av} for 2 min) was used as the source of co-lipase.

The partially purified enzyme after DEAE-cellulose fractionation showed a ninefold purification in terms of activity with monogalactosyl diglyceride and an 81-fold purification in terms of activity with digalactosyl diglyceride, both assayed in the presence of co-lipase. The addition of co-lipase caused only a marginal enhancement of activity with monogalactosyl diglyceride, but a twofold increase was noted with digalactosyl diglyceride. At all stages of purification the specific activity and the degree of purification were similar with methyl oleate and

monogalactosyl diglyceride. The partially purified enzyme, however, was active towards phosphatidylcholine as well as triacylglycerol. These results suggest that the enzyme preparation may contain more than one lipase activity. It also appears that the enzyme activity with methyl oleate and monogalactosyl diglyceride may be the same, whereas separate enzymes may be involved in the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride. Further separation and purification will be necessary to enable definite conclusions to be drawn with regard to the specificity of the glycosyl glyceride acyl hydrolases.

Distribution of galactosyl diglyceride acyl hydrolase activity in the pancreas and the intestine of different species

A comparative study was made of the enzyme activity in saline extracts of acetone-dried powder preparations from sheep, rat and guinea-pig pancreas and intestinal mucosa (Table 3). Of the species tested, guinea-pig tissues showed the highest activity with respect to both monogalactosyl diglyceride and digalactosyl diglyceride. In all the species, pancreas showed higher activity than did intestine.

Discussion

In the present study it has been shown that saline extracts of sheep pancreas acetone-dried powder contain an active glycosyl glyceride acyl hydrolase. In addition, evidence was obtained for the occurrence, in this tissue, of galactosidases that can degrade these glycolipids. Pancreatic lipase is known to be activated by bile salts and Ca^{2+} (Wills, 1965). It has been observed in the present work that sodium deoxycholate enhances the ester hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride 3–4-fold. By contrast Ca^{2+} , although having no action on monogalactosyl diglyceride hydrolysis, increased the rate of hydrolysis of digalactosyl diglyceride. However, when both sodium deoxycholate and Ca^{2+} were added together, there was considerably less hydrolysis than when sodium deoxycholate was used alone. The enzyme activity varied in the different pancreatic acetone-dried powder preparations, but usually the acyl hydrolase activities towards both monogalactosyl diglyceride and digalactosyl diglyceride were in the range 130–190 nequiv. of ester/h per mg of protein when assayed in the presence of sodium deoxycholate. In brain tissue the rate of hydrolysis of ester bonds in both monogalactosyl diglyceride and digalactosyl diglyceride has been reported to be about 1.6 nequiv. of ester/h per mg of protein (Subba Rao *et al.*, 1970). Thus the activity in pancreas is about 80 times that in brain. However, the specific activity of pancreatic galactosyl

glyceride acyl hydrolase is comparable with that of crude runner-bean extracts (Helmsing, 1969), but much less than that of the potato enzyme (Galliard, 1971). Unlike the plant enzymes the activity of the pancreas acyl hydrolase was maximum at pH 7.2 with either substrate when assayed in the presence of sodium deoxycholate.

An analysis of the lipid-soluble products clearly showed the formation of monogalactosyl monoglyceride and digalactosyl monoglyceride as intermediates in the hydrolysis of monogalactosyl diglyceride and digalactosyl diglyceride respectively. This confirms the observations made by Noda & Fujiwara (1967). This is in marked contrast with the hydrolysis of these lipids in plants, where the formation of monoacyl intermediates was not observed (Sastry & Kates, 1964b; Helmsing, 1969), except for minor amounts of monogalactosyl monoglyceride isolated from the products of monogalactosyl diglyceride hydrolysis by potato acyl hydrolase (Galliard, 1970). The formation of such an intermediate in the hydrolytic products of glycosyl glycerides has not been reported in brain. Further, monogalactosyl diglyceride was detected as a product in the hydrolysis of digalactosyl diglyceride, indicating the existence of α -galactosidase acting on this lipid in pancreas. A similar enzyme has previously been shown to be present in rat brain (Subba Rao & Pieringer, 1970). It has also been observed in the present study that monogalactosyl diglyceride is hydrolysed by a β -galactosidase, forming a diglyceride that is further broken down to monoglyceride and fatty acid, but this appears to be a minor pathway. Analysis of the water-soluble products by paper chromatography showed the presence of monogalactosyl- and digalactosyl-glycerol as well as of free sugars as products. From these observations and time-course studies, the probable pathways for metabolic degradation of glycosyl glycerides in pancreas may be envisaged as shown in Scheme 1.

Pancreas is well known to show lipolytic activity on a variety of substrates. Thus pancreatic juice will hydrolyse emulsified long-chain triacylglycerols, cholesteryl esters and phospholipids. This is attributed to the presence of glycerol ester hydrolase (EC 3.1.1.3), sterol ester hydrolase (EC 3.1.1.13) and phospholipase (EC 3.1.1.4) respectively. In addition, a carboxylic ester hydrolase (EC 3.1.1.1) that hydrolyses water-soluble esters (Szafran *et al.*, 1959) and another enzyme that is responsible for the hydrolysis of esters of secondary alcohols (Mattson & Volpenhein, 1966) are known to occur in this tissue. There are several reports of the separation and purification of various lipolytic enzymes (Wills, 1965). Two enzymes that hydrolyse glycerides have been purified from rat pancreatic juice (Morgan *et al.*, 1968). One of these hydrolyses emulsified triacylglycerol and monoacylglycerol and is probably

Scheme 1. Pathways for the enzymic degradation of glycosyl glycerides by pancreas

—, Predominant route; ———, minor route; ·····, reactions not observed.

the lipase (glycerol ester hydrolase, EC 3.1.1.3). The other hydrolyses micellar solutions of monoacylglycerol, triacylglycerol and cholesteryl esters. On the other hand, two molecular species with lipase activity have also been obtained from pig pancreas. These appear to be closely related in terms of their specific activities in hydrolysing long-chain and short-chain fatty acid esters of glycerol (Verger *et al.*, 1969). However, the hydrolysis of glycosyl glycerides with the purified enzymes was not determined in the studies of Morgan *et al.* (1968) or of Verger *et al.* (1969). Attempts were made in the present work to separate the pancreatic glycosyl glyceride acyl hydrolase from other lipolytic enzymes by using such treatments as protamine sulphate, gel filtration and DEAE-cellulose column chromatography. This procedure gave a partially purified preparation that showed an 8–9-fold higher specific activity towards monogalactosyl diglyceride. However, the partially purified preparation still hydrolysed methyl oleate, digalactosyl diglyceride, phosphatidylcholine and triacylglycerol. But the specific activity and degree

of purification as well as the effect of co-lipase, differed considerably with the various substrates at different stages of purification. This indicates that more than one acyl hydrolase activity may be present in the partially purified enzyme preparation. It is noteworthy that the acyl hydrolase activity towards monogalactosyl diglyceride and methyl oleate showed similar specific activities at various stages of purification. This suggests that these two substrates may be hydrolysed by the same enzyme.

Glycosyl glyceride acyl hydrolase activity seems to be widely distributed in animal tissues and has now been found in the pancreas and intestines of rat, sheep and guinea pig. It is significant that the guinea-pig tissues showed much higher activity than did the sheep or rat tissues. From these results it may be concluded that pancreatic tissue is equipped with enzymes necessary for the complete degradation of glycosyl glycerides. In addition, intestinal mucosa is also capable of hydrolysing the ester bonds in these glycolipids. In ruminants, the hydrolysis of the acyl ester bonds of galactosyl glycerides as well as the

extensive hydrogenation of unsaturated fatty acids by rumen micro-organisms has been reported (Viviani, 1970). It is therefore reasonable to suppose that glycosyl glycerides obtained from plant foods are digested by animals either in rumen or in the gastrointestinal tract.

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