Biochem. J. (1971) **121**, 379–390
Printed in Great Britain

The Enzymic Deacylation of Phospholipids and Galactolipids in Plants

PURIFICATION AND PROPERTIES OF A LIPOLYTIC ACYL-HYDROLASE FROM POTATO TUBERS

By T. GALLIARD
Agricultural Research Council Food Research Institute, Colney Lane, Norwich, NOR 70F, U.K.

(*Received* 27 *July* 1970)

An enzyme preparation that catalyses the deacylation of mono- and di-acyl phospholipids, galactosyl diglycerides, mono- and di-glycerides has been partially purified from potato tubers. The preparation also hydrolyses methyl and *p*-nitrophenyl esters and acts preferentially on esters of long-chain fatty acids. Triglycerides, wax esters and sterol esters are not hydrolysed. The same enzyme preparation catalyses acyl transfer reactions in the presence of alcohols and also catalyses the synthesis of wax esters from long-chain alcohols and free fatty acids. Gel filtration, DEAE-cellulose chromatography and free-flow electrophoresis failed to achieve any separation of the acyl-hydrolase activities towards different classes of acyl lipids (phosphatidylcholine, monogalactosyl diglyceride, mono-olein, methyl palmitate and *p*-nitrophenyl palmitate) or any separation of these activities from a major protein component. For each class of lipid the acyl-hydrolase activity was subject to substrate inhibition, was inhibited by relatively high concentrations of di-isopropyl phosphorofluoridate and the pH responses were changed by Triton X-100. The hydrolysis of phosphatidylcholine was stimulated 30–40-fold by Triton X-100. The specific activities of the potato enzyme with galactolipids were at least 70 times higher than those reported for a homogeneous galactolipase enzyme purified from runner bean leaves. The possibility that a single lipolytic acyl-hydrolase enzyme is responsible for the deacylation of several classes of acyl lipid is discussed.

Little is known at present about the mechanisms of catabolism of polar lipids in higher plants. Phospholipase D (phosphatidylcholine phosphatidohydrolase; EC 3.1.4.4) occurs widely in the plant kingdom (Kates, 1960; Quarles & Dawson, 1969*a*) but enzymes that deacylate phospholipids have not been well characterized (Kates, 1960; Ansell & Hawthorne, 1964). Phospholipase A (phosphatide acyl-hydrolase; EC 3.1.1.4) and lysophospholipase (lysolecithin acyl-hydrolase; EC 3.1.1.5) have been reported to occur in barley and rice grains (Contardi & Ercoli, 1933); no confirmation of this early work is available. The enzymic deacylation of galactosyl diglycerides has been described in leaves of some *Phaseolus* species (Sastry & Kates, 1964) and spinach (Helmsing, 1967) and a galactolipase has been purified from leaves of *Phaseolus multiflorus* (Helmsing, 1969). However, the substrate specificity of the galactolipase has not been well defined.

Homogenization of potato tuber tissue results in a rapid enzymic breakdown of endogenous phospholipids and galactolipids to form free fatty acids and fatty acid hydroperoxides (Galliard, 1970). Lipolytic acyl-hydrolase activity and lipoxygenase were responsible for the observed effects and it became increasingly likely during the course of the work that these naturally occurring lipids, as well as some other classes of acyl lipids, were hydrolysed by a single enzyme from the tubers.

EXPERIMENTAL

Materials

Potato tubers (*Solanum tuberosum*, var. Majestic) were obtained at commercial harvest and stored for 1–6 months at 10°C.

Phosphatidylcholine and esterified sterol glucoside were isolated from potato tuber lipids (Galliard, 1968); mono- and di-galactosyl diglycerides were prepared from spinach leaf lipids (Galliard, 1969). Phosphatidylglycerol was purified from an acetone-insoluble fraction from cabbage leaf lipids by chromatography on DEAE-cellulose columns (Galliard, 1968). Phosphatidylethanolamine (from egg lipids), lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphorylcholine) and phosphatidic acid prepared from egg phosphatidylcholine with phospholipase A and phospholipase D respectively were purchased from Koch–Light

Laboratories Ltd., Colnbrook, Bucks., U.K. Mono-olein (glycerol 1-monoleate), diolein (a mixture of glycerol 1,2-dioleate and glycerol 1,3-dioleate) and triolein (glycerol trioleate) were obtained from Phase Separations Ltd., Queen's Ferry, Flints., U.K., and were purified on columns of silicic acid by the procedure of Hirsch & Ahrens (1958). Ethyl stearate was prepared by acid-catalysed esterification of stearic acid with excess of ethanol and crystallization of the product. Octadecyl acetate and octadecyl palmitate were prepared by treating octadecanol with the respective acid chlorides (Swell & Treadwell, 1955) and the esters formed were extracted by the method described by Deykin & Goodman (1962). Methyl esters of long-chain fatty acids were purchased as such or were prepared from the corresponding fatty acids with diazomethane. The 2-naphthyl and *p*-nitrophenyl esters were purchased from Sigma Chemical Co. St Louis, Mo., U.S.A. The purity of all lipid substrates was checked by t.l.c.; all preparations used were at least 90% pure on this basis.

[1-^{14}C]Octadecanol was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Tetraethyl pyrophosphate and diethyl *p*-nitrophenyl thiophosphate were kindly given by the Murphy Chemical Co. Ltd., Wheatampstead, St Albans, Herts., U.K. Pure proteins and enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K., except for soya-bean lipoxygenase (Sigma Chemical Co.). Triton X-100 (scintillation grade) was purchased from Koch–Light Laboratories Ltd. All solvents were redistilled before use.

Methods

Preparation of substrate dispersions. For routine assays in the presence of Triton X-100, the lipid substrate and Triton X-100 (2mg/μequiv. of ester in substrate) were mixed in chloroform solution. After removing the solvent under a stream of N_2, the residual mixture was warmed with a few drops of water to obtain an emulsion; further water or buffer solution was then added slowly with continuous agitation on a vortex mixer until the required volume was achieved (normally 2μequiv. of ester and 4mg of Triton X-100/ml). This method produced stable optically clear dispersions of substrate. In experiments without detergent, dispersions of substrate in aqueous media were obtained by ultrasonic treatment (Galliard, 1970).

Incubation systems. When a recording spectrophotometer was used to follow the hydrolysis of *p*-nitrophenyl esters, the total volume in the cell was 3ml; in all other incubation systems the final volume was 2.4ml.

Enzyme assays. Continuous measurement of the *p*-nitrophenol released by hydrolysis of *p*-nitrophenyl esters was obtained with a recording spectrophotometer and the E_{400} related to a standard curve for *p*-nitrophenol measured at the pH of the incubation. Most assays were performed by analysis of the reaction products at the end of the 10min incubation period. Lipid substrates, except *p*-nitrophenyl and 2-naphthyl esters, were extracted and the ester content was assayed by the method of Galliard (1970). *p*-Nitrophenyl esters were extracted with 0.1M-glycine–NaOH buffer, pH9.5, in place of the acetate buffer and the *p*-nitrophenol in the upper aqueous methanol phase was determined from the E_{400} after centrifuging the solution. A standard curve was constructed with known amounts of *p*-nitrophenol. Naphthol released by hydrolysis of 2-naphthyl esters was determined by coupling with tetra-azotized *o*-dianisidine (Seligman & Nachlas, 1963). To determine the effect of substrate concentration on hydrolysis rates, hydrolysis was measured by analysis of liberated free fatty acids (Duncombe, 1963). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Radioactivity counting. Radioactive bands on t.l.c. plates were detected and the radioactivity was counted by using an automatic thin-layer-chromatogram scanner (Panax Equipment Ltd., Redhill, Surrey, U.K.).

Disc electrophoresis. The methods of Ornstein (1964) and Davis (1964) were used. Polyacrylamide (7%, w/v) gels (7cm × 0.5cm) were formed by chemical polymerization. Samples containing approx. 20μg of protein were applied in 10–20μl of 25% (w/v) sucrose solution. Electrophoresis was performed in 0.025M-tris–glycine buffer, pH8.3, at 4mA/gel and stopped when a Bromophenol Blue marker band applied to a parallel separate gel had travelled the length of the gel. Protein was detected by staining the gel with 0.25% Coomassie Blue in acetic acid–methanol–water (7:25:70, by vol.). Esterase activity in the developed gels was detected by incubating the gels with 2-naphthyl acetate and tetra-azotized *o*-dianisidine (Paul & Fottrell, 1961). Attempts to use long-chain fatty acid esters of 2-naphthol as substrates with acrylamide gels have been only partially successful. Positive results have been obtained by adding 2-naphthyl laurate and Triton X-100 to the gel substrates before polymerization and treating the developed gel after electrophoresis with tetra-azotized *o*-dianisidine; under these conditions coincident areas of acyl-hydrolase activity and protein were obtained but resolution was less satisfactory and high background staining was obtained. As shown below acetate esters were hydrolysed at a lower rate by the same lipolytic acyl-hydrolase enzyme preparation.

Free-flow electrophoresis. This was performed at 4°C with the continuous-electrophoresis apparatus developed by Hannig (1964) (Elphor model VaP II; Bender und Hobgin G.m.b.H., München, Germany). Samples were applied at entry point 6 (above collection tube 35) at a rate of approx. 2ml/h in 1–2ml of the electrophoresis buffer, namely 0.1M-tris–0.05M-acetic acid, pH8.3. An applied voltage of 1725V (35.5V/cm) produced a current of 170mA. The total flow-rate was 100ml/h and the eluate was collected in 50 fractions. The electrophoresis was stopped after 90min when the volume of each fraction was approx. 3ml.

RESULTS

Purification of lipolytic acyl-hydrolase

The purification stages used in the present work are summarized in Table 1. All operations were at 0–1°C unless otherwise stated.

Stage 1. Potato tubers were peeled, washed, diced and homogenized for 1min with 2 vol. of 2mM-sodium metabisulphite. The homogenate was left for 15min to precipitate starch, then filtered through Miracloth (Evans, Adlard and Co. Ltd., Cheltenham, Gloucs., U.K.).

Table 1. *Purification of lipolytic acyl-hydrolase activity from potato tuber*

Stage	Total protein (mg)	Total activity (units*)	Recovery (%)	Sp. activity (units*/mg of protein)	Purification
(1) Homogenate	915	2620	100	2.87	1.0
(2) High-speed supernatant	798	2540	97	3.19	1.1
(3) Acetone precipitation (80%, v/v)	293	1150	44	3.92	1.4
(4) Sephadex G-150 column (fractions 56–66)	85	990	38	11.6	4.0
(5) First DEAE-cellulose column (fractions 51–61)	51	640	24	12.5	4.4
(6) Second DEAE-cellulose column (fractions 20–28)	31	425	16	13.7	4.8
(7) Suspensions in 2.2M-$(NH_4)_2SO_4$	22.7	352	13.5	15.5	5.4

* *p*-Nitrophenyl palmitate as substrate.

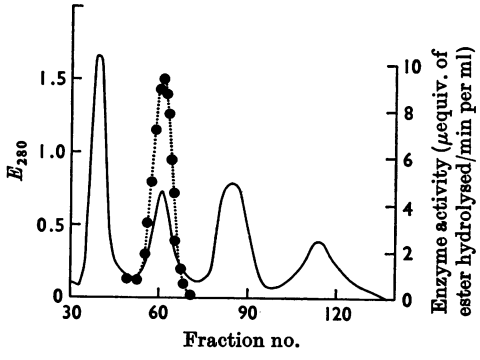

Fig. 1. Gel filtration of acetone-dried preparation on Sephadex G-150. Fractions (15ml) were collected and assayed for protein content (——, E_{280}) and acyl-hydrolase activity with *p*-nitrophenyl palmitate as substrate (●). Fractions 56–66 were combined for further purification.

Stage 2. The filtrate from stage 1 was centrifuged for 90min at 65000*g*. The clear colourless supernatant was retained.

Stage 3. Acetone (4vol.) at −50°C was stirred into the supernatant from stage 2. The precipitated material was collected by centrifugation, washed twice with dry acetone and finally with diethyl ether. The resulting powder was stored under anhydrous conditions at 0°C.

Stage 4. A column (90cm×4.5cm) of Sephadex G-150 was equilibrated with 0.05M-potassium phosphate buffer, pH7.0. The powder from stage 3 was dissolved in 4.4ml of 0.05M-potassium phosphate buffer, pH7.0, to give a slightly opaque solution and applied to the column. The same buffer solution was passed through the column at a flow rate of 30ml/h (2ml/h per cm^2). Fractions (15ml) were collected and analysed for protein content and enzyme activity. Fig. 1 illustrates the separation obtained; the acyl-hydrolase activity closely followed the protein content of one peak in the elution pattern when *p*-nitrophenyl palmitate, monogalactosyl diglyceride or phosphatidylcholine was used as substrate for the assay, and in all cases a single peak of activity was obtained in the same position. Fractions containing the acyl-hydrolase activity were combined.

Stage 5. DEAE-cellulose (Whatman DE-23, H. Reeve Angel and Co. Ltd., London E.C.4, U.K.) was pre-cycled according to the manufacturers' instructions, equilibrated with 0.05M-potassium phosphate buffer, pH6.5, and packed into a column (20cm×1.5cm). The enzyme solution from stage 4 was adjusted to pH6.5 and applied to the column at a flow rate of 30ml/h. After washing the column with 30ml of 0.05M-potassium phosphate buffer, pH6.5, a linear sodium chloride gradient was used to elute the enzyme. The gradient was achieved by using a mixing chamber containing 0.05M-potassium phosphate buffer, pH6.5 (150ml), connected with a siphon to a reservoir containing 0.5M-sodium chloride in 0.05M-potassium phosphate buffer, pH6.5 (150ml). Fractions (5.5ml) were collected at a flow rate of 30ml/h and assayed for protein content and acyl-hydrolase activity. Fig. 2 shows the results obtained with *p*-nitrophenyl palmitate as substrate. Again, a close relationship between protein content in the major peak and enzyme activity was obtained. Fractions containing acyl-hydrolase activity were combined and dialysed overnight against 0.05M-potassium phosphate buffer, pH6.5 (3litres).

Stage 6. The preparation from stage 5 was subjected to chromatography on a second DEAE-cellulose column under conditions identical with those used in stage 5, but little purification was achieved (Table 1). Moreover, there appeared to be some modification of the enzyme since re-chromatography caused some spreading of both protein and enzyme activity. Fractions containing the main peak of enzyme activity were combined.

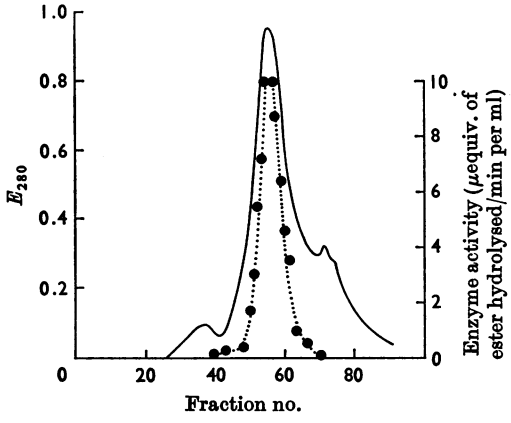

Fig. 2. DEAE-cellulose chromatography of enzyme preparation after gel filtration. After loading the sample, 30ml of 0.05M-potassium phosphate buffer, pH6.5, was passed through the column before commencing a sodium chloride gradient at fraction 33. Fractions (5.5ml) were collected and assayed for protein content (——, E_{280}) and acyl-hydrolase activity with *p*-nitrophenyl palmitate as substrate (●). Fractions 51–61 were combined and dialysed against 0.05M-potassium phosphate buffer, pH6.5, for 16h.

Stage 7. Concentration by freeze-drying or ammonium sulphate precipitation was unsatisfactory and the solution from stage 6 was concentrated tenfold by dialysis against aq. 50% (w/v) polyethylene glycol 4000 (Shell Chemical Co. Ltd., London S.E.1, U.K.). The cellulose tubing containing the concentrated enzyme solution was washed several times with water to remove polyethylene glycol before dialysis against 2.2M-ammonium sulphate, pH7.0, for 16h.

The enzyme preparation thus obtained was stored at 4°C and used as the purified enzyme. The preparation was a turbid suspension which, on dilution, gave a clear solution. A small increase in specific activity of the enzyme was consistently obtained by the final concentration procedure (Table 1).

Free-flow electrophoresis. A portion of the purified enzyme preparation was subjected to free-flow electrophoresis, but protein content and acyl-hydrolase activity of the fractions again showed coincidence between the major protein peak and enzyme activity towards phosphatidylcholine, monogalactosyl diglyceride, mono-olein and *p*-nitrophenyl palmitate. A small but significant amount of acyl-hydrolase activity was associated with a small protein peak in fractions remote from the major peak and separate from it.

Polyacrylamide-gel electrophoresis. The purified enzyme preparation was subjected to disc electrophoresis on polyacrylamide gels and, after staining for protein with Coomassie Blue and for acyl-hydrolase activity with 2-naphthyl acetate and tetra-azotized *o*-dianisidine, showed very similar patterns of protein and enzyme distribution (Fig. 3).

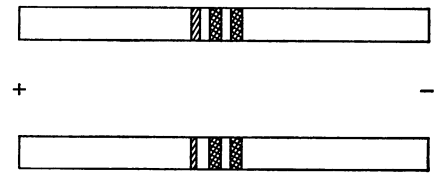

Fig. 3. Disc electrophoresis on 7% (w/v) polyacrylamide gels of purified lipolytic acyl-hydrolase preparation. Samples containing 20μg of protein were subjected to electrophoresis in 0.025M-tris–glycine buffer, pH8.3.

Determination of molecular size of the enzyme by gel filtration. The column of Sephadex G-150 used in the purification procedure was calibrated by applying a mixture of proteins of known molecular weights and developing the column as described above. A linear relationship was obtained between elution volume and log (molecular weight) for the following: cytochrome *c* from horse heart, mol.wt. 12400 (Margoliash, 1962), assayed by E_{412}; bovine serum albumin, mol.wt. 67000 (Phelps & Putnam, 1960), assayed by E_{280}; lipoxygenase (EC 1.13.1.13) from soya beans, mol.wt. 102000 (Theorell, Holman & Akeson, 1947), assayed with linoleic acid (Galliard, 1970); pyruvate kinase (EC 2.7.1.40) from rabbit muscle, mol.wt. 237000 (Warner, 1958), assayed by E_{280}; the void volume of the column was determined with Blue Dextran, mol.wt. 2×10^6. The elution of the peak of lipolytic acyl-hydrolase activity corresponded to that of a globular protein with a molecular weight of 107000.

Reactions catalysed by the lipolytic acyl-hydrolase

Hydrolysis of compounds containing fatty acyl ester bonds. The acetone-dried preparation and the purified enzyme preparation were examined for catalytic activities in the deacylation of a range of lipid classes; phospholipids, galactolipids and several other classes of acyl lipid were hydrolysed (Table 2). The diacyl phospholipids studied were hydrolysed at similar rates but all were significantly lower than that at which monoacylphosphatidylcholine (lysophosphatidylcholine) was hydrolysed. Glycerides were hydrolysed in the order monoglyceride > diglyceride > triglyceride; monoglycerides were the most labile substrates investigated with this system.

With methyl esters of C_{18} fatty acids, there was a small increase in activity with increased unsatura-

Table 2. *Substrate specificity of lipolytic acyl-hydrolase activity*

Incubation mixtures contained substrates (2 μequiv. of ester), Triton X-100 (4mg), potassium phosphate (240 μmol) and enzyme preparation (sufficient, where possible, to liberate 1 μequiv. of fatty acid in 10min) in a total volume of 2.4ml at pH 7.5. The mixtures were incubated at 25°C for 10min.

	Enzyme activity (units/mg of protein)	
	Purified enzyme	Acetone-dried powder
Substrate		
Phosphatidylcholine	5.1	1.2
Lysophosphatidylcholine	28.0	
Phosphatidylethanolamine	4.2	
Phosphatidylglycerol	4.8	
Phosphatidic acid	4.8	
Monogalactosyl diglyceride	12.0	3.0
Digalactosyl diglyceride	6.8	1.2
Mono-olein	39.0	9.4
Diolein	8.2	2.0
Triolein	<0.1	<0.01
Tributyrin	<0.2	<0.06
Methyl palmitate		4.0
Methyl stearate		1.3
Methyl oleate	10.8	2.1
Methyl linoleate		2.7
Methyl linolenate		3.0
Methyl behenate		0.3
Ethyl stearate		1.1
Octadecyl acetate		<0.01
Octadecyl palmitate		<0.01
p-Nitrophenyl stearate	9.0	
p-Nitrophenyl palmitate	15.5	3.9
p-Nitrophenyl laurate	9.3	
p-Nitrophenyl octanoate	17.9	
p-Nitrophenyl acetate	0.14	0.02
2-Naphthyl myristate		0.9
2-Naphthyl acetate		0.05
Cholesteryl oleate		<0.01
Esterified sterol glucoside		<0.01

tion and a decrease of activity with higher-chain-length acids. With *p*-nitrophenyl esters of fatty acids, C_{16} and C_8 acids showed similar rates of hydrolysis whereas the ester of the C_{12} acid was hydrolysed more slowly; *p*-nitrophenyl acetate was a relatively poor substrate. Although the above results suggest a low substrate specificity of the acyl-hydrolase, some specificity with respect to the alcoholic moiety of the carboxyl ester obviously exists. For example, fatty acid esters of long-chain alcohols or of sterols were not hydrolysed at a detectable rate (Table 2). Also, esterified glucoside, in which the fatty acid is esterified to position 6 of the glucose moiety, was not hydrolysed. The specificity for a long-chain acyl moiety in the substrate ester, in contrast with a long-chain alcoholic group, is illustrated by the marked difference in rates of hydrolysis of ethyl stearate and octadecyl acetate (Table 2).

When the activity of the purified enzyme preparation towards a range of lipid substrates was compared with that of the crude acetone-dried preparation, a similar degree of purification with respect to phospholipids, galactolipids, monoglycerides and the methyl and *p*-nitrophenyl esters of fatty acids was observed (Table 3). Also, the activity towards *p*-nitrophenyl acetate, although lower than towards long-chain esters, was increased by a similar factor in the purified preparation. On the other hand, although the acetone-dried preparation contained significant activity towards *p*-nitrophenyl phosphate at acid pH, the purified acyl-hydrolase preparation showed no activity towards this phosphate ester. Several phosphate ester hydrolase activities have been identified in potato tubers and have been shown, by electrophoresis, to be distinct from carboxyl ester hydrolase activities (Jaaska, 1969).

Synthesis of wax esters. The formation of wax esters by an enzyme preparation from broccoli leaves and from potato tubers was shown to occur by the reverse reaction of an acyl-hydrolase when a long-chain acid and a long-chain alcohol were used as substrates (Kolattukudy, 1967). Both the acetone-dried preparation and the purified lipolytic acyl-hydrolase preparation catalysed the formation of octadecyl palmitate from octadecanol and palmitic acid (Table 4). Examination of the reaction products by t.l.c. showed that radioactivity was present only in the wax ester fraction and in unchanged octadecanol. Some evidence for the possibility that the same enzyme is involved in wax synthesis and lipolytic deacylation is provided by the fact (Table 4) that the ratio of activities of the purified enzyme preparation and the acetone-dried powder for wax synthesis is similar to that obtained for lipid hydrolysis (Table 3).

Acyltransferase activity. Galliard (1970) established that an acyltransferase activity was associated with the acyl-hydrolase activity in extracts of potato tuber. The purified lipolytic acyl-hydrolase preparation described above was found to exhibit acyltransferase activity. When methanol (0.2ml) was added to incubation systems described above for assay of lipolytic acyl-hydrolase activity on phosphatidylcholine, galactosyl diglycerides and mono-olein, the incubation products contained fatty acid methyl esters in addition to free fatty acids. Thus the acyltransferase activity may be associated with the same enzyme responsible for the breakdown and formation of fatty acyl ester bonds.

Table 3. *Hydrolysis of various substrates catalysed by enzyme preparations from potato tubers*

Incubation mixtures with acyl ester substrates contained substrate (2μequiv. of ester), Triton X-100 (4mg) and enzyme as indicated in 0.1M-potassium phosphate buffer, pH7.5. With *p*-nitrophenyl phosphate (2μmol) Triton was omitted and 0.1M-sodium acetate buffer, pH5.0, was used. Incubation was for 10min in a total volume of 2.4ml.

	Acetone-dried powder preparation			Purified acyl-hydrolase preparation			
Substrate	Enzyme added (μg of protein)	Ester hydrolysed (μequiv./10min)	Sp. activity (units)	Enzyme added (μg of protein)	Ester hydrolysed (μequiv./10min)	Sp. activity (units)	Purification factor*
Phosphatidylcholine	80	0.93	1.16	20	1.02	5.1	4.4
Monogalactosyl diglyceride	16	0.48	3.00	4	0.48	12.0	4.0
Mono-olein	16	1.50	9.40	4	1.55	39.0	4.2
Methyl oleate	40	0.83	2.10	10	1.08	10.8	5.2
p-Nitrophenyl palmitate	16	0.63	3.90	4	0.62	15.5	4.0
p-Nitrophenyl acetate	800	0.17	0.02	133	0.18	0.14	7.0
p-Nitrophenyl phosphate	160	0.17	0.11	133	0	0	—

* Purification factor; sp. activity of purified preparation/sp. activity of acetone dried preparation.

Table 4. *Formation of wax ester from palmitic acid and* [1-^{14}C]*octadecanol catalysed by enzyme preparations from potato tubers*

Duplicate incubation mixtures contained [1-^{14}C]octadecanol (0.5μCi; 0.02μmol), palmitic acid (0.1μmol), Triton X-100 (0.5mg), potassium phosphate (240μmol) and enzyme in a total vol of 2.4ml at pH7.5. After incubation for 2h at 25°C, non-radioactive octadecanol and palmitic acid (1μmol each) were added as carriers and the total lipid products extracted into chloroform. Approx. 10% of each lipid extract was applied to a t.l.c. plate, which was developed in light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (50:50:1, by vol.). Radioactivity was counted by automatic scanning; octadecanol, palmitic acid and octadecyl palmitate bands were detected under u.v. light after spraying the t.l.c. plates with dichlorofluorescein. Results of duplicate incubations are shown.

Enzyme preparation	Enzyme added (μg of protein)	^{14}C radioactivity in t.l.c. bands (c.p.m.) Octadecanol	Wax ester	Conversion into wax ester (% of total radioactivity)	Ester formed* (μequiv./2h per mg of protein)	Mean	Purification factor†
Acetone-dried powder	160	4925	1346	21.6	27	25	
		4797	1137	19.2	23		
							5.2
Purified enzyme	30.8	6973	1674	19.4	127	130	
		5672	1464	20.6	133		
Enzyme omitted	—	7194	0	0	0		—

* Assumes ester formation is proportional to enzyme concentration.
† Activity of purified enzyme/activity of acetone-dried powder.

Properties of the lipolytic acyl-hydrolase

Effects of pH *and wetting agents.* In studies with potato tuber extracts Galliard (1970) the deacylation of phospholipids and galactolipids was obtained with substrate dispersions prepared by sonication in aqueous media. In the present work, to study the substrate specificity of the acyl-hydrolase, sonication was not satisfactory with the less polar lipids. The use of Triton X-100 as a dispersing agent facilitated the preparation of stable emulsions in aqueous media and gave optically clear preparations at the concentrations used.

With most lipid substrates, the minimum amount of Triton X-100 that would produce a clear dispersion of substrate in aqueous media was 1–2mg of Triton/μequiv. of ester in the substrate; substrate dispersions have been prepared with 2mg of Triton X-100/μequiv. of ester as a routine. Excess of Triton was inhibitory. Studies on the progress

curves of the enzymic hydrolysis of *p*-nitrophenyl palmitate (Fig. 4) illustrate this effect. Optimum amounts of substrate and Triton X-100 gave a progress curve that was linear after the first minute of incubation, but which showed a slight lag phase (curve *A*, Fig. 4); decreasing the amount of Triton (curve *D*) accentuated the lag phase; increasing the amount of Triton (curve *B*) decreased the lag phase but also decreased the linear rate. The lag phase was also shortened when the substrate concentration was lowered (curve *E*). Increased substrate concentrations (curve *C*) produced an increased lag phase and a lower linear rate; this substrate-inhibition effect is considered in the Discussion section. Under the conditions used for routine assay of lipolytic acyl-hydrolase activity with the other substrates (see the Experimental section), progress curves were linear for at least 10 min.

The use of Triton X-100 resulted in changes in the pH characteristics for the enzymic deacylation of the lipids. When sonicated substrate preparations were used in the absence of Triton X-100, maximal rates of deacylation were obtained at pH values less than 6.0 (Fig. 5). The pH curves for deacylation of the two galactolipids and of mono-olein were obtained by using an acetone-dried preparation of the enzyme because this preparation had little activity towards phosphatidylcholine in the absence of detergent (see below); the pH curve for the phospholipid was obtained with a crude 15000*g* supernatant from potato tubers, as described by Galliard (1970). The pH characteristics shown in Fig. 5 are similar to those observed for the breakdown of endogenous phospholipids and galactolipids in crude homogenates of potato tubers (Galliard, 1970). However, when Triton X-100 was used to disperse the substrate, the pH characteristics were markedly changed for all substrates investigated. Fig. 6 shows pH responses obtained with phosphatidylcholine, mono- and di-galactosyl diglyceride, mono-olein and also *p*-nitrophenyl stearate, which was not dispersed satisfactorily without added detergent. With all substrates, maximal rates of deacylation were observed at pH values above 7.0. The effects of added Triton X-100 and of pH for some substrates are summarized in Table 5. Phosphatidylcholine, which in the absence of Triton X-100 was hydrolysed only slowly by both acetone-dried preparations and the purified acyl-hydrolase, was deacylated 30–40 times more rapidly when the detergent was present. The same stimulation by Triton X-100 was obtained when phosphatidylcholine was emulsified with the detergent or when Triton was added to the substrate dispersed by sonication. Other

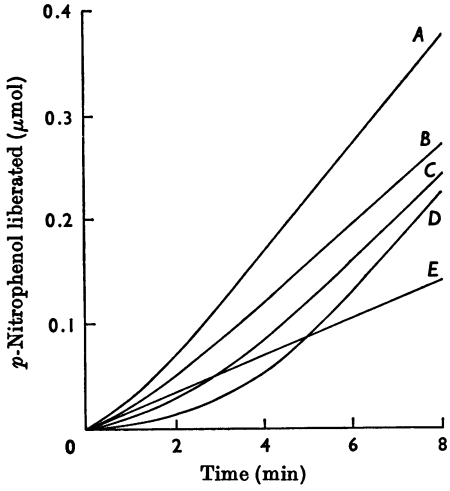

Fig. 4. Progress curves for the enzymic hydrolysis of *p*-nitrophenyl palmitate. Incubation mixtures (total volume of 3 ml in 1 cm cells at 25°C) contained purified enzyme preparation (2.7 μg of protein), potassium phosphate buffer, pH 7.5 (0.3 mmol), *p*-nitrophenyl palmitate as follows: 0.67 mM (*A*, *B* and *D*), 2 mM (*C*) and 0.13 mM (*E*), and Triton X-100 as follows: 1.33 mg/ml (*A*, *C* and *E*), 6 mg/ml (*B*) and 0.67 mg/ml (*D*).

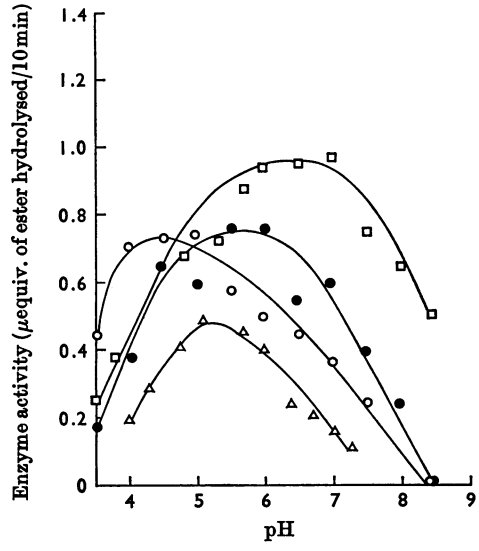

Fig. 5. pH-response curves for lipolytic acyl-hydrolase activity. Incubation mixtures contained substrate (2 μequiv.) and sufficient acetone-dried preparation to hydrolyse 0.5–1.5 μequiv. of ester at optimum pH in a 10 min incubation at 25°C. The following buffer solutions were used (0.1 M): sodium acetate (pH 3.5–5.5); potassium phosphate (pH 6.0–7.0); tris–HCl (pH 7.5–9.5). Incubation volume was 2.4 ml. Substrates used were: ●, mono-galactosyl diglyceride; ○, digalactosyl diglyceride; △, phosphatidylcholine; □, mono-olein.

substrates investigated showed less stimulation of hydrolysis at pH 7.5 by Triton X-100 than did phosphatidylcholine (Table 5). The effect of Triton is unlikely to be due solely to a decrease in the size of substrate micelles, because the greatest effect was observed with phosphatidylcholine, which forms water-clear aqueous dispersions on sonication, and little stimulation occurred with monogalactosyl diglyceride, even though the detergent produced water-clear dispersions compared with the opaque preparations produced by sonication.

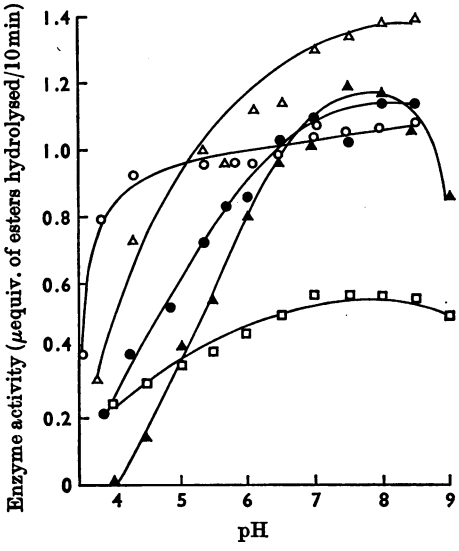

Fig. 6. Effect of Triton X-100 on pH-response curves for lipolytic acyl-hydrolase activity. Conditions were as described in Fig. 5 except for addition of Triton X-100 (4mg) to each incubation mixture. Substrates used were: ●, monogalactosyl diglyceride; ○, digalactosyl diglyceride; △, phosphatidylcholine; □, mono-olein; ▲, *p*-nitrophenyl stearate.

The rate of hydrolysis was increased fivefold when 2-naphthyl laurate was dispersed in Triton X-100 instead of acetone (Seligman & Nachlas, 1963) by using an acetone-dried preparation from potato tuber. Other wetting agents were tested in this system at the same concentration as that used for Triton (i.e. 4mg/2.4ml); Tween 20, sodium cholate and sodium deoxycholate gave no stimulation; sodium dodecyl sulphate and cetylpyridinium salts were slightly inhibitory.

Effect of concentration of enzyme substrate. The rates of hydrolysis of phospholipid, galactolipid, monoglyceride and *p*-nitrophenyl esters were linear with respect to enzyme concentration under the conditions used for routine assays of enzyme activity, i.e. at concentrations that catalysed the hydrolysis of 1 μequiv. of ester in 10min. The lipolytic acyl-hydrolase enzyme activity was found to be inhibited by high substrate concentrations. This substrate inhibition was observed with both acetone-dried and purified enzyme preparation. Fig. 7 illustrates the substrate inhibition produced by increasing amounts of galactolipid, monoglyceride and *p*-nitrophenyl esters. The same effect was shown in the progress curves in Fig. 4. For all the substrates containing long-chain fatty acids, inhibition occurred at substrate concentrations above 1–2 μequiv. of ester/ml. The hydrolysis of *p*-nitrophenyl acetate, however, was linear with respect to substrate concentrations up to at least 3mM, suggesting that the substrate inhibition may

Table 5. *Effect of Triton X-100 on acyl-hydrolase activity with various lipid substrates*

Incubation mixtures contained substrate (2 μequiv. of ester) and acetone-dried preparation (sufficient to catalyse the hydrolysis of approx. 1 μequiv. of ester in 10min) in 0.1M buffer solution (potassium phosphate, pH 7.5, or sodium acetate, pH 4.5–5.5). Substrates were prepared by ultrasonication or treatment with Triton X-100. Incubation was for 10min at 25°C in a total volume of 2.4ml.

		Ester hydrolysed (μequiv./min per mg)	
Substrate	pH	Substrate emulsified by ultrasonication	Substrate emulsified with Triton X-100
Phosphatidylcholine	5.5	0.05	0.8
	7.5	0.03	1.1
Monogalactosyl diglyceride	5.5	2.5	2.0
	7.5	2.3	3.0
Digalactosyl diglyceride	4.5	0.6	0.9
	7.5	0.2	1.2
Mono-olein	5.5	6.2	6.0
	7.5	6.2	9.4
Methyl oleate	5.5	0.6	0.8
	7.5	0.6	2.1

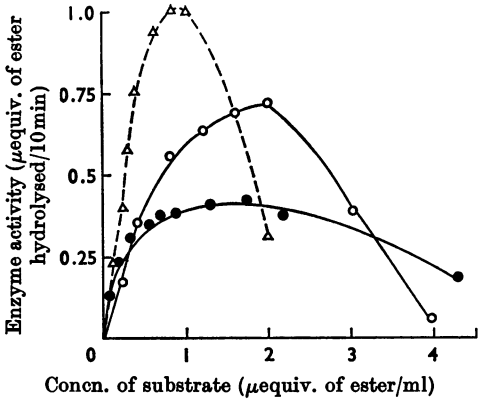

Fig. 7. Substrate concentration curves for the enzymic deacylation of acyl lipids by preparations from potato tuber. Incubation mixtures contained Triton X-100 (4mg), potassium phosphate buffer, pH7.5 (240μmol), substrates as indicated and either purified lipolytic acyl-hydrolase preparation (2.7μg of protein) with mono-olein substrate (△) or acetone-dried preparation (24μg of protein) with *p*-nitrophenyl stearate (●) and monogalactosyl diglyceride (○). Incubation time was 10min; the volume was 2.4ml.

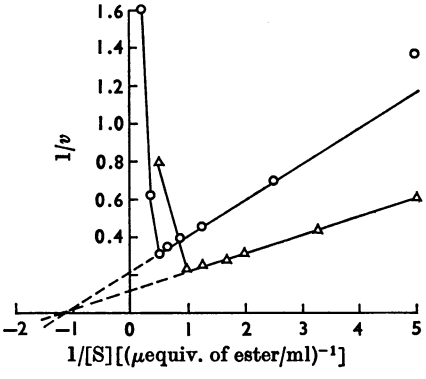

Fig. 8. Lineweaver–Burk curves obtained from substrate concentration curves. Experimental data were as given in Fig. 7 for mono-olein with purified enzyme preparation (△) and monogalactosyl diglyceride with acetone-dried preparation (○).

be due to interaction between enzyme and the long-chain fatty acid components of lipids.

At sub-optimum substrate concentrations the kinetics followed the reciprocal Lineweaver–Burk relationship (Fig. 8) but gave marked deviations at higher concentrations. Extrapolation of the linear portions of the Lineweaver–Burk plots (Fig. 8) enabled values to be calculated for K_m with each substrate. Because the lipid substrates are not water-soluble and were present in micellar dispersions, the absolute K_m values probably have little meaning, but the relative values give some information about the enzyme affinity for different substrates. Values determined for the apparent K_m for some substrates were as follows: monogalactosyl diglyceride (0.8mM ester), mono-olein (0.7mM), *p*-nitrophenyl palmitate (0.5mM), *p*-nitrophenyl stearate (0.7mM), *p*-nitrophenyl acetate (2.0mM). Thus lipids containing long-chain fatty acids showed similar affinities which were greater than with the acetate ester.

The maximum rate of hydrolysis obtained in practice with each substrate was lower than the theoretical maximum velocity determined from the Lineweaver–Burk curves. Thus it was not possible to run enzyme assays at saturating concentrations of substrate without causing substrate inhibition. For this reason, assays were always run with substrate concentrations in the range 0.67–0.8μequiv. of ester/ml.

Inhibition of enzyme activity. Organophosphorus compounds are potent inhibitors of many acyl-hydrolase enzymes (see Hofstee, 1960). The lipolytic acyl-hydrolase from potato was also inhibited by these compounds but only at relatively high concentrations of inhibitors. Table 6 presents results obtained with di-isopropyl phosphorofluoridate. With each substrate studied, little inhibition was obtained at inhibitor concentrations below 1mM. Table 6 shows that 5mM-di-isopropyl phosphorofluoridate caused approximately 50% inhibition of hydrolysis of each substrate. Tetraethyl pyrophosphate was less inhibitory than di-isopropyl phosphorofluoridate; diethyl *p*-nitrophenyl phosphate and diethyl *p*-nitrophenyl thio-

Table 6. *Inhibition of lipolytic acyl-hydrolase activity by di-isopropyl phosphorofluoridate (DFP)*

Incubation and assay conditions were as described in the text.

Substrate	Enzyme activity (units/mg of protein) Control	+DFP (5mM)	Inhibition by DFP (%)
Phosphatidylcholine	6.3	3.2	49
Monogalactosyl diglyceride	11.9	6.5	45
Mono-olein	35.0	19.6	44
p-Nitrophenyl palmitate	15.0	7.5	50

phosphate (parathion) caused inhibition at concentrations above 1mM but their limited solubility at this concentration prevented quantitative studies.

No effects on the lipolytic acyl-hydrolase activity were obtained with *p*-chloromercuribenzoate (0.1mM), cysteine, mercaptoethanol or dithioerythritol (all at 1mM).

In contrast with the phosphatide acyl-hydrolase from snake venoms (Hanahan, 1952; Long & Penny, 1957), the activity of the lipolytic acyl-hydrolase from potatoes on phosphatidylcholine was not stimulated by diethyl ether or by Ca^{2+} ions. No effects were observed with Ca^{2+} and Mg^{2+} or EDTA (all at 1 and 10mM) on the deacylation of monogalactosyl diglyceride, *p*-nitrophenyl palmitate and phosphatidylcholine.

Enzyme stability. Although the lipolytic acyl-hydrolase activity was stable for several months in crude acetone-dried preparations, freeze-drying of the partially purified enzyme preparations, after dialysis against water to remove salts, resulted in denaturation of the protein. Only 45% of the freeze-dried powder could be redissolved in water and the lipolytic activity of the protein that redissolved was, for each substrate, lower than that of the enzyme before freeze-drying. The percentage decrease in specific activity (μequiv. of ester hydrolysed/min per mg of protein) for each substrate was as follows: phosphatidylcholine (75); monogalactosyl diglyceride (69); mono-olein (76); methyl oleate (75); *p*-nitrophenyl palmitate (78).

Storage of the partially purified enzyme preparation as a suspension in 2.2M-ammonium sulphate, pH 7.0 (1–2mg of protein/ml), at 4°C for 3 months resulted in retention of most of the original activity. However, the enzyme was rapidly inactivated at 100°C and approx. 50% loss of activity towards *p*-nitrophenyl palmitate occurred after treatment for 3min at 60°C.

DISCUSSION

The activity of the lipolytic acyl-hydrolase preparations from potato tubers towards several different classes of naturally occurring lipids (phospholipids, galactolipids and mono- and diglycerides), as well as some artificial substrates, shows many similarities. (*a*) Acyl-hydrolase activity towards phospholipids, galactolipids, mono- and di-glycerides and *p*-nitrophenyl palmitate is located in the particle-free supernatant fraction of potato tuber homogenates. (*b*) Attempts to separate these activities by successive purification procedures were unsuccessful and the activity profiles towards each substrate were coincident on gel filtration, DEAE-cellulose chromatography and free-flow electrophoresis. In addition, the purified preparation/crude acetone-dried preparation activity ratio was very similar for each substrate (Table 3). Thus no selective purification of any specific lipolytic acyl-hydrolase was achieved. (*c*) Di-isopropyl phosphorofluoridate (5mM) caused the same degree of inhibition with each class of substrate (Table 6). (*d*) Enzyme activity was lost when the purified preparation was freeze-dried but for each class of substrate there occurred a similar decrease in specific activity. (*e*) In the absence of detergent (Fig. 5) the pH optima for deacylation of different lipid classes were all acidic. [Differences were observed in the actual values of the pH optima, but this property was also shown by mono- and di-galactosyl diglyceride with the galactolipase from bean leaves, which has been purified to homogeneity by Helmsing (1969).] In the presence of Triton X-100 (Fig. 6), the hydrolysis of each class of lipid exhibited a wide pH response with maximum activity at alkaline pH values. (*f*) Acyltransferase activity was associated with the acyl-hydrolase activity for each class of lipid, fatty acid methyl esters being produced in the presence of methanol as acceptor. (*g*) The deacylation of each class of lipid studied was subject to inhibition by excess of substrate at similar concentrations (Fig. 7) that, in each case, were below the theoretical concentrations giving maximum rates as calculated from Lineweaver–Burk plots.

The above evidence suggests that, if a single enzyme is not responsible for the deacylation of the different lipid classes described, then the enzymes involved are similar with respect to subcellular localization, molecular size and charge and their behaviour with substrates, inhibitors and detergents. However, the observation that the purified enzyme preparation gave three distinct bands of esterase activity on gel electrophoresis raises the possibility of isoenzymic forms of the lipolytic acyl-hydrolase. The appearance of isoenzymic forms of the esterase activity in potato tubers was shown by Schwartz, Biedron, von Holdt & Rehm, (1964) and, on the basis of genetic studies, Desborough & Peloquin (1967) have postulated that the esterase isoenzymes are tetramers in which the assembly of monomeric subunits is under genetic control.

The results also indicate that the formation of wax esters by reversal of acyl-hydrolase activity and the acyltransferase activity in the presence of alcoholic acceptors may also be catalysed by the same lipolytic acyl-hydrolase enzyme. The reversibility and acyltransferase activities of carboxylic acid esterases and of lipases are well known (Hofstee, 1960). A mechanism for wax ester formation by the reverse of acyl-hydrolase action has been described for leaves (Kolattukudy, 1967) and mammalian liver (Friedberg & Greene, 1967).

Although the lipolytic acyl-hydrolase preparation deacylates different classes of lipid substrates, specificity for certain types of acyl lipids exists. Esters containing short-chain fatty acids are poor substrates; triglycerides and lipids in which long-chain fatty acids are esterified with large alcoholic groups, e.g. sterol esters and wax esters, are not deacylated (Table 2). Presumably both polarity and steric factors are involved in the enzyme–substrate interactions. As the substrates were insoluble in water, the physical form of the micellar dispersions could influence the rates of hydrolysis of the different substrates.

The effects of Triton X-100 on the pH characteristics of the lipolytic acyl-hydrolase are similar in some respects to those of bile salts with pancreatic lipase (Borgström, 1954) and of anionic detergents on phospholipase D (Quarles & Dawson, 1969*b*). The most marked effect of Triton was the stimulation of phospholipid hydrolysis. Triton X-100 is a non-ionic detergent which would not affect the net charge of phospholipid substrate micelles but could influence the physical form of these micelles to facilitate enzyme–substrate interactions. The effects of Triton on the progress curves for the enzymic hydrolysis of *p*-nitrophenyl palmitate (Fig. 4) suggest that the detergent can interact with the enzyme to influence the binding of substrate. The fact that crude extracts from potato tubers will deacylate phospholipids (Galliard, 1970), whereas little activity was found with the purified preparation in the absence of Triton, suggests that a naturally occurring component in the crude extracts was activating the phospholipid deacylation. An activating agent of this type has been isolated in this laboratory from homogenates of plant tissues and has been identified as a free fatty acid (T. Galliard, unpublished work). It is possible that enzymes similar to the lipolytic acyl-hydrolase described here are involved in the production of free fatty acids in homogenates of plant tissues and their inhibitory effect on activities of chloroplasts (McCarty & Jagendorf, 1965; Constantopoulas & Kenyon, 1968; Friedlander & Neumann, 1968) and of mitochondria (Dalgarno & Birt, 1963; Baddeley & Simon, 1969).

The fact that the monoacyl intermediate monogalactosyl monoglyceride was isolated from the products formed during the enzymic hydrolysis of monogalactosyl diglyceride with the potato enzyme (Galliard, 1970) indicates that a sequential removal of fatty acids takes place. However, no monoacyl intermediates were detected in the products when phosphatidylcholine, digalactosyl diglyceride or diolein were used as substrates; in these cases the rate-limiting step could be the removal of the first fatty acid since the results showed that lysophosphatidylcholine and mono-olein were hydrolysed more rapidly than their diacyl analogues.

The activity of the lipolytic acyl-hydrolase from potato tubers towards galactosyl diglycerides suggested that the enzyme was similar to that described as a galactolipase and which has been found in the leaves of some *Phaseolus* species (Sastry & Kates, 1964; Helmsing, 1969) and spinach (Helmsing, 1967). Many similarities between the potato enzyme and the galactolipase are evident: (*a*) both enzymes are found in the particle-free supernatant fractions; (*b*) both have similar molecular weights as determined by gel filtration; (*c*) the ratio of activities towards mono- and di-galactosyl diglyceride is similar with each enzyme; (*d*) both are inhibited by substrate concentrations above 2 μequiv. of ester/ml. The purified lipolytic acyl-hydrolase preparation from potato tuber has a specific activity towards monogalactosyl diglyceride of 12 μequiv. of ester hydrolysed/min per mg of protein at 25°C. This is approximately 70 times greater than that of the purified homogeneous galactolipase enzyme from *Phaseolus multiflorus* (10.2 μequiv. of ester hydrolysed/h per mg of protein at 30°C; Helmsing, 1969). No detailed studies on the substrate specificity of the galactolipase have been published, but Sastry & Kates (1964) found some activity towards phosphatidylcholine and diglycerides in their partially purified preparation of galactolipase from *Phaseolus multiflorus* leaves.

The substrate-inhibition effect shown by the lipolytic acyl-hydrolase preparation from potato is relevant to the assay system used. Some conventional techniques for determination of lipolytic activity involve the use of dispersions of substrate at relatively high concentrations. Results from the present work show that at substrate concentrations above 2 μequiv. of ester/ml the enzyme is inhibited.

In addition to the question posed in this work on whether one enzyme, or a number of similar enzymes, in potato has lipolytic acyl-hydrolase activity, a further problem is evident. The overall purification of the enzyme activity was low, about fivefold. This apparently poor degree of purification was due to a close association, throughout the purification procedures, of enzyme activity with a major protein component from the particle-free supernatant fraction and was not caused by loss of enzyme activity during purification. Only the gel-filtration stage produced a significant increase in specific activity of the acyl-hydrolase. However, none of the purification procedures used to date has given any evidence of even a partial separation of enzyme activity from a major protein component. I have used gel filtration and DEAE-cellulose chromatography both at different pH values and salt concentrations, free-flow electrophoresis and disc gel electrophoresis. In all cases, coincidence of protein

and enzyme activity was observed. When the enzyme from a DEAE-cellulose column was rechromatographed under the same conditions, some trailing of the protein peak occurred, possibly owing to modification of the protein by the salt concentration; however, even in this case, enzyme activity and protein remained together. Freeze-drying of the enzyme preparation caused both denaturation of the enzyme and loss of acyl-hydrolase activity. It appears that, not only are the enzyme activities towards several classes of lipid substrate similar in many respects, but also these activities are associated with protein which has similar physical properties to those of a significant proportion (10–20%) of the proteins in the original acetone-dried preparation. What proportion of the protein in the partially purified preparation represents acyl-hydrolase activity is not yet known. Nevertheless, the specific activity of the preparation is reasonable, e.g. 39 μmol of mono-olein hydrolysed/min per mg of protein. Assuming an average molecular weight of 10^5 for the protein in the preparation, a minimum value for the molecular activity of the enzyme towards mono-olein would be 4000 mol hydrolysed/min per mg of enzyme. It is hoped that further study of the lipolytic acyl-hydrolase preparations by isoelectric focusing and analytical ultracentrifugation techniques will provide further information on the nature of the enzyme(s) involved.

I am grateful to Mrs J. A. Matthew and Mr D. R. Phillips for experimental assistance and to Mr W. M. Laird who kindly performed the free-flow electrophoresis experiments.

REFERENCES

Ansell, G. B. & Hawthorne, J. N. (1964). *Phospholipids*, p. 150. Amsterdam: Elsevier Publishing Co.
Baddeley, M. S. & Simon, E. W. (1969). *J. exp. Bot.* **20**, 94.
Borgström, B. (1954). *Biochim. biophys. Acta*, **13**, 149.
Constantopoulas, G. & Kenyon, C. M. (1968). *Pl. Physiol., Lancaster*, **43**, 531.
Contardi, A. & Ercoli, A. (1933). *Biochem. Z.* **261**, 275.
Dalgarno, L. & Birt, L. M. (1963). *Biochem. J.* **87**, 586.
Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.
Desborough, S. & Peloquin, S. J. (1967). *Phytochemistry*, **6**, 989.
Deykin, D. & Goodman, D. S. (1962). *J. biol. Chem.* **237**, 3649.
Duncombe, W. G. (1963). *Biochem. J.* **88**, 7.
Friedberg, S. J. & Greene, R. C. (1967). *J. biol. Chem.* **242**, 234.
Friedlander, M. & Neumann, J. (1968). *Pl. Physiol., Lancaster*, **43**, 1249.
Galliard, T. (1968). *Phytochemistry*, **7**, 1905.
Galliard, T. (1969). *Biochem. J.* **115**, 335.
Galliard, T. (1970). *Phytochemistry*, **9**, 1725.
Hanahan, D. J. (1952). *J. biol. Chem.* **195**, 199.
Hannig, K. (1964). *Hoppe-Seyler's Z. physiol. Chem.* **338**, 211.
Helmsing, P. J. (1967). *Biochim. biophys. Acta*, **144**, 470.
Helmsing, P. J. (1969). *Biochim. biophys. Acta*, **178**, 519.
Hirsch, J. & Ahrens, E. H. (1958). *J. biol. Chem.* **233**, 311.
Hofstee, B. H. J. (1960). In *The Enzymes*, vol. 4, p. 485. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Jaaska, V. (1969). *Eesti NSV Tead. Akad. Toim.* **18**, 55; cited in *Chem. Abstr.* (1969). **70**, 74575.
Kates, M. (1960). In *Lipide Metabolism*, p. 165. Ed. by Bloch, K. New York: John Wiley and Sons Inc.
Kolattukudy, P. E. (1967). *Biochemistry, Easton*, **6**, 2705.
Long, C. & Penny, I. F. (1957). *Biochem. J.* **65**, 382.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McCarty, R. E. & Jagendorf, A. T. (1965). *Pl. Physiol. Lancaster*, **40**, 725.
Margoliash, E. (1962). *J. biol. Chem.* **237**, 2161.
Ornstein, L. (1964). *Ann. N.Y. Acad. Sci.* **121**, 321.
Paul, J. & Fottrell, P. F. (1961). *Biochem. J.* **78**, 418.
Phelps, R. A. & Putnam, F. W. (1960). In *The Plasma Proteins*, vol. 1, p. 143. Ed. by Putnam, F. W. New York: Academic Press Inc.
Quarles, R. H. & Dawson, R. M. C. (1969*a*). *Biochem. J.* **112**, 787.
Quarles, R. H. & Dawson, R. M. C. (1969*b*). *Biochem. J.* **112**, 795.
Sastry, P. S. & Kates, M. (1964). *Biochemistry, Easton*, **3**, 1280.
Schwartz, H. M., Biedron, S. I., von Holdt, M. M. & Rehm, S. (1964). *Phytochemistry*, **3**, 189.
Seligman, A. M. & Nachlas, M. M. (1963). In *Methods of Enzymatic Analysis*, p. 776. Ed. by Bergmeyer, H. U. London: Academic Press (Inc.) Ltd.
Swell, L. & Treadwell, C. R. (1955). *J. biol. Chem.* **212**, 141.
Theorell, H., Holman, R. T. & Akeson, A. (1947). *Acta chem. scand.* **1**, 571.
Warner, R. C. (1958). *Archs Biochem. Biophys.* **78**, 498.