Phytochemistry, 1970, Vol. 9, pp. 1725 to 1734 Pergamon Press. Printed in England.

THE ENZYMIC BREAKDOWN OF LIPIDS IN POTATO TUBER BY PHOSPHOLIPID- AND GALACTOLIPID-ACYL HYDROLASE ACTIVITIES AND BY LIPOXYGENASE*

T. GALLIARD

Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, NOR 7OF

(*Received* 12 *December* 1969)

Abstract—Homogenization of potato tubers at 0° results in rapid enzymic hydrolysis of the endogenous phospholipids and galactolipids to produce free fatty acids and fatty acid hydroperoxides. The enzymes responsible for these effects have been identified as acyl hydrolases and lipoxygenase. Monogalactosyl diglyceride is particularly susceptible to hydrolysis and monogalactosyl monoglyceride was detected as an intermediate product. Acyl transferase activity was also associated with the acyl hydrolase action. Properties of the various enzyme activities are described; all are present mainly in the particle-free supernatant fraction and have acidic pH optima. Evidence is presented which indicates that free lipids are hydrolysed more readily than the lipids of lipoprotein membranes. The results are discussed with particular reference to the lipid composition and enzyme activities of subcellular organelles prepared from tissue homogenates.

INTRODUCTION†

ALTHOUGH phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) is widespread in plant tissue,[1] little information is available on the occurrence in plants of phospholipid acyl hydrolase enzymes which liberate free fatty acids. In leaves, the presence of galactolipases which deacylate MGDG and DGDG to produce free fatty acids, has recently been demonstrated.[2–4] It is also known that free fatty acids have inhibitory effects on the activities *in vitro*, of chloroplasts[5–7] and mitochondria from plants[8,9] and, possibly, have similar effects *in vivo*.[9]

Recently, in this laboratory, it was consistently observed that the lipid composition of aqueous homogenates of potato tubers showed marked differences from the lipids extracted directly from the same tissue.[11] It was unlikely that phospholipase D activity was responsible for any lipid breakdown since potato tubers do not contain significant amounts of this enzyme.[12] The requirement for the addition of bovine serum albumin to the extraction

* Part III in the series "Aspects of Lipid Metabolism in Higher Plants"; for Part II, see *Phytochem.* 7, 1915 (1968).

† *Abbreviations used.* PC—phosphatidyl choline; PE—phosphatidyl ethanolamine; MGMG—monogalactosyl monoglyceride; MGDG—monogalactosyl diglyceride; DGDG—digalactosyl diglyceride.

[1] M. KATES, in *Lipide Metabolism* (edited by K. BLOCH), p. 165, John Wiley, New York (1960).
[2] P. S. SASTRY and M. KATES, *Biochem.* 3, 1280 (1964).
[3] P. J. HELMSING, *Biochim. Biophys. Acta* 144, 470 (1967).
[4] P. J. HELMSING, *Biochim. Biophys. Acta* 178, 519 (1969).
[5] R. E. MCCARTY and A. T. JAGENDORF, *Plant Physiol.* 40, 725 (1965).
[6] M. FRIELANDER and J. NEUMANN, *Plant Physiol.* 43, 1249 (1968).
[7] G. CONSTANTOPOULAS and C. M. KENYON, *Plant Physiol.* 43, 531 (1968).
[8] L. DALGARNO and L. M. BIRT, *Biochem. J.* 87, 586 (1963).
[9] M. S. BADDELEY and E. W. SIMON, *J. Exptl. Botany* 20, 94 (1969).
[10] S. DRAPER, *Phytochem.* 8, 1641 (1969).
[11] T. GALLIARD, *Phytochem.* 7, 1907 (1968).
[12] R. H. QUARLES and R. M. C. DAWSON, *Biochem. J.* 112, 787 (1969).

medium used in the preparation of active mitochondria from potatoes[13] suggested that free fatty acids were present in these preparations. The present paper shows that, in fact, free fatty acids and their oxidation products are rapidly formed from phospholipids and galactolipids in homogenates of potato tubers and describes the phospholipid- and galactolipid-acyl hydrolase activities which, together with lipoxygenase, are responsible for this breakdown of endogenous lipids.

RESULTS

Loss of Glycerolipids on Homogenization of Potato Tubers

The compositions of lipid extracts obtained directly from tuber tissue and from homogenates of the same tissue are illustrated in the TLC separations in Fig. 1. Marked differences were observed even between tissue lipids (A) and the lipids extracted immediately following a 30 sec homogenization of the tissue of 0° (B). Further changes were visible in the lipids extracted from the homogenate which had been held at 0° for 10 min and, after incubation of the homogenate at 25° for 10 min, most of the glycerolipids (particularly phospholipids and galactolipids and, to a smaller extent triglycerides) had disappeared (C). However, other lipids, e.g. sterol-containing lipids and cerebroside, showed no observable changes in amount. The loss of glycerolipids (Figs. 1a and 1b) was associated with an increase in free fatty acids and their oxidation products (Fig. 1c). A consistent finding (Fig. 1b) was that MGDG disappeared more rapidly than DGDG or the phospholipids.

The qualitative results obtained by TLC were supported by analytical data (Table 1) which showed that the fall in acyl ester content of the total lipid was associated with an increase in the free fatty acid content. On the basis of fresh weight of tissue used, the sterol content was found to be constant and thus served as an "internal standard".

TABLE 1. QUANTITATIVE ANALYSES OF ACYL ESTER, FREE FATTY ACID AND STEROL CONTENT OF WHOLE TISSUE AND HOMOGENATES OF POTATO TUBER

	μ Eq./g fr. wt.		
	Acyl ester	Free fatty acid	Sterol*
Whole tissue	2·34	0·70	0·13
Homogenate, zero time	2·04	1·40	0·14
Homogenate, incubated 10 min at 0°	1·72	1·75	0·13
Homogenate, incubated 10 min at 25°	0·54	2·90	0·13

* Relative to a standard of β-sitosterol.

Chloroform-soluble Products formed on Breakdown of Endogenous Lipids

TLC examination of the lipids extracted from homogenates was used to identify the enzyme activities causing the lipid breakdown. The presence of free fatty acids indicated the action of acyl hydrolase enzymes and the fact that fatty acid hydroperoxides were also formed suggested the involvement of lipoxygenase. No lysophospholipids, lysogalactolipids,

[13] W. D. BONNER, in *Methods in Enzymology* (edited by S. P. KOLOWICK and N. O. KAPLAN), Vol. 10, p. 126, Academic Press, New York (1967).

phosphatidic acid or diglycerides were observed in the homogenates; these observations indicated that phospholipases A, C and D were either absent or that their products were rapidly deacylated by further acyl hydrolase action.

Gas chromatography of the fatty-acid methyl esters showed that the content of palmitic, stearic and oleic acids in the total lipids extracted from homogenates remained constant whereas the linoleic acid and linolenic acids fell during autolysis—as would be expected if the polyunsaturated fatty acids, when released, were attacked by lipoxygenase. The identity of some of the products formed on further conversions of the fatty acid hydroperoxides are discussed later.

Differential Rates of Breakdown of Free and Membrane-bound Lipids

The qualitative and quantitative results on the breakdown of endogenous lipids (Fig. 1 and Table 1) indicated that a rapid loss of lipids during a 30 sec homogenization at 0° was followed by a slower rate of breakdown when the homogenate was held at 0° for 10 min. However, since almost all the glycerolipids were lost if the homogenate was kept at 25° for 10 min, the lipids remaining after homogenization must also be attacked. These results suggested that the lipids which were rapidly lost during homogenization were in a different "pool" from those hydrolysed more slowly during subsequent incubation. If, during homogenization, some lipids were present in a free form, or were liberated from a lipoprotein environment by disruption of membrane structures, then it might be expected that these "exposed" lipids would be hydrolysed more readily. Support for this possibility was obtained from an experiment (Table 2) in which ^{14}C (acyl)-labelled phospholipids (mainly ^{14}C-PE) were added to potato tissue immediately before homogenization. If the added free ^{14}C-labelled lipid were hydrolysed more rapidly than the total (free + bound) endogenous lipid, then the specific radioactivity of a given lipid should decrease during homogenization and subsequent incubation of the homogenate. Table 2 shows that with phosphatidyl ethanolamine, for example, the specific radioactivity of this lipid fell with the extent of hydrolysis.

TABLE 2. HYDROLYSIS OF ^{14}C(ACYL)-PHOSPHOLIPID ADDED TO POTATO TISSUE BEFORE HOMOGENIZATION

	% Hydrolysis of ^{14}C-lipid (counts in free fatty acid as % total lipid counts)	Specific radioactivity of PE (dpm/μg phosphorus)
Whole tissue	0·4	$4{\cdot}3 \times 10^4$
Homogenate, zero time	43	$2{\cdot}7 \times 10^4$
Homogenate, incubated 10 min at 0°	60	$1{\cdot}1 \times 10^4$
Homogenate, incubated 10 min at 25°	84	—

^{14}C(acyl)-phospholipid (mainly PE) was added to potato tissue immediately before homogenization (10^5 dpm of ^{14}C-lipid added per g fr. wt. of potato) Incubation and extraction methods as in Experimental. Radioactivity and phosphorus analyses were made on individual lipids separated by TLC.

Extraction Conditions for Lipolytic Activity in Potato Tubers

Maximal rates of deacylation of endogenous lipids were obtained when the tissue was homogenized at pH 4·8–5·6 in acetate buffer. The addition of a reducing agent was necessary

to inhibit the oxygenase-mediated phenolic polymerization reactions. When sodium metabisulphite (2×10^{-3} M) was added to the extraction media,[14] homogenates from potato tubers at all stages of development remained colourless and retained lipolytic activity for at least 24 hr at 0°. Although mercaptobenzathiazole (10^{-4} M) recommended by Palmer[15] was satisfactory when fully developed dormant tubers were used, this reductant (even at 10^{-3} M) did not prevent the discoloration of extracts prepared from immature developing tubers.

Characterization of Enzymes Responsible for Lipolysis in Potato Tubers

Identification of the products formed in homogenates of potatoes indicated that the following enzyme activities were present: (*a*) a galactolipase, galactolipid acyl hydrolase,[2-4] (*b*) a phospholipid acyl hydrolase and (*c*) lipoxygenase (EC 1.99.2.1). In order to study further these enzyme activities, the action of potato tuber extracts on the following substrates was investigated: (*a*) MGDG and DGDG, prepared from potato tubers and spinach leaves, (*b*) phosphatidylcholine, isolated from potato tubers and (*c*) commercial samples of linoleic and linolenic acids. Substrates prepared from potato tissue were favoured as these would have similar fatty acid compositions to the endogenous lipids in the homogenates described above.

Subcellular Distribution of Enzyme Activities

Incubation of the various substrates with fractions obtained by differential centrifugation of homogenates prepared in hypertonic media indicated that the enzyme activities were concentrated almost completely in the 100,000 *g*–60 min supernatant fraction (Table 3). For routine enzyme preparations, a 15,000 *g*–30 min supernatant fraction of a homogenate prepared in the presence of $Na_2S_2O_5$ (2×10^{-3} M) was used.

TABLE 3. SUBCELLULAR LOCALIZATION OF ENZYME ACTIVITIES

	Enzyme activity		
	Acyl hydrolase (% loss of acyl ester content)		Lipoxygenase (mμmoles O_2 consumed/min)
Substrate:	PC	DGDG	Linoleic acid
(1) 1000 *g*–10 min pellet	3	0	0
(2) 15,000 *g*–30 min pellet	4	3	7
(3) 100,000 *g*–60 min pellet	0	0	1
(4) 100,000 *g*–60 min supernatant	74	56	147
(5) *Boiled* 100,000 *g*–60 min supernatant	4	0	0
(4) + (3) + (2) combined	63	50	177

Acyl hydrolase assay systems (2·4 ml) contained subcellular fraction (equivalent to 250 mg fr. wt. of potatoes), mannitol (0·1 M) and acetate buffer (0·1 M) at pH 5·5 for PC (1·6 μ eq. ester) or pH 4·5 for DGDG (1·0 μ eq. ester). Incubations at 25° for 10 min. Lipoxygenase was assayed in a vol. of 5 ml containing linoleic acid (ammonium salt, 10 μmoles), acetate buffer, pH 5·5 (0·05 M), and subcellular fraction (equivalent to 25 mg fr. wt. of potato). Incubations at 25° were monitored with the O_2-electrode.

[14] D. M. STOKES, J. W. ANDERSON and K. S. ROWAN, *Phytochem.* 7, 1509 (1968).
[15] J. K. PALMER and J. B. ROBERTS, *Science* 157, 200 (1967).

Properties of Enzymes

The pH optima for phospholipid acyl hydrolase activity on PC and for lipoxygenase activity were, in both cases, at pH 5·5–5·6. The pH curves for galactolipid–acyl hydrolase activities differed with the substrate used. The hydrolysis of MGDG showed a broad optimum in the range pH 5·0–7·0 whereas DGDG hydrolysis was optimal at pH 4·5.

Enzyme concentration curves for the deacylation of phospholipid and galactolipids (Fig. 2) showed that the activity of the acyl hydrolase was much greater towards MGDG than to DGDG, but that both galactolipids were hydrolysed more efficiently than the phospholipid. Little glycerol ester hydrolase activity was observed at pH 5·5.

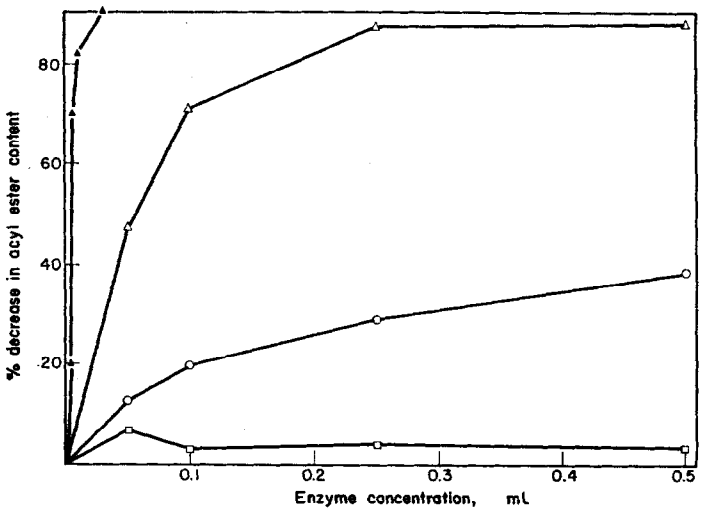

FIG. 2. ENZYME CONCENTRATION CURVES FOR THE HYDROLYSIS OF MGDG, DGDG, PC AND TRIGLYCERIDE

Incubation mixtures (2·4 ml) contained enzyme (15,000 × *g* supernatant fraction), 0·1 M acetate buffer and substrate (1 μ eq. acyl ester): ▲—MGDG, o—PC, □—trilinolein (all at pH 5·5) and △—DGDG at pH 4·5. The mixtures were incubated at 25° for 10 min.

In preliminary attempts to separate and purify the enzymes, dialysis, ammonium sulphate precipitation and chromatography on Sephadex G-200 columns did not yield any separation of the phospholipid- and galactolipid-acyl hydrolase activities. Acetone powders prepared from 15,000 *g* supernatant fractions retained both phospholipid- and galactolipid-acyl hydrolase activities, but did not show lipoxygenase activity.

Further specific properties of the 15,000 *g* supernatant fractions with the different substrates are described below:

(a) *Phospholipid acyl hydrolase.* Activity on PC was not affected by Ca^{2+} (10^{-3} M) and was not inhibited by EDTA (10^{-3} M) nor by F^- (2×10^{-2} M). Examination of reaction products by TLC showed that free fatty acids and their oxidation products were formed but no lyso-PC was observed.

(b) *Galactolipid acyl hydrolase.* Action on MGDG and DGDG produced mainly free fatty acids and their oxidation products. However, when MGDG was used as substrate and the enzyme concentration was limiting, a further product was observed by TLC analysis, which was identified as *monogalactosyl monoglyceride* (MGMG, i.e. the lyso-derivative of MGDG) by the following evidence: (1) the lipid was more polar than MGDG [R_{MGDG}

on TLC in $CHCl_3$–CH_3OH–CH_3COOH–H_2O (170:30:20:5) was 0·50] and gave a positive periodate—Schiff's reaction; (2) after purification of the product by preparative TLC, mild alkaline hydrolysis and analysis of deacylation products,[16] the water-soluble deacylation product had the same properties on paper chromatography as monogalactosyl glycerol[16] and that fatty acids were liberated (as methyl esters) as the only hexane-soluble hydrolysis products.

When galactolipids were added to incubation mixtures as methanolic solutions (as used by previous workers),[2–4] the loss of substrate as observed by TLC analysis of the products and by liberation of water-soluble mono- or di-galactosyl glycerol, was greater than the observed decrease in acyl ester content as assayed by the hydroxamate method. Further analysis of the products by TLC showed that, when methanol was present in the incubation mixtures, *fatty acyl methyl esters* were formed in addition to free fatty acids. This presumably indicated a *transferase* activity by which fatty acids were transferred from the substrate to form an ester with the alcohol present in the mixture. Approximately equal amounts of free fatty acids and methyl esters were formed when the methanol concentration in the incubation mixture was 4%, v/v. Preliminary experiments have shown that ethanol and *n*-propanol can also act as acceptors in the transacylation reaction but that propan-2-ol and glycerol cannot.

(c) *Lipoxygenase.* Activity showed equal rates of oxidation of linoleic and linolenic acids and no activity towards oleic acid. Ca^{2+} CN^-, F^- and EDTA (all at 10^{-3} M) had no effect on the lipoxygenase activity.

The major product was shown to be the corresponding conjugated *cis–trans* hydroperoxide derivative which had an absorption maximum at 235 mμ, gave a positive reaction with *N*,*N*-dimethyl-*p*-phenylenediamine[17] and which, as the free acid and methyl ester, had identical properties on TLC to the analogous products formed when pure lipoxygenase was used. Other products which gave positive fatty acid reactions were examined by specific spray reagents on TLC and by their u.v. spectra. One product which ran on TLC just behind free fatty acid gave a positive carbonyl reaction and had an absorption maximum at 275 nm was tentatively identified as a conjugated ketodiene fatty acid. Additional products which were more polar than the hydroperoxides and which also had 235 nm maxima were presumably hydroxylated diene fatty acid derivatives.

Investigations with other Plant Tissues

Sastry and Kates[2] surveyed a range of plants for galactolipase but found marked activities only in several species of *Phaseolus*. In the present work, galactolipid- and phospholipid-acyl hydrolase activities were observed in potato tubers at all stages of development from newly formed tubers (4–5 g) through growth and dormancy to the onset of sprouting, although no quantitative comparisons were obtained. Some other tissues, e.g. cauliflower florets, spinach leaves, tomato and apple fruits, were examined for a rapid breakdown of endogenous lipids in homogenates, but no activities comparable with that in potato tubers or bean leaves[2] were observed.

It has been shown that some plant tissues contain inhibitors of lipolytic enzymes.[18,19] To determine whether the absence of rapid breakdown of endogenous lipid in, for example,

[16] T. GALLIARD, *Biochem. J.* **115**, 335 (1969).
[17] E VIOQUE and R. T. HOLMAN, *Arch. Biochem. Biophys.* **99**, 522 (1962).
[18] M. KATES, *Can. J. Biochem. Physiol.* **32**, 571 (1954).
[19] H. L. TOOKEY and A. K. BALLS, *J. Biol. Chem.* **218**, 213 (1956).

spinach leaf, was due to the presence of inhibitors, a 15,000 *g* supernatant preparation from potato tubers (equivalent to 250 mg fr. wt. of potato) was added to a water-homogenate of spinach leaves containing 1 g fr. wt. in 4 ml. Rapid loss of phospholipids and galactolipids from the spinach homogenate was obtained in the presence of the potato extract, whereas little breakdown (as observed by TLC analysis) was seen in the leaf homogenate incubated for 2 hr at 25° in the absence of added potato enzymes. Thus, in spinach leaf homogenates, the absence of lipid breakdown is due, not to the presence of inhibitors, but to the absence of hydrolase activity.

DISCUSSION

The results described show that potato tubers, at all stages of development, contain very active phospholipid- and galactolipid-acyl hydrolase activities which manifest themselves immediately the cells are broken by homogenization.

TABLE 4. SUMMARY OF ENZYME ACTIVITIES IN POTATO TUBER EXTRACTS WITH LIPID SUBSTRATES

	Enzyme activity			
	Phospholipid acyl hydrolase	Galactolipid acyl hydrolase		Lipoxygenase
Substrate used:	PC	MGDG	DGDG	Linoleic acid
Subcellular localization	← 100,000 g supernatant →			
pH Optimum	5·5–5·6	5·0–7·0	4·5	5·5–5·6
Relative enzyme activities*	1·4	15·0	2·4	11·0
Major $CHCl_3$-soluble products	Free fatty acids + fatty acid hydroperoxide			Fatty acid hydroperoxide
Minor $CHCl_3$-soluble products	—	MGMG	—	Conjugated dienes + keto dienes

* Enzyme activities (mμ eq. substrate reacted/min/mg fr. wt. of tissue) under optimal conditions at 25° with 15,000 *g* supernatant as enzyme source.

The fact that no monoacyl phospholipids (lysophospholipids) are observed in autolysates, or after incubation of added phospholipid with cell-free extracts, suggests that the enzyme activity is of the phospholipase B type (or combined phospholipases A and B).[1] There is little information on the occurrence of this type of phospholipase activity in plants[1,20] although phospholipase B activity has been reported in rice grains.[21] The results of the present work do not show whether the hydrolysis is caused by a single enzyme which removes both fatty acids from the phospholipid or whether an initial, rate-limiting attack by phospholipase A (phosphatide acyl hydrolase, EC 3.1.1.4) is followed by phospholipase B action (lysolecithin acyl hydrolase, EC 3.1.1.5). Further purification of the enzymes (now in progress) should give this information.

The galactolipid acyl hydrolase in potato shows some marked differences from the galactolipase recently purified from bean leaves.[2,4] In potato, the enzyme activity is particularly high with MGDG as substrate compared with DGDG and the activity is confined to the cytoplasmic fraction. The pH optima also differ from those observed in bean[2] and spinach[3] leaves. The observation that monogalactosyl monoglyceride is formed from MGDG

[20] E. J. BARRON, in *Modern Methods of Plant Analysis* (edited by H. F. LINSKENS, B. D. SANWAL and M. V. TRACEY), Vol. 7, p. 454, Springer-Verlag, Berlin (1964).
[21] A. CONTARDI and A. ERCOLI, *Biochem. Z.* **261**, 275 (1933).

suggests that the removal of the two fatty acids from the galactolipids is a step-wise process. Again elucidation of the mechanism must await further purification of the enzyme(s).

That the polyunsaturated fatty acids liberated by acyl hydrolase action are immediately attacked by lipoxygenase, raises interesting aspects of integrated lipid catabolism. However, under the conditions of the present experiments, alternative pathways of fatty acid breakdown (e.g. α- or β-oxidation) would not be operative due to dilution of necessary co-factors. Although lipoxygenase activity is widespread in plants,[22] its physiological role is not understood.

Previous workers[2-4] have added galactolipid substrates as solutions in methanol to galactolipase preparations and assayed enzyme activity by measuring the loss of acyl ester on hydrolysis of the galactolipids. However, the potato galactolipase activity was shown to be associated with transferase activity which transferred acyl moieties from lipid to an alcohol and thus preserved acyl ester function. This activity is analogous to the transphosphatidylation activity shown by phospholipase D[23] and which is responsible for the formation of phospholipid artefacts in some plant extracts, e.g. phosphatidyl methanol in methanolic extracts of Crown gall.[24] Transacylation reactions of phospholipases are well known in animal tissues;[25] they are known to catalyse fatty acid exchange reactions *in vivo* and have also been suggested as responsible for the formation of sterol esters.[26] Possibly, a similar physiological role may be played by these enzymes in plants.

Since there is a large loss of lipids even during homogenization and holding at 0°, the lipid composition and enzymic activity of organelles prepared from homogenates must reflect the action of lipolytic enzymes during the preparation and isolation of cell fractions. Free fatty acids, which inhibit the activities of mitochondria and chloroplasts, are found in preparations of these organelles from several plant tissues.[5-9] The inhibitory effects of fatty acids can be diminished if bovine serum albumin is added to the preparations, particularly if added to the extraction medium before homogenization.[6] However, bovine serum albumin acts by binding free fatty acids *already formed* by lipid breakdown. The present work has shown that, although at pH 7·5 (recommended for the preparation of potato mitochondria)[13,27] the lipolytic enzymes are less active, there is still significant lipid breakdown at this pH and this is not inhibited by EDTA.

Since most of the phospholipids and galactolipids disappear from potato homogenates, it must be concluded that the lipids in lipoprotein membrane structures are susceptible to attack but the results show that these lipids are less readily hydrolysed than the free lipids. Similar observations were made on the action of snake venom phospholipase A on intact and damaged mitochondria[28] and with the proteolytic enzymes on membrane proteins.[29]

EXPERIMENTAL

Materials. White potatoes (*Solanum tuberosum*, cv. "Majestic") were grown in our fields and developing tubers were used immediately after picking. Mature tubers were harvested in September and stored at 10° Other plants were grown in greenhouses or purchased locally.

[22] A. L. TAPPEL, in *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBACK), Vol. 8, p. 275, Academic Press, New York (1963).
[23] S. F. YANG, S. FREER and A. A. BENSON, *J. Biol. Chem.* **242**, 477 (1967).
[24] R. DOUCE, *C. R. Acad. Sci. Paris* Series D, **262**, 1549 (1966).
[25] H. GOLDFINE, *Ann. Rev. Biochem.* **37**, 303 (1968).
[26] A. RAZ, F. A. KUMMEROW and T. NISHIDA, *Biochim. Biophys. Acta* **176**, 591 (1969).
[27] J. D. VERLEUR, *Plant Physiol.* **40**, 1003 (1965).
[28] E. C. WEINBACH and J. GARBUS, *Biochim. Biophys. Acta* **162**, 500 (1968).
[29] J. A. RUPLEY, in *Methods in Enzymology* (edited by S. P. KOLOWICK and N. O. KAPLAN), Vol. 11, p. 905, Academic Press, New York (1967).

MGDG and DGDG were prepared from potato tubers and spinach leaves by methods previously described.[11,16] PC was obtained from potato tubers and purified by DEAE-cellulose and silcic acid chromatography.[11] Each lipid preparation gave one major spot on TLC analysis and was estimated to be >90% pure. The MGDG preparations contained small amounts of pigments and esterified sterol glycosides; glucocerebroside was a minor component of the DGDG and the PC contained some sterol glucoside. None of these minor impurities interfered with the methods used in the present work. Linoleic acid and linolenic acid and lipoxygenase were purchased from the Sigma Chemical Co. Trilinolein was a gift from Dr. P. A. T. Swoboda of this Institute. Sodium acetate-2-^{14}C (55 mc/mmole) was obtained from the Radiochemical Centre, Amersham.

Preparation of Enzyme Extracts

(a) Crude homogenates for the study of the breakdown of endogenous lipids were prepared by adding 10 g diced potato tuber tissue to 15 ml of a solution containing 2×10^{-3} M $Na_2S_2O_5$ and 0·1 M acetate buffer, pH 5·5. The mixture was cooled to 0° and homogenized for 30 sec at 0° using an Ultraturax homogenizer (Type TP. 18/2N, Janke & Kunel KG, Staufen, Germany) and the crude homogenate used without filtration.

(b) Subcellular fractionations were performed by differential centrifugation of homogenates prepared at 0° in an Omnimix blender (M.S.E. Ltd.) with diced tuber tissue in 3 vol. of a solution containing 0·3 M mannitol, 2×10^{-3} M $Na_2S_2O_5$ adjusted before and after homogenization to pH 7·5. The homogenate was filtered through muslin and centrifuged at 0° as indicated in the text. Pellets were washed by suspending in the extraction medium and re-centrifuging.

(c) For routine enzyme assays, tuber tissue was homogenized at 0° in a blender with 3 vol. of 2×10^{-3} M $Na_2S_2O_5$ solution in water. After filtration through muslin and centrifugation at 15,000 *g* for 30 min, the supernatant was used as the source of enzyme.

Enzyme Assays

(a) *Endogenous lipolysis.* An assay was accomplished by adding samples of the crude homogenate corresponding to a known weight of tissue into 5 vol. of refluxing methanol–H_2O (4:1, v/v). After refluxing for a further 5 min, the mixture was cooled and methanol (1 vol.) and $CHCl_3$ (2·5 vol.) were added to form the uniphasic mixture of Bligh and Dyer.[30] The mixture was allowed to stand for 30 min before adding $CHCl_3$ (2·5 vol.) and 0·2 M acetate buffer, pH 4·0 (2·5 vol.). The acid buffer was necessary to prevent the formation of emulsions caused by free fatty acids liberated during lipolysis. The $CHCl_3$ phase was removed for analysis. The initial lipid content of tuber tissue was determined by "killing" a known wt. of dried tuber tissue in refluxing aq. methanol before homogenization and extraction of the lipids as above.

(b) *Phospholipid- and galactolipid-acyl hydrolase.* Activities on added substrates were routinely assayed in a system containing 0·5–1·0 μmoles of lipid (1·0–2·0 μ eq. acyl ester) and enzyme solution in 0·1 M acetate buffer at optimal pH in a total vol. of 2·4 ml. Incubations at 25° were stopped by boiling and the lipids were extracted as above. Enzyme activities were assayed by determination of the decrease in acyl ester content of the lipids or, in some cases, by estimation of water-soluble hydrolysis products from galactolipids in the aq. methanol phase. All lipids were introduced to incubation mixtures as stable emulsions formed by ultrasonication in water.

(c) *Lipoxygenase.* Activity was monitored by polarographic measurement of O_2-uptake using an oxygen electrode system (Yellow Springs Instrument Co., Ohio, U.S.A.). The incubation mixture at 25° contained fatty acid (added as ammonium salt, 10^{-3} M) and enzyme with acetate buffer, pH 5·5 (10^{-1} M), in a total volume of 5 ml of air-saturated solution.

Preparation of ^{14}C-labelled Lipid

Disks (1 cm dia. × 1 mm) were cut from potato tubers and shaken for 18 hr at 25° in 10^{-3} $CaSO_4$ solution. These "aged" disks actively incorporate ^{14}C-acetate into the fatty acid moieties of lipids.[31,32] Forty disks (*ca.* 2 g fr. wt.) were then incubated at 25° for 24 hr with 50 μc sodium acetate-2-^{14}C in 3 ml of 0·1 M phosphate buffer at pH 5·5. The disks were killed in refluxing aq. methanol and the lipids extracted as described above. A portion of the ^{14}C-labelled lipids was chromatographed on a silicic acid column. After eluting neutral lipids with chloroform, the phospholipids were obtained by washing the column with $CHCl_3$–methanol (2:1, v/v). The composition of the labelled phospholipid mixture was determined by scanning TLC separations for radioactivity (Model TLRS 1A, Panax Equipment Ltd.). ^{14}C-(acyl) PE was the major ^{14}C-labelled lipid in the fraction used in the present work.

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Analytical Methods

Acyl ester was determined by a slight modification[33] of the hydroxamate method.[34] In some cases, a half-scale method was used to give a final volume of 2 ml, the colour of which was read in semi-micro cells. Carbohydrate was analysed by the phenol-sulphuric method[35] relative to a glucose standard. Total sterol was measured by the Liebermann–Burchard reaction,[36] relative to β-sitosterol. Free fatty acids were measured as coloured complexes formed between their copper soaps and 1,5-diphenylcarbohydrazide,[37] after silicic acid treatment to remove interfering phospholipid.[37]

TLC

Silica gel G plates were prepared and activated as previously described.[11] Two systems were used: $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:5, v/v) for polarlipids and petrol. ether (B.P. 60–80°–Et_2O–HOAc (70:30:1, v/v) for neutral lipids. Total lipids were visualized with I_2 vapour or by charring after a 50% H_2SO_4 spray. Specific spray reagents used were: molybdate reagent for phospholipids,[38] periodate—Schiff's reagent for glycolipids,[39] *N,N*-dimethyl-*p*-phenylenediamine for fatty acid hydroperoxides,[17] 2,4-dinitrophenylhydrazine for carbonyl functions; free fatty acids were detected by the method of Dudzinki.[40] In experiments with ^{14}C-labelled lipids, TLC plates were scanned for radioactivity and, after I_2 treatment to visualize the lipids, areas of the TLC plate containing each labelled lipid were scraped off, extracted with solvent[41] and the extract analysed for organic phosphate[42] and radioactivity (Automatic Liquid Scintillation Analyser, Philips N.V., Eindhoven, The Netherlands).

Acknowledgements—I am grateful to Mr. A. S. A. Pryke, Mr. D. R. Phillips and Mrs. J. Matthew for experimental assistance, to Mr. S. Frey for photographic work and to Drs. A. C. Hulme and M. J. C. Rhodes for valuable discussions.

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