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The Hydrolysis of Phosphatidylinositol by Lysosomal Enzymes of Rat Liver and Brain

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1. Lysosomes from rat liver contain two enzymic systems for hydrolysing phosphatidylinositol: a deacylation via lysophosphatidylinositol producing glycerophosphoinositol and non-esterified fatty acid, and a phospholipase C-like cleavage into inositol 1-phosphate and diacylglycerol. 2. The separate enzyme systems involved can be distinguished by gel filtration, differential temperature-stability and the inhibitory action of detergents. 3. The enzyme systems both have pH optima at 4.8 and their attack on a pure phosphatidylinositol substrate is inhibited by many bivalent metals including Ca^{2+} and Mg^{2+}, and cationic drugs. 4. Whereas the deacylation system will attack other glycerophospholipids, the phospholipase C shows a marked specificity towards phosphatidylinositol, although it will also slowly attack phosphatidylcholine with the liberation of phosphocholine. 5. Gel filtration and temperature-stability distinguish the phospholipase C from lysosomal phosphatidic acid phosphatase, but not from sphingomyelinase. 6. Evidence is presented that an EDTA-insensitive phospholipase C degrading phosphatidylinositol is present in rat brain.

The metabolism of inositolphospholipids is often markedly different from that of the major phospholipid fractions and is specifically altered in a wide range of physiologically stimulated tissues (Hokin, 1969; Michell, 1975). These changes in metabolism are usually thought to be the result of increased catabolism of phosphatidylinositol by the Ca^{2+}-dependent enzyme that specifically cleaves phosphatidylinositol in a phospholipase C-like manner; this enzyme is widely distributed in animal tissues and is predominantly cytosolic in location (Dawson, 1959; Kemp *et al.*, 1961; Atherton & Hawthorne, 1968).

While investigating the catabolism of phosphatidylinositol by rat liver lysosomes, we observed that breakdown took place by two distinct pathways. Lysosomes can completely deacylate phosphatidylinositol to yield glycerophosphoinositol (Irvine *et al.*, 1977*a*) in a manner similar to the deacylation of other glycerophospholipids by these organelles (Franson *et al.*, 1971). However, the predominant mode of degradation is by an EDTA-insensitive phospholipase C-like activity that cleaves phosphoinositol directly from the substrate, and we have been able to distinguish this activity clearly from the Ca^{2+}-dependent enzyme in the cytosol (Irvine *et al.*, 1977*a*,*b*).

All of the tissues that exhibit stimulated phosphatidylinositol turnover are also likely to show enhanced lysosomal activity, either through direct involvement of lysosomes in secretion (Dingle & Fell, 1969; Novikoff, 1976) or through their probable role in recycling of membrane vesicles within the cell (Hokin, 1969; Lloyd, 1977). It is clear therefore that, if lysosomes do specifically break down phosphatidylinositol by cleavage of phosphoinositol from the substrate, stimulated turnover of this phosphoinositol moiety could be a direct result of increased lysosomal activity (Dawson & Irvine, 1978). For this reason we have begun a detailed study of the manner in which lysosomes catabolize phosphatidylinositol, and this paper reports the results of the first stage of investigations on the enzymes concerned, with pure [^{32}P]phosphatidylinositol as substrate.

Methods

Preparation of substrates

^{32}P-labelled phospholipids were prepared from baker's yeast by the methods of Hazlewood & Dawson (1975). However, the initial fractionation on the alumina column was modified to give a better recovery of phosphatidylinositol. The column was successively eluted with chloroform/ethanol/water mixtures with the compositions (1) 2:5:1 (by vol.), (2) 4:10:3 (by vol.), (3) 2:5:3 (by vol.), which eluted phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol respectively. The latter, after purification by t.l.c. (Hazlewood & Dawson, 1975), was examined by alkaline degradation (Dawson *et al.*, 1962). No evidence for the presence of ceramidephosphoinositol (Smith & Lester, 1974) in the

[^{32}P]phosphatidylinositol could be obtained; alkali-stable phospholipid was present at less than 0.02%.

[^{32}P]Phosphatidic acid was prepared from [^{32}P]-phosphatidylcholine (200μg of P), which was mixed with 0.2ml of 0.2M-sodium acetate buffer pH5.4, 0.1ml of 0.5M-$CaCl_2$ and 1.5mg of phospholipase D (Sigma, London S.W.6, U.K.; type 1) in 0.6ml of water. The mixture was shaken with 0.15ml of diethyl ether and incubated for 30min at 37°C. The reaction was terminated by heating for 5min at 100°C and adding 4ml of chloroform/methanol (2:1, v/v). After shaking, the lower phase was separated, and the [^{32}P]phosphatidic acid in it was isolated by preparative t.l.c. (Merck, Darmstadt, Germany; silica gel F254; chloroform/methanol/acetic acid/water, 65:50:1:4, by vol.) with the use of radioautography and markers for location of the phosphatidic acid strip (yield, 100μg of P).

Preparation of lysosomal enzymes

Lysosomes were prepared by the method of Trouet (1974); usually three rats (approx. 200g) were each injected with 2ml of 20% (w/v) Triton WR-1339, and their livers (total 40g) removed 4 days later. The distribution of marker enzymes in the fractions isolated was determined as described by Irvine *et al.* (1977*a*). The lysosomal preparation was dialysed overnight against water.

Large-granule soluble proteins were prepared by homogenizing the livers of two rats in 80ml of cold 0.25M-sucrose. Nuclei and cell debris were removed by centrifugation at 640*g* for 12min, and the pellet was washed once in 80ml of 0.25M-sucrose. The combined supernatants were then centrifuged at 32000*g* for 20min. The pellet was washed once and resedimented as before in 160ml of 0.25M-sucrose and then resuspended in 40ml of glass-distilled water and frozen and thawed 10–12 times. Membranes were removed by centrifugation at 100000*g* for 60min, and the supernatant proteins were concentrated by precipitation in 70%-satd. $(NH_4)_2SO_4$, followed by solubilization of the precipitate in 5–10ml of water and dialysis overnight against water.

Enzyme determinations

The incubation of [^{32}P]phosphatidylinositol was as described previously (Irvine *et al.*, 1977*a*), except that the Na^+ in the medium was replaced by K^+ that allowed a higher rate of substrate degradation (see below). The isolation and determination of [^{32}P]-phosphoinositol and glycero[^{32}P]phosphoinositol is also as stated in this previous publication. Ca^{2+}-activated phosphatidylinositol inositolphosphohydrolase was measured by determining water-soluble ^{32}P released from the [^{32}P]phosphatidylinositol in a similar incubation system. Phosphatidate phosphatase (EC 3.1.3.4) was assayed in the same medium with [^{32}P]phosphatidic acid as the substrate. The water-soluble product was shown to be exclusively [^{32}P]P_i by ionophoresis (Dawson & Clarke, 1972), even in the presence of an excess of non-radioactive glycerophosphate, and was estimated by assaying a portion of the water-soluble products for [^{32}P]P_i by scintillation counting. Sphingomyelin phosphodiesterase (EC 3.1.4.12) was determined by the method of Heller & Shapiro (1966).

Examination of water-soluble products of phospholipid digestion

^{32}P-labelled water-soluble products cleaved from phosphatidylinositol were separated by the method of Dawson & Clarke (1972). Phosphocholine, glycerophosphocholine, phosphoethanolamine and glycerophosphoethanolamine were separated two dimensionally on paper by ionophoresis (2h, pH3.6, 40V/cm) in one direction and chromatography (phenol-saturated with water/ethanol/17M-NH_3, 10:3:2, by vol.) in the other. The substances did not move appreciably on ionophoresis, but buffer salts were removed. In the chromatography, the phosphate esters previously mentioned had R_F values of 0.59, 0.83, 0.11 and 0.37 respectively.

Examination of lipid products of phosphatidylinositol digestion

To examine the lipid products formed from phosphatidylinositol by lysosomal enzymes, the incubation was terminated by the addition of 3.6ml of chloroform/methanol (2:1, v/v) and 0.15ml of 1M-$CaCl_2$. After shaking and centrifuging, the lower chloroform-rich phase was taken to low volume and a portion applied for t.l.c. on silica gel (Merck F254). For examining lysophosphatidylinositol production, the solvents chloroform/methanol/acetic acid/water (65:50:1:4, by vol.) and chloroform/methanol/17M-NH_3 (110:50:13, by vol.) were used. To investigate neutral lipids, the solvent was light petroleum (b.p. 60–80°C)/diethyl ether/acetic acid (50:50:1, by vol.) in which fatty acid, diacylglycerol and monoacylglycerol had R_F values of 0.46, 0.25 and 0.03 respectively.

Results

General properties of the two lysosomal enzyme preparations

The two enzyme preparations used in the present investigation were a dialysed preparation of liver lysosomes prepared by the Triton WR-1339 technique, and a soluble-protein preparation obtained by freeze–thawing a washed large-granule fraction from

liver (see the Methods section). Although the latter contained soluble lysosomal proteins, it also contained proteins from the mitochondrial matrix and a small amount of contaminating cytoplasmic proteins not removed by washing the large granules. The Triton WR-1339-prepared lysosomes, although free of these contaminants, contained Triton WR-1339, which, as shown below, inhibits the enzymes hydrolysing phosphatidylinositol. Both preparations produced a cleavage of phosphoinositol from [^{32}P]phosphatidylinositol in the presence of EDTA. On the addition of $CaCl_2$ the activity of the lysosomal enzyme preparation was markedly inhibited, whereas the large-granule soluble proteins showed appreciable activity consistent with the presence of residual Ca^{2+}-stimulated cytoplasmic phospholipase C active against phosphatidylinositol. In further experiments, it was shown that this latter enzyme in rat liver supernatant fractions could be totally suppressed by EDTA. Thus the large-granule soluble-protein fraction in the presence of EDTA could be used to study the lysosomal phospholipases active against phosphatidylinositol. Such preparations have the advantage of being very easily obtained with a much higher yield of the lysosomal enzymes than that obtained by the Triton WR-1339 method. Measurements showed that, when freshly prepared, the large-granule lysosomal enzyme preparation liberated phosphoinositol and glycerophosphoinositol in a concentration ratio of greater than 10 but on storage, even at −10°C, this ratio decreased. This is due to the greater instability of the phospholipase C enzyme compared with the deacylating phospholipases, which will be discussed below. The freshly prepared liver lysosomes, obtained after the injection of detergent, liberated phosphoinositol at 3–6 times the rate of glycerophosphoinositol. This lower ratio can probably be attributed to the selective inhibition of the phospholipase C enzyme by Triton WR-1339 present in lysosomes (see below).

Under the standard conditions of assay the liberation of both products from the substrate was linear for up to 1 h. On dialysis of the lysosomal preparation against water overnight, the activity was recovered in the supernatant obtained on centrifuging at 100000*g* for 1 h; the pellet was devoid of activity.

Products of the hydrolysis of phosphatidylinositol by lysosomal enzymes

The main water-soluble ^{32}P-labelled product formed from labelled phosphatidylinositol was identified as inositol 1-phosphate by paper ionophoresis, chromatography and radioautography (Dawson & Clarke, 1972). Small amounts of glycerophosphoinositol were produced. No glycerophosphate and only traces of P_i were detected. Inositol cyclic phosphate, which is a product of phosphatidylinositol hydrolysis by the cytoplasmic phospholipase C (Dawson *et al.*, 1971), was not found. The lysosomal enzyme preparation when incubated under the present conditions with inositol 1,2-cyclic phosphate slowly attacked this phosphodiester, liberating inositol monophosphate possibly because of the acid-pH-optimum phosphodiesterases present (Brightwell & Tappel, 1968). However, the addition of excess of non-radioactive inositol cyclic phosphate did not significantly effect the liberation of labelled inositol monophosphate from [^{32}P]phosphatidylinositol and, on isolation, the inositol cyclic phosphate showed no label. Thus inositol cyclic phosphate is unlikely to be an intermediary in the formation of phosphoinositol from phosphatidylinositol.

Examination of the lipids extracted from the incubation medium by t.l.c. showed, as well as the original substrate, a well-defined spot in the position of lysophosphatidylinositol (acid solvent, R_F for phosphatidylinositol 0.48, for lysophosphatidylinositol 0.19; alkaline solvent, R_F for phosphatidylinositol 0.20, for lysophosphatidylinositol 0.08). Mild alkaline degradation of this substance (Dawson *et al.*, 1962) produced glycerophosphoinositol as the sole water-soluble product, consistent with the identification of the original lipid as lysophosphatidylinositol. This latter lipid has previously been identified as a component of the pancreas (Keenan & Hokin, 1962).

The finding of phosphoinositol as the main hydrolysis product of phosphatidylinositol suggested that this had been formed by direct cleavage of the phosphatidylinositol producing diacylglycerol. No evidence was obtained that the enzyme preparations could liberate phosphoinositol from glycerophosphoinositol as has been shown to occur with many tissue membrane fractions, e.g. kidney, liver, spleen and pancreas (Dawson & Hemington, 1977). However, t.l.c. of the neutral lipids of the incubation medium showed the almost exclusive production of non-esterified fatty acid.

It has been reported that liver lysosomes contain a neutral lipid lipase whose activity is markedly stimulated by the addition of anionic amphiphiles such as phosphatidylinositol (Karija & Kaplan, 1973). In confirmation, the present enzyme preparations were found to deacylate diacylglycerol, a reaction that was markedly stimulated by the addition of phosphatidylinositol. Thus it seems certain that any diacylglycreol formed by the cleavage of phosphoinositol from phosphatidylinositol by the lysosomal enzymes would itself be rapidly deacylated. However, by using short periods of incubation either with the Triton WR-1339-prepared enzymes, or with large-granules soluble enzymes partially purified on Sephadex G-150 columns (see below), it has been possible to show by t.l.c. the liberation of small amounts of diacylglycerol and monoacylglycerol, although any

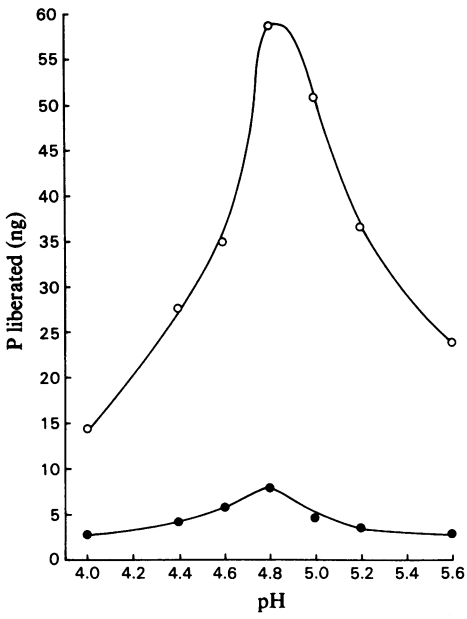

Fig. 1. *Effect of pH on the hydrolysis of phosphatidylinositol by lysosomal enzymes*

The pH was varied with a series of acetic acid/potassium acetate buffers (0.02M). Incubation was as described in the Methods section with a lysosomal preparation (Triton WR-1339). Graphs show the liberation of phosphoinositol (○) and glycerophosphoinositol (●). Similar results were obtained from an identical experiment.

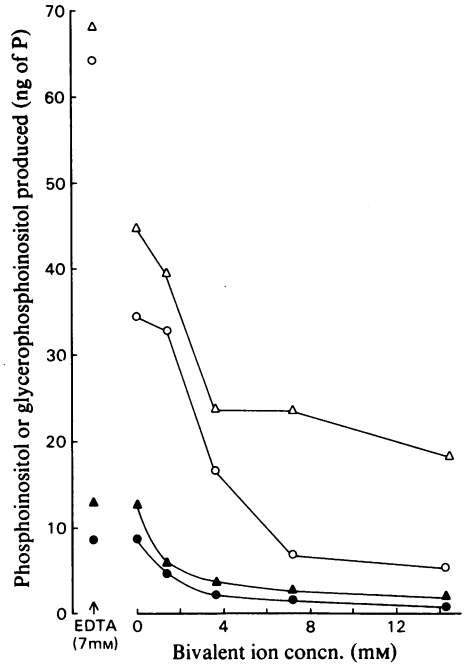

Fig. 2. *Effect of EDTA, Ca^{2+} and Mg^{2+} on lysosomal hydrolysis of phosphatidylinositol*

Lysosomes were used as the enzyme preparation. Incubation conditions were as described in the Methods section, but EDTA was not added, except as indicated. Similar results were obtained in an identical experiment. For phosphoinositol release: △, $MgCl_2$ added; ○, $CaCl_2$ added. For glycerophosphoinositol release: ▲, $MgCl_2$ added; ●, $CaCl_2$ added.

demonstration of stoicheiometry between diacylglycerol and the phosphoinositol formed will have to await further purification of the phospholipase C.

pH optimum of the hydrolysis

The pH optimum for phosphatidylinositol hydrolysis measured in a series of acetate buffers was pH4.8 for both the production of phosphoinositol and glycerophosphoinositol (Fig. 1). This can be compared with a pH optimum of 4.5 for the deacylating lysosomal phospholipases attacking phosphatidylethanolamine (Waite *et al.*, 1976) and of 5.6 for the splitting of phosphoinositol from phosphatidylinositol by the Ca^{2+}-stimulated cytoplasmic phospholipase C of rat liver (Kemp *et al.*, 1961); also Allan & Michell (1974) reported optima of 5.5 and 7.0 in lymphocyte supernatants.

Effect of metal ions

The hydrolysis of phosphatidylinositol consistently occurred more rapidly (20–40%), when the Na^+ in the buffer and other solutions added for the incubation was replaced by K^+. Consequently all the experiments were performed with potassium salts.

The formation of both glycerophosphoinositol and phosphoinositol was substantially inhibited by both Ca^{2+} and Mg^{2+} (Fig. 2), although with the phosphoinositol release, the inhibition was less at high Mg^{2+} concentration than with the equivalent concentration of Ca^{2+}. Both of these bivalent cations also inhibited the accumulation of lysophosphatidylinositol from the same substrate. Mn^{2+} and Fe^{2+} were even more effective inhibitors of the hydrolysis (Fig. 3), 4mM producing complete cessation of hydrolysis; Zn^{2+} and Fe^{3+} (4mM) also produced total inhibition of the phospholipases.

EDTA consistently stimulated the lysosomal release of phosphoinositol from phosphatidylinositol (Fig. 2) by a factor that varied with each preparation (40–110%). The corresponding stimulation of the glycerophosphoinositol release was small or non-existent. To test whether the EDTA stimulation was

caused by the removal of inhibitory heavy metals accumulated by the lysosomes during isolation (Sternlieb & Goldfischer, 1976), two preparations were made in which all the solutions used, for homogenization, washing etc., were prepared from doubly distilled water and precautions were taken to avoid contamination of the subcellular fractions with metal ions from equipment. EDTA still stimulated the phosphoinositol release by 39 and 22% in the two preparations, suggesting that although contamination of the lysosomes with metals during isolation might be part of the phenomenon, this accumulation of metals may have occurred *in vivo*. It is known that lysosomes are involved in both the physiological metabolism of heavy metals and the sequestration of potentially toxic metals picked up from the food and environment (Sternlieb & Goldfischer, 1976).

Specificity for glycerophospholipids

Lysosomes were incubated separately with equivalent concentrations of [^{32}P]phosphatidylinositol, [^{32}P]phosphatidylcholine and [^{32}P]phosphatidylethanolamine, and the water-soluble phosphorus products examined. With pure phospholipids the deacylating system has a preferential specificity for phosphatidylethanolamine (Table 1) in agreement with the results of Waite *et al.* (1976). On the other hand the phospholipase C has a substantial preference for phosphatidylinositol with phosphatidylcholine and phosphatidylethanolamine being hydrolysed at 5–6% and 1–2% of this rate respectively. Confirmation of the production of [^{32}P]phosphocholine was obtained by exact correspondence of the radioactive and marker spots on radioautography and by its stability to acid hydrolysis (3.5M-HCl, 100°C, 20min). The release of phosphocholine from phosphatidylcholine was not increased by solubilization of the phospholipid by Triton X-100 (0.2%).

Inhibition by cationic drugs, detergents and other substances

The effect of three cationic drugs, tetracaine, mepacrine and chlorpromazine, on the hydrolysis was tested (Fig. 4). All were potent inhibitors of the hydrolysis, with chlorpromazine being especially effective: a concentration of 0.5mM resulted in almost total suppression of both glycerophosphoinositol and phosphoinositol release. The formation of the latter product was less sensitive to the inhibitory effect of all three drugs.

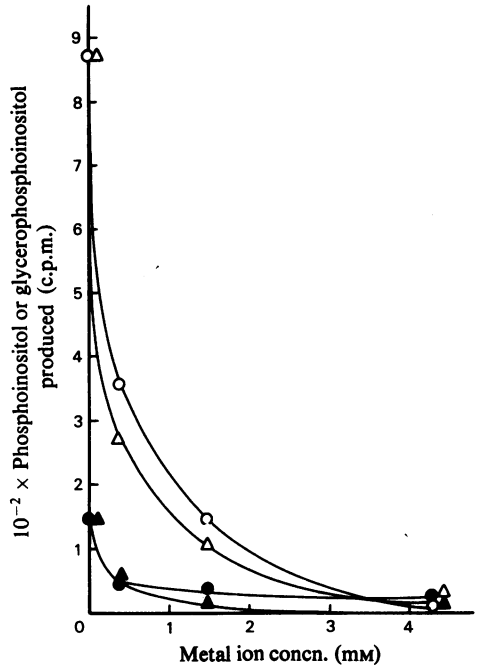

Fig. 3. *Inhibition of lysosomal hydrolysis of phosphatidylinositol by Fe^{2+} and Mn^{2+}*

Lysosomes were used as the enzyme preparation. Incubation conditions were as described in the Methods section, but no EDTA was added. Similar results were obtained in an identical experiment. For phosphoinositol release: ○, $FeSO_4$ added; △, $MnCl_2$ added. For glycerophosphoinositol release: ●, $FeSO_4$ added; ▲, $MnCl_2$ added.

Table 1. *Water-soluble radioactive products formed on hydrolysis of individual ^{32}P-labelled phosphoglycerides by the lysosomal phospholipases*

[^{32}P]Phosphatidylinositol, [^{32}P]phosphatidylcholine, and [^{32}P]phosphatidylethanolamine (44μg) were incubated with a lysosomal fraction under the standard conditions. After incubation the water-soluble products were separated as stated in the Methods section, and the radioactivity in the 'phospho' and 'glycerophospho' derivatives counted. Similar relative amounts of products were obtained in an identical experiment with a different enzyme preparation.

Substrate	Products (ng of P liberated)	
Phosphatidylinositol	Phosphoinositol (91.4)	Glycerophosphoinositol (14.8)
Phosphatidylcholine	Phosphocholine (5.3)	Glycerophosphocholine (5.3)
Phosphatidylethanolamine	Phosphoethanolamine (1.4)	Glycerophosphoethanolamine (20.0)

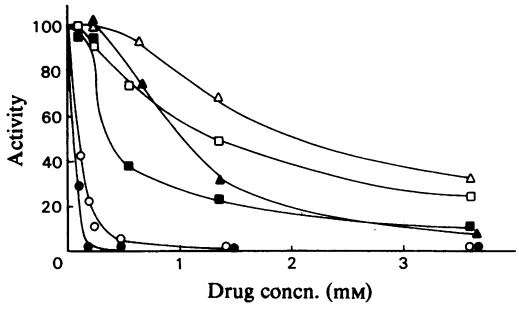

Fig. 4. *Inhibition of lysosomal phosphatidylinositol hydrolysis by cationic drugs*

A liver large-granule soluble-protein fraction was used as the source of lysosomal phospholipases. The incubations were carried out as described in the Methods section with increasing concentrations of drug solutions (at pH4.8) added. Similar results were obtained in two identical experiments. For phosphoinositol release: △, tetracaine; □, mepacrine; ○, chlorpromazine. For glycerophosphoinositol release: ▲, tetracaine; ■, mepacrine; ●, chlorpromazine.

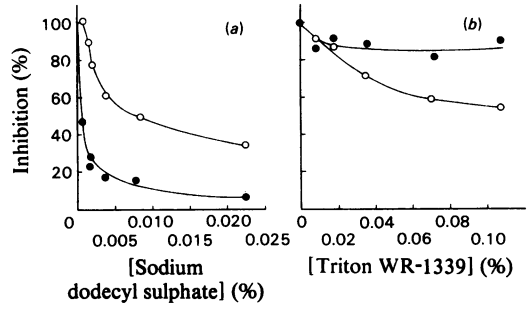

Fig. 5. *Inhibition of lysosomal phosphatidylinositol hydrolysis by detergents*

The activities were assayed as described in the Methods section with a large-granule soluble-protein fraction, as the source of lysosomal phospholipases. Similar results were obtained in an identical experiment. Symbols: ○, phosphoinositol release; ●, glycerophosphoinositol release. (*a*) Effect of sodium dodecyl sulphate. (*b*) Effect of Triton WR1339.

The liberation of both products was inhibited by detergents, although some differences existed in relative sensitivity. The glycerophosphoinositol release was more sensitive to detergents such as sodium dodecyl sulphate (Fig. 5), sodium deoxycholate, cetyltrimethylammonium bromide and Triton X-100. In contrast, the Triton WR-1339 used to prepare lysosomes, gave only a small inhibition of phosphoinositol release (Fig. 5) and even sometimes slight stimulation of glycerophosphoinositol formation.

Both phospholipases were inhibited by iodoacetate (14mM), glycerophosphoinositol release by 38% and phosphoinositol release by 29%. Cysteine (35mM) also inhibited both types of hydrolysis, phosphoinositol release (44%) and glycerophosphoinositol release (68%).

Behaviour of lysosomal phospholipases hydrolysing phosphatidylinositol on gel filtration

To investigate more thoroughly whether the supposed lysosomal enzyme systems hydrolysing phosphatidylinositol had a separate identity, and their relationship to other phospholipases previously reported in lysosomes, fractionation of a large-granule soluble-protein preparation was carried out on a Sephadex G-150 column (Fig. 6). The enzyme causing release of phosphoinositol from phosphatidylinositol was completely excluded from the column and was clearly distinguishable from the deacylating phospholipases of lower molecular weight forming lysophosphatidylinositol and glycero-

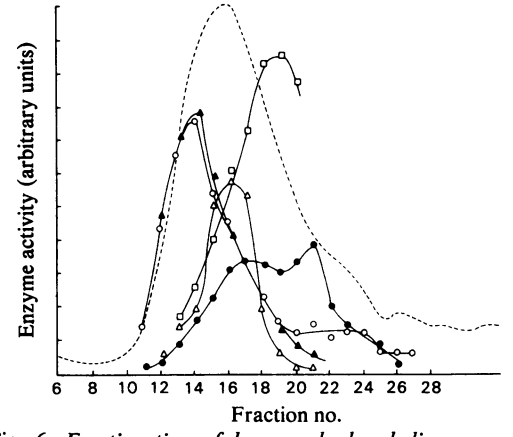

Fig. 6. *Fractionation of lysosomal phospholipases on a Sephadex G-150 column*

Large-granule soluble proteins were fractionated on a Sephadex G-150 column (110cm × 1cm) in 0.02M-potassium acetate/acetic acid buffer, pH4.8. Fractions of about 3.5ml were collected and assayed for the liberation of phosphoinositol (○), glycerophosphoinositol (●) and lysophosphatidylinositol (□) from [^{32}P]phosphatidylinositol (see the Methods section), phosphatidate phosphatase (△) and sphingomyelin phosphodiesterase (▲). The broken line represents protein (A_{280}). Similar results were obtained in an identical experiment.

phosphoinositol. In addition, the phospholipase C enzyme could be distinguished from phosphatidate phosphatase, another phospholipase in lysosomes that hydrolyses the glycerol phosphate bond of

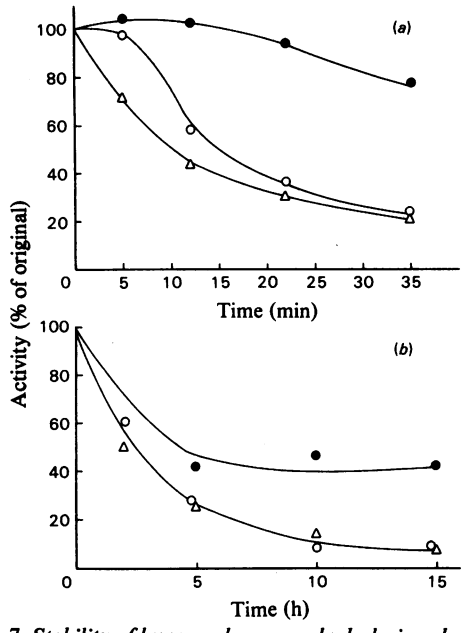

Fig. 7. *Stability of lysosomal enzymes hydrolysing phosphatidylinositol and sphingomyelin*

A large-granule soluble-protein fraction was used as a source of lysosomal enzymes. The activities of the phospholipases were assayed as indicated in the Methods section. ●, Glycerophosphoinositol release from phosphatidylinositol; ○, phosphoinositol release from phosphatidylinositol; △, sphinogomyelin phosphodiesterase. (*a*) The enzyme preparation diluted 5 times with water was kept at 52°C for various times. (*b*) The enzyme preparation was diluted 5 times with 0.1% Triton X-100 in 0.02M-potassium acetate/acetic acid buffer, pH4.8 (final concentrations). The mixture was frozen at −15°C for various times. These experiments were repeated twice. Although the absolute stability of the enzymes varied with the preparation, their relative stability was the same as that shown.

phosphatidic acid (Sedgwick & Hübscher, 1965). However, the phospholipase C active against phosphatidylinositol was not separated from lysosomal sphingomyelin phosphodiesterase, an enzyme that cleaves sphingomyelin into ceramide and phosphocholine (Heller & Shapiro, 1966). These high-molecular-weight enzymes were also not separated on a Sephadex G-200 column; again both appeared to be totally excluded from the column. The same was true if the column was run in the presence of 0.1% Triton X-100; it has been suggested that such non-ionic detergents can prevent or dissociate aggregation of lysosomal sphingomyelin phosphodiesterase (Pentchev *et al.*, 1977).

Differential stability of lysosomal phospholipases

Many indications were obtained that the phospholipase C active against phosphatidylinositol was less stable than the corresponding deacylating phospholipases. On storage, the activity of the phospholipase C was lost more rapidly. Thus when fractions eluted from a Sephadex G-150 column (Fig. 6) were stored for 1 month at 4°C in 0.02M-acetate buffer (pH4.8), the phospholipase C had diminished to 10% of the original value, whereas the deacylase system was little changed.

When the large-granule soluble proteins were heated at 52°C for various times the deacylating phospholipases were more stable than the phospholipase C (Fig. 7). The latter showed an unusual form of instability curve, which could suggest initial protection or activation that is also observed with the deacylating enzymes in the early stages. Apart from this early unusual behaviour, the fall off in phospholipase C is quite similar to that of the sphingomyelin phosphodiesterase activity (Fig. 7). In further experiments it was shown that at 59°C the stability of phosphatidate phosphatase was greater than the deacylation system that was, in turn, more stable than the phospholipase C. We also used the instability of the phospholipase C enzyme frozen at −15°C in the presence of 0.1% Triton X-100 to compare it with the other enzymes. The loss of activity of the phospholipase C and sphingomyelin phosphodiesterase was very similar and the deacylating system was again more stable (Fig. 7).

Occurrence of the phospholipase C degrading phosphatidylinositol in brain lysosomes

Since many of the reports of enhanced turnover of phosphatidylinositol on stimulation have been connected with the nervous system, it was important to establish whether a phospholipase C equivalent to that found in liver lysosomes occurred in brain. This is more difficult to ascertain, because of the very high content of cytoplasmic Ca^{2+}-activated phospholipase C active against phosphatidylinositol in rat brain compared with liver and because of the problems involved in preparing lysosome-enriched fractions from brain. Consequently, we prepared three soluble-protein fractions from brain tissue, derived from a total homogenate, a lysosomal concentrate prepared by the method of Koenig (1974) and from the supernatant. The ratios between the activities of the Ca^{2+}-activated release of phosphoinositol and the EDTA-insensitive formation of phosphoinositol were measured. Although the specific activity of the Ca^{2+}-activated phospholipase C was very large compared with that of the EDTA-insensitive enzyme, there was nevertheless in two experiments an appreciable enrichment of the latter enzyme in the lysosomal

Table 2. *Comparison of the Ca^{2+}-activated and EDTA-insensitive formation of phosphoinositol from phosphatidylinositol by soluble proteins from various brain fractions*

The brain fractions were isolated as described by Koenig (1974). They were dialysed overnight against water, and then frozen and thawed and centrifuged at 100000*g* to prepare the soluble proteins. Enzymic activities (release of phosphoinositol from [^{32}P]phosphatidylinositol) in the latter were determined as described in the Methods section.

Brain fraction (soluble proteins)	Ratio (Ca^{2+}-activated phospholipase C)/(EDTA-insensitive phospholipase C)		Enrichment of EDTA-insensitive phospholipase	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Total homogenate	537	979	1	1
Supernatant	261	595	2.1	1.6
Lysosome concentrate	74	107	7.3	9.1

concentrate compared with that of the soluble proteins from either the total homogenate or the supernatant (Table 2). This result suggested the existence of an EDTA-insensitive phospholipase C in brain distinct from that of the Ca^{2+}-activated cytoplasmic enzyme. Confirmatory evidence was obtained by fractionation of the soluble proteins from a brain homogenate on Sephadex G-150. The peak of the EDTA-insensitive phospholipase C corresponded to its total exclusion as had been found previously. The Ca^{2+}-activated phospholipase C eluted from the column emerged later, although an absolute separation was not achieved.

Discussion

The present results show that the glycerophosphoinositol and phosphoinositol, formed by incubating preparations of liver lysosomes with a pure phosphatidylinositol substrate, are obtained by different enzyme systems. This is indicated by the partial separation achieved by gel filtration on Sephadex G-150 columns, the far greater instability of the phosphoinositol-producing enzyme to storage under various conditions and the differential inhibition of the two enzyme systems by detergents. Under the present conditions of incubation, it is clear that when lysosomal phospholipases hydrolyse phosphatidylinositol, phosphoinositol formation is heavily favoured, occurring at more than ten times the rate of that of glycerophosphoinositol release.

The latter hydrolysis, representing as it does complete deacylation of the substrate through the intermediary lysophosphatidylinositol, is presumably carried out by the phospholipases A_1 and A_2 characterized in liver lysosomes by Waite *et al.* (1976). They showed that the activity of these enzymes against the *N*-containing glycerophospholipids was inhibited by Ca^{2+} and other metals, as we have found for the liberation of lysophosphatidylinositol and glycerophosphoinositol from phosphatidylinositol. Waite *et al.* (1976) also found a pH optimum of 4.5 and a favoured substrate in phosphatidylethanolamine, which is equivalent to our own observations. However, purified lysosomal phospholipase A_1 hydrolysed phosphatidylinositol at only a few per cent of the rate of phosphatidylcholine at optimal substrate concentrations, whereas in our own experiments glycerophosphocholine was liberated more slowly than glycerophosphoinositol. This could possibly be due to a different substrate specificity of lysosomal phospholipase A_2 against the intact phospholipids.

The evidence obtained suggests that the phospholipase C attacking phosphatidylinositol causes a direct cleavage into diacylglycerol and inositol 1-phosphate without the additional formation of inositol 1,2-cyclic phosphate obtained with the Ca^{2+}-dependent cytoplasmic phospholipase C. The inability to isolate stoicheiometric amounts of diacylglycerol can be adequately explained by the presence of an active lysosomal lipase in the enzyme preparations, a lipase that we have confirmed is markedly stimulated by phosphatidylinositol.

The substrate specificity of the phospholipase C towards various glycerophospholipids was quite different from the deacylation system. With the pure phospholipid substrates, the enzyme had a marked preference for phosphatidylinositol, whereas phosphatidylcholine was only slowly hydrolysed and phosphatidylethanolamine even less readily. However, exact descriptions of specificity of phospholipases with pure substrates are of doubtful significance (Dawson, 1973), and a better physiological picture of the relative activities of the various enzymes must await studies on membranous substrates. An EDTA-insensitive formation of phosphoethanolamine by brain homogenates that could not be accounted for by reversal of the metabolic pathway synthesizing phosphatidylethanolamine has been reported by Williams *et al.* (1973) and the present enzyme could be responsible for this.

The question arises as to whether the phospholipase C hydrolysing phosphatidylinositol can be equated to

any of the known phospholipases present in lysosomes. Phosphatidate phosphatase, which cleaves phosphatidic acid into diacylglycerol and P_I, is clearly different, since it can be separated from the phospholipase C on Sephadex G-150 columns, and its stability at elevated temperatures is appreciably greater. With sphingomyelin phosphodiesterase the difference is not as clear-cut. Like the present enzyme, sphingomyelin phosphodiesterase is totally excluded from Sephadex G-200, which would indicate that both enzymes have a high molecular weight, either as a monomer or in an active polymeric form. Both enzymes show rather similar characteristics of instability when stored or maintained at elevated temperatures. However, purified lysosomal sphingomyelin phosphodiesterase is reported to be highly specific for sphingomyelin, and even phosphatidylcholine is not hydrolysed (Pentchev *et al.*, 1977), whereas in our experiments phosphocholine is clearly liberated from this latter substrate by lysosomal enzymes (Table 1). It would be therefore remarkable if sphingomyelin phosphodiesterase could attack a substrate such as phosphatidylinositol, whose structure was even more different from sphingomyelin than phosphatidylcholine.

The marked inhibitory action of various metal ions and cationic drugs on both the phospholipase C and deacylation systems can probably be ascribed to the dislike of the enzymes concerned for positive charges on the surface of the substrate. Such agents would either alter the ξ-potential of the substrate by counter-ion binding (metals) or by actual absorption into and orientation within the phospholipid bilayer of the drugs (Hauser & Dawson, 1968). It is known that phospholipases can be very dependent for their activity on the precise electrokinetic conditions on the surface of substrate (Dawson, 1968). Hauser & Dawson (1968) have shown that chlorpromazine, the most inhibitory drug of those tested, was extremely surface-active when tested for its effect on unimolecular films of phosphatidylinositol.

There is little information about the role that the present lysosomal phospholipases active against phosphatidylinositol play in the turnover of phosphatidylinositol in cells. Both glycerophosphoinositol (Koch & Diringer, 1974) and phosphoinositol (Hübscher & Hawthorne, 1957) have been found as components of tissues. It has already been pointed out that phosphatidylinositol turnover can markedy increase when a tissue is stimulated into physiological activity and it is now believed that this turnover is turned on by a stimulation of the catabolism of phosphatidylinositol by a mechanism that does not necessarily involve the entrance of Ca^{2+} into the cell. The present enzymes could fulfil this requirement, since, unlike the cytoplasmic phospholipase C, they have no requirement for Ca^{2+}. We have recently pointed out that the enhanced turnover of phosphatidylinositol in many tissues in response to stimulation is associated with enhanced lysosomal activity (Dawson & Irvine, 1978). This stimulated turnover is generally assumed to involve only the phosphoinositol moiety of the phosphatidylinositol molecule (Michell, 1975), so the further hydrolysis of diacylglycerol by the lipase in liver lysosomes (see the Results section) might appear to preclude a role for lysosomes in this phenomenon. We emphasize, however, first that stimulated turnover is not always confined to the phosphoinositol group (for example, Scott *et al.*, 1968), and secondly that hydrolysis of diacylglycerol in a membrane may be very much slower than when presented as the activated substrate.

At first sight, the activity of the lysosomal phosphatidylinositol-specific phospholipase C may seem minimal compared with the cytoplasmic phospholipase C. But it should be stressed that the former suffers an enormous dilution on preparation, compared with its likely concentration in the lysosome. Thus the relative activities of the two enzymes are between 50:1 and 100:1 in rat liver in favour of the cytoplasmic enzyme (Irvine *et al.*, 1977*a*). As the cytoplasm has a volume approximately fifty times greater than lysosomes in rat liver (Weibel *et al.*, 1969), the actual concentration of the two activities *in vivo* must be similar.

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References

Allan, D. & Michell, R. H. (1974) *Biochem. J.* **142**, 599–604
Atherton, R. S. & Hawthorne, J. N., (1968) *Eur. J. Biochem.* **4**, 68–75
Brightwell, R. & Tappel, A. L. (1968) *Arch. Biochem. Biophys.* **124**, 325–332
Dawson, R. M. C. (1959) *Biochim. Biophys. Acta* **33**, 68–77
Dawson, R. M. C. (1968) in *Biological Membranes* (Chapman, D., ed.), pp. 203–232, Academic Press, London
Dawson, R. M. C. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 97–116, Elsevier, Amsterdam, New York and London
Dawson, R. M. C. & Clarke, N. (1972) *Biochem. J.* **127**, 113–118
Dawson, R. M. C. & Hemington, N. (1977) *Biochem. J.* **162**, 241–245
Dawson, R. M. C. & Irvine, R. F. (1978) *Adv. Prostaglandin Thromboxane Res.* **3**, 47–54
Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962) *Biochem. J.* **84**, 497–501
Dawson, R. M. C., Freinkel, N., Jungalwala, F. B. & Clarke, N. (1971) *Biochem. J.* **122**, 605–607
Dingle, J. T. & Fell, H. B. (eds.) (1969) *Lysosomes in Biology and Pathology*, vol. 2, North-Holland, Amsterdam and London

Franson, R., Waite, M. & Lavia, M. (1971) *Biochemistry* **10**, 1942–1946
Hauser, H. & Dawson, R. M. C. (1968) *Biochem. J.* **109**, 909–916
Hazlewood, G. P. & Dawson, R. M. C. (1975) *J. Gen. Microbiol.* **89**, 163–174
Heller, M. & Shapiro, B. (1966) *Biochem. J.* **98**, 763–769
Hokin, L. E. (1969) in *Structure and Function of Nervous Tissue* (Bourne, G., ed.), vol. 3, pp. 161–184, Academic Press, New York
Hübscher, G. & Hawthorne, J. N. (1957) *Biochem. J.* **67**, 523–527
Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1977*a*) *Biochem. J.* **164**, 277–280
Irvine, R. F., Hemington, N. & Dawson, R. M. C. (1977*b*) *Biochem. Soc. Trans.* **5**, 1297–1299
Karija, M. & Kaplan, A. (1973) *J. Lipid Res.* **14**, 243–249
Keenan, R. W. & Hokin, L. E. (1962) *Biochim. Biophys. Acta* **60**, 428–430
Kemp, P., Hübscher, G. & Hawthorne, J. N. (1961) *Biochem. J.* **79**, 193–200
Koch, M. A. & Diringer, H. (1974) *Biochem. Biophys. Res. Commun.* **58**, 361–367
Koenig, H. (1974) *Methods Enzymol.* **31A**, 457–475
Lloyd, J. B. (1977) *Biochem. J.* **164**, 281–282
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Novikoff, A. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2781–2787
Pentchev, P. G., Brady, R. O., Gal, A. E. & Hibbert, S. R. (1977) *Biochim. Biophys. Acta* **488**, 312–321
Scott, T. W., Mills, S. C. & Freinkel, N. (1968) *Biochem. J.* **109**, 325–332
Sedgwick, B. & Hübscher, G. (1965) *Biochim. Biophys. Acta* **106**, 63–77
Smith, S. S. & Lester, R. L. (1974) *J. Biol. Chem.* **249**, 3395–3405
Sternlieb, I. & Goldfischer, S. (1976) in *Lysosomes in Biology and Medicine* (Dingle, J. T. & Dean, R., eds.), vol. 5, pp. 185–200, North-Holland, Amsterdam
Trouet, A. (1974) *Methods Enzymol.* **31A**, 323–329
Waite, M., Griffin, H. D. & Franson, R. (1976) in *Lysosomes in Biology and Medicine* (Dingle, J. T. & Dean, R., eds.), vol. 5, pp. 257–305, North-Holland, Amsterdam
Weibel, E. R., Staubli, W., Gnagni, H. R. & Hess, F. A. (1969) *J. Cell Biol.* **42**, 68–91
Williams, D. J., Spanner, S. & Ansell, G. B. (1973) *Biochem. Soc. Trans.* **1**, 466–467