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Lysosomal Phospholipases A_1 and A_2 of Bovine Adrenal Medulla

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1. [^{32}P]Lecithin and [^{32}P]phosphatidylethanolamine were prepared by incubating rat liver mince with [^{32}P]phosphate. With these ^{32}P-labelled phospholipids conditions for the quantitative assay of phospholipase A activity were established. 2. The distribution of phospholipase A activity between subcellular fractions of the bovine adrenal medulla was determined. Phospholipases A_1 and A_2, with pH optima at 4·2 and 6·5 respectively, were found in the large-granule fraction. By means of sucrose-density-gradient centrifugation it was shown that both these phospholipases were localized in lysosomes. 3. Lysosomal phospholipase A_1 catalysed the hydrolysis of [^{32}P]lecithin and [^{32}P]phosphatidylethanolamine at the same rate. The enzymic activity was inhibited by 70% in the presence of 10mM-calcium chloride. 4. Lysosomal phospholipase A_2 catalysed the hydrolysis of [^{32}P]phosphatidylethanolamine more rapidly than it hydrolysed [^{32}P]lecithin. The hydrolysis of [^{32}P]phosphatidylethanolamine, but not that of [^{32}P]lecithin, by phospholipase A_2 was activated by 0·8mM-calcium chloride. However, the hydrolysis of both substrates was inhibited by 8mM-calcium chloride. 5. The significance of the presence of phospholipase activity in lysosomes is discussed in relation to the functions of lysosomes in general and in the adrenal medulla.

The large-granule fraction obtained from homogenates of adrenal medulla contains three different types of cell particle: mitochondria, lysosomes and chromaffin granules (for a review see Smith, 1968). The chromaffin granules, i.e. the particles which contain the hormones, are characterized by a high content of lysolecithin (Blaschko, Firemark, Smith & Winkler, 1967*b*; Winkler, Strieder & Ziegler, 1967*b*). Lysolecithin can be formed in tissues by the action of phospholipase A on lecithin (for a review see Dawson, 1966). Such an enzyme has been reported to be present in bovine adrenal glands (Francioli, 1933) and, recently, in bovine adrenal medulla (Winkler, Strieder & Ziegler, 1967*c*). In further studies, with radioactively labelled substrates, we examined the large-granule fraction of bovine adrenal medulla for phospholipase A activity. Two enzymes, which are distinguished by their different pH optima (Blaschko, Smith, Winkler, van den Bosch & van Deenen, 1967*a*) and by their sites of attack on the lecithin molecule (Winkler, Smith, Dubois & van den Bosch, 1967*a*), were found in the large-granule fraction. One phospholipase A has a pH optimum at 4·2 and is specific for the 1-position of lecithin, whereas the other enzyme acts at pH 6·5 and preferentially removes the 2-acyl residue from lecithin. In accordance with current usage (van Deenen & de Haas, 1966) these enzymes have been called respectively phospholipase A_1 and phospholipase A_2 (Winkler *et al.* 1967*a*).

Phospholipase A_1 is present in the lysosomes of bovine adrenal medulla (Blaschko *et al.* 1967*a*). The present paper gives a detailed account of the use of ^{32}P-labelled phospholipids for the determination of small amounts of phospholipase A activity, provides evidence for the localization of the phospholipase A_2 in lysosomes and describes some of the properties of the two lysosomal phospholipases.

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METHODS

Isolation and analysis of subcellular fractions. Bovine adrenal medullae were homogenized and the homogenate was fractionated by differential and sucrose-density-gradient centrifugation as described by Smith & Winkler (1966). The sucrose density gradient was made up in centrifuge tubes of the SW 39 rotor (Spinco ultracentrifuge). Each tube contained 0·25ml. of 2·5M-sucrose solution at the bottom followed by 0·5ml. each of 2·0M-, 1·9M-, 1·8M-, 1·7M-, 1·6M-, 1·5M-, 1·4M- and 1·3M-sucrose solution. For some experiments the supernatant remaining after sedimentation of the large granules was centrifuged at 110000*g* for 60min. to obtain a microsomal fraction and a final supernatant. To lyse the subcellular particles and to remove the sucrose, the fractions obtained after centrifugation were dialysed against 25mM-glycylglycine–NaOH buffer, pH 6·5, for 16hr. at 3° before assay of enzymic

activities. Visking tubing of diameter $\frac{18}{32}$ in. was used for dialysis.

Catecholamines were determined by the method of Euler & Hamberg (1949), but with citrate–phosphate buffers (McIlvaine, 1921) instead of acetate buffers. Fumarase activity was measured by the method of Racker (1950) and glucose 6-phosphatase activity by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Acid ribonuclease activity was assayed by the method of de Duve (1955) with modifications (Smith & Winkler, 1966).

Preparation of ^{32}P*-labelled phospholipids.* For the preparation of ^{32}P-labelled substrates a method similar to that described by van den Bosch & van Deenen (1965) was used. About 800mg. of rat liver was chopped with scissors and suspended in 1ml. of Krebs solution (pH7·3), which contained 1·5mg. of sodium acetate, 1·5mg. of CTP and 10mg. of glucose. A portion (1ml.) of sodium [^{32}P]phosphate (Injection B.P.; The Radiochemical Centre, Amersham, Bucks.), with a specific radioactivity of 5mc/mg. of P, was added. This mixture was incubated for 6hr. at 37° in a water bath with shaking and under a stream of O_2+CO_2 (95:5). Afterwards the suspension was transferred with 20ml. of methanol to a Potter–Elvehjem homogenizer and homogenized. Then 10ml. of methanol, 15ml. of chloroform and 12ml. of water were added to the homogenate; this mixture was stirred overnight. On the next day 15ml. of chloroform and 15ml. of 0·06M-sodium phosphate buffer, pH7·4, were added to give two phases, according to the procedure of Bligh & Dyer (1959). The mixture was centrifuged for 15min. at 800*g*. The upper phase and the interphase containing precipitated proteins were sucked off and the surface of the lower phase was rinsed with the phosphate buffer. Finally, the lower phase was evaporated under reduced pressure and redissolved in 0·6ml. of chloroform. This solution (0·3ml.) was applied to a thin-layer chromatography plate (20cm. × 20cm.), covered with a layer (1mm. thick) of a mixture of starch and silica gel (MN-Kieselgel S; Macherey, Nagel and Co., Düren, Germany). Ascending chromatography was performed with the solvent system chloroform–methanol–water (45:15:2, by vol.). Chromatography was continued for about 30min. after the solvent had reached the top of the plate. The plate was then briefly left to dry and dipped into a solution of Ponceau red and uranyl acetate (Hooghwinkel & Niekerk, 1960). The stained phospholipids were identified by their R_F values and the respective areas of silica gel were scraped off into centrifuge tubes. The phospholipids were extracted from the silica gel according to the procedure of Bligh & Dyer (1959); the chloroform phase was filtered and kept at −15° for up to 4 weeks. The amounts and purity of the phospholipids were determined by quantitative thin-layer chromatography according to the procedure of Skipski, Peterson & Barclay (1964).

Assay of phospholipase A activity. After evaporation of the chloroform the ^{32}P-labelled substrate was dispersed in water by ultrasonic treatment for 20sec. at 20kHz and amplitude 4μ (MSE 100w ultrasonic disintegrator). The final concentration of the ^{32}P-labelled phospholipid was 110μg./ml. The phospholipid dispersion (0·2ml.) was incubated with up to 0·5ml. of a subcellular fraction with a buffer solution to give a final volume of 1ml. The final concentrations of the buffers were 50mM for glycylglycine–NaOH, 12·5mM for sodium acetate and 5mM for tris–sodium succinate.

It can be calculated from previous work (Winkler *et al.* 1967*a*) and from the results given below that probably all the phospholipase A activity at pH4·2 is due to that of phospholipase A_1 and that about 94% of the activity at pH6·5 (with phosphatidylethanolamine as substrate) is due to the activity of phospholipase A_2. Accordingly, it has been assumed in the present study that the phospholipase A activities measured at pH4·2 and pH6·5 represent respectively the activities of phospholipases A_1 and A_2. Unless otherwise indicated, sodium acetate buffer, pH4·2, was used for the assay of phospholipase A_1 activity and glycylglycine buffer, pH6·5, was used for the assay of phospholipase A_2 activity. In each experiment control tubes, containing only substrate and buffer solution, were incubated. After incubation at 37° for 1hr. 4ml. of chloroform–methanol (1:2, v/v) was added to each tube. After 15min. any protein precipitate was removed by centrifugation and the solvents were evaporated *in vacuo*. Methanol (0·3ml.) was added to dissolve the residue.

The ^{32}P-labelled phospholipids and their breakdown products were separated by thin-layer chromatography according to the procedure of Skipski *et al.* (1964). Phospholipids from rat liver, to which lysolecithin had been added, were co-chromatographed with each sample to act as markers. The phospholipids were made visible by briefly exposing the developed plate to iodine vapour, and areas of the silica gel were scraped off for analysis. In experiments with [^{32}P]lecithin, area 4 (see Fig. 1) was used for lecithin and area 2 was used for lysolecithin. In experiments with [^{32}P]phosphatidylethanolamine, areas 6 and 7 were used for the substrate and areas 4 and 5 for lysophosphatidylethanolamine. The radioactive material remaining on the origin (area 1) was also estimated, and was presumed to be glycero[^{32}P]phosphorylcholine and glycero[^{32}P]phosphorylethanolamine respectively. Glycerophosphorylcholine [Sigma (London) Chemical Co. Ltd., London, S.W. 6] was used as a marker. The silica was placed in plastic vials: 1ml. of methanol and 4ml. of the scintillation fluid described by Bray (1960) were added and the radioactivity was measured with a Beckman liquid-scintillation spectrometer (model LS-200B). In several experiments the ^{32}P-labelled phospholipids were located after thin-layer chromatography by means of a radiochromatogram scanner (Panax Equipment Ltd.).

RESULTS

Use of [^{32}P]*lecithin and* [^{32}P]*phosphatidylethanolamine as substrates.* These two ^{32}P-labelled phospholipids were isolated by preparative thin-layer chromatography from the lipids extracted from a rat liver mince that had been incubated with [^{32}P]phosphate. A radiochromatogram scan of a sample of purified [^{32}P]phosphatidylethanolamine is shown in Fig. 1(*a*) and illustrates the radiochemical purity of the ^{32}P-labelled phospholipid. A similar result was obtained with [^{32}P]lecithin. By the more sensitive methods of scintillation counting and phosphate analysis small amounts of impurities could be detected, ranging from 2 to 15% for different preparations of the two phospholipids. This contamination with other phospholipids could be decreased by rechromatography. The samples

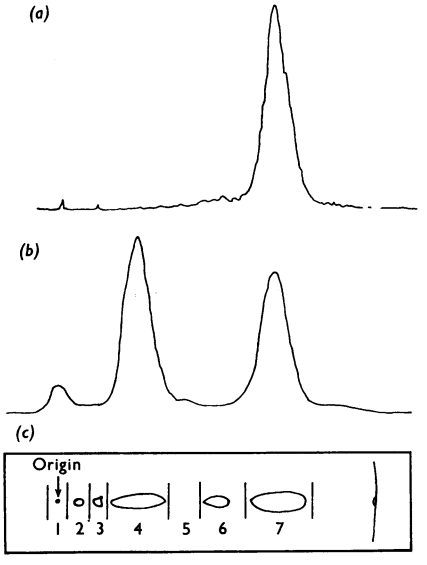

Fig. 1. (*a*) Radiochromatogram scan of a sample of [^{32}P]-phosphatidylethanolamine. (*b*) Radiochromatogram scan of ^{32}P-labelled compounds after incubation of [^{32}P]phosphatidylethanolamine with phospholipase A_1. The peaks of radioactivity correspond, from left to right, to glycero[^{32}P]-phosphorylethanolamine, [^{32}P]lysophosphatidylethanolamine and [^{32}P]phosphatidylethanolamine respectively. (*c*) Drawing of a thin-layer chromatography plate showing the rat liver phospholipids (+lysolecithin) used as markers; the plate was stained with I_2 vapour. The spots, from left to right, correspond to lysolecithin, sphingomyelin, lecithin, phosphatidylserine+phosphatidylinositol and phosphatidylethanolamine. The vertical lines indicate the regions of silica gel, numbered 1–7, which were scraped off for scintillation counting (see the Methods section).

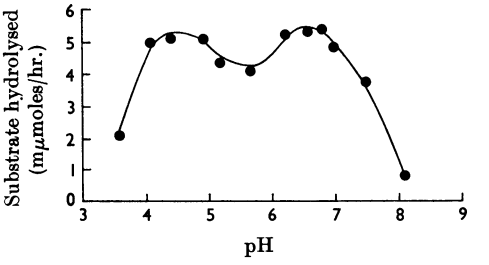

Fig. 2. Effect of pH on phospholipase A activity in large-granule fraction. An amount of the large-granule fraction corresponding to 9mg. of original tissue was incubated with 22μg. of [^{32}P]phosphatidylethanolamine in tris–sodium succinate buffers for 1hr.

of [^{32}P]lecithin or [^{32}P]phosphatidylethanolamine that were used as substrates for phospholipase A assay contained less than 5% of other phospholipids. The initial specific activities of [^{32}P]lecithin and of [^{32}P]phosphatidylethanolamine varied for each preparation, but were about 3000counts/min./μg. and 6000counts/min./μg. respectively.

The effect of incubating [^{32}P]phosphatidylethanolamine with tissue containing phospholipase A_1 is shown in Fig. 1(*b*), which is a tracing of a radiochromatogram scan after chromatography of a lipid extract of the incubation mixture. Three peaks of radioactivity can be seen, corresponding (from left to right) to glycero[^{32}P]phosphorylethanolamine, [^{32}P]lysophosphatidylethanolamine and [^{32}P]phosphatidylethanolamine respectively. By means of scintillation counting it was found that the recovery of radioactivity after chromatography was the same whether or not enzyme was present in the incubation mixture. This showed that no radioactive products were formed that were not soluble in methanol and therefore that measurements of the radioactivity from areas of the thin-layer chromatography plates could be used to calculate the degree of breakdown of the substrate.

For quantitative studies it was necessary to demonstrate that the rate of breakdown of the labelled substrates was proportional both to incubation time and enzyme concentration. Such zero-order conditions were demonstrable for only a limited degree of hydrolysis; eventually, above 10–20% hydrolysis, the amount of substrate broken down became constant in spite of an increase in time or of enzyme concentration. The effect of enzyme concentration is shown below (see Figs. 4 and 5). In the experiments described below zero-order conditions were always checked by using at least two enzyme concentrations and incubation periods not exceeding 1hr.

Effect of pH *on phospholipase A activity of large-granule fraction.* Portions of the dialysed large-granule fraction were incubated with [^{32}P]phosphatidylethanolamine in buffers ranging from pH3·6 to pH8·1: the result of this experiment is shown in Fig. 2. Breakdown of the substrate was maximal between pH 4·0 and pH5·0 and between pH6·0 and pH7·0, presumably owing to the presence of two enzymes. [^{32}P]Lysophosphatidylethanolamine comprised more than 80% of the hydrolysis products at both pH optima, indicating that both enzymes are of the phospholipase A type (EC 3.1.1.4). Since it has already been shown that the enzymes acting at pH4·2 and at pH6·5 have different positional specificities (Winkler *et al.* 1967*a*), they will be called phospholipases A_1 and A_2 respectively.

Lysosomal localization of phospholipase A_2. The distribution of phospholipase A_1 and A_2 activities between the low-speed supernatant, large-granule fraction and the second supernatant was first studied. Each of these fractions had been dialysed

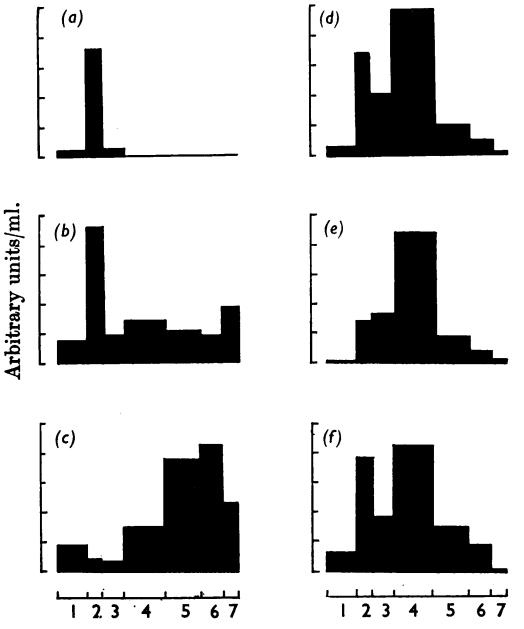

Fig. 3. Analysis of fractions from a sucrose density gradient. The large-granule fraction was centrifuged on a sucrose density gradient and fractions were obtained by cutting the centrifuged tube (see the Methods section). The abscissa in each histogram is divided according to the volumes of the fractions and the numbers represent the fractions described by Smith & Winkler (1966). The columns in each histogram from the left to right correspond to the fractions from the top to the bottom of the centrifuge tube. The ordinates are divided into arbitrary units/ml. of fraction and the actual value of one arbitrary unit is shown in parenthesis below; the values for enzymes are expressed in μmoles of substrate/hr./ml. of fraction. (*a*) Fumarase (5·6); (*b*) glucose 6-phosphatase (0·75); (*c*) catecholamines (1·2μmoles/ml.); (*d*) acid ribonuclease (3·9); (*e*) phospholipase A_1 (0·07μmole of lecithin); (*f*) phospholipase A_2 (0·035μmole of phosphatidylethanolamine). The recoveries of the enzymes from the gradient ranged from 70% to 103%.

before use. The enzymic activities in the low-speed supernatant were, in terms of the amount of substrate hydrolysed, 2·6μmoles and 1·6μmoles of [^{32}P]phosphatidylethanolamine/g. of original tissue/hr. for phospholipase A_1 and phospholipase A_2 respectively. The large-granule fraction contained 84% of the activity of phospholipase A_1 present in the low-speed supernatant and 76% of that of phospholipase A_2 (the recoveries were respectively 98% and 102%). It can be concluded that these two enzymes are localized in particles. The evidence for the localization of the phospholipase A_1 in lysosomes was given by Blaschko *et al.* (1967*a*).

To determine which of the three types of particle present in the large-granule fraction contained phospholipase A_2 activity, sucrose-density-gradient centrifugation was carried out. The fractions from a sucrose density gradient were analysed for fumarase activity, acid ribonuclease activity, glucose 6-phosphatase activity and catecholamines. The results of these analyses are shown in Fig. 3, together with the results of assays of phospholipase A_2 activity. For comparison, the distribution of phospholipase A_1 activity in a different experiment (Blaschko *et al.* 1967*a*) is also shown. The distribution of fumarase, acid ribonuclease and catecholamines is in agreement with what is known about the localization of these constituents in the different particulate fractions of the adrenal medulla, i.e. mitochondria, lysosomes and chromaffin granules respectively (Smith & Winkler, 1966). The activity of glucose 6-phosphatase, a microsomal enzyme, was mainly found at the top of the gradient, but some activity was also present in the other fractions. (The microsomal localization of this enzyme in the bovine adrenal medulla is shown by the fact that only 25% of the glucose 6-phosphatase activity present in the low-speed supernatant was recovered in the large-granule fraction, whereas 70% was sedimented by centrifuging at 110000*g* for 60min., in the microsomal pellet.)

The distribution of phospholipase A_2 activity in the gradient differed from that of typical constituents of mitochondria, microsomes and chromaffin granules, but was very similar to that of acid ribonuclease. The distribution given by these two enzymes, like that of several other acid hydrolases (Smith & Winkler, 1966), was bimodal, with peaks in fractions 2 and 4. The pH optimum of the phospholipase A activity was 6·5 in both these fractions. It is therefore likely that phospholipase A_2, as well as phospholipase A_1, is a constituent of lysosomes.

Properties of lysosomal phospholipase A_1. For these experiments the fraction from the density gradient with the highest specific activity was used. The enzymic activity in sodium acetate buffers, with [^{32}P]lecithin as substrate, was optimum between pH 4·0 and 4·6. This is similar to the result obtained with [^{32}P]phosphatidylethanolamine in tris–sodium succinate buffers (see Fig. 2). The rate of breakdown was the same when the substrate concentration was doubled. When the dialysed lysosomal fraction was centrifuged at 110000*g* for 60min., it was found that 78% of the total phospholipase A_1 activity remained in the supernatant.

The breakdown of [^{32}P]lecithin and of [^{32}P]-phosphatidylethanolamine by different concentrations of the lysosomal fraction was measured, and the results are shown in Fig. 4. At low concentrations of the lysosomal fraction each substrate was hydrolysed at the same rate: the

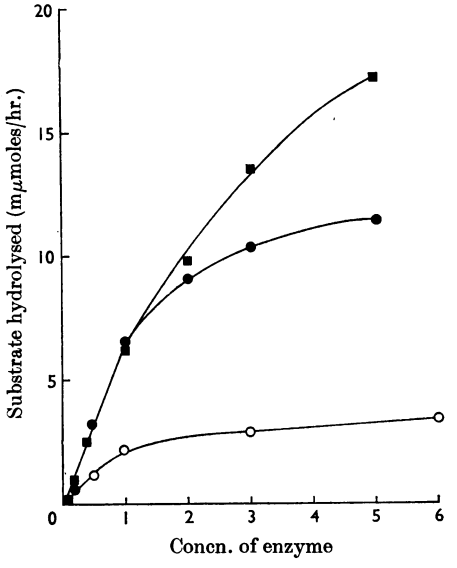

Fig. 4. Effect of enzyme concentration on the breakdown of substrates by phospholipase A_1. One division of the abscissa indicates an amount of the lysosomal fraction containing 4·1 μg. of acid-precipitable N and an amount of the large-granule fraction corresponding to 1·7 mg. of original tissue. ■, [^{32}P]Phosphatidylethanolamine incubated with lysosomal fraction; ●, [^{32}P]lecithin incubated with lysosomal fraction; ○, [^{32}P]lecithin incubated with large-granule fraction.

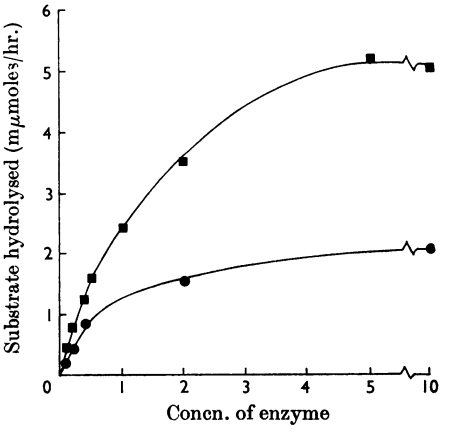

Fig. 5. Effect of enzyme concentration on breakdown of substrates by phospholipase A_2. One division on the abscissa indicates an amount of lysosomal fraction containing 9·4 μg. of acid-precipitable N. ■, [^{32}P]Phosphatidylethanolamine; ●, [^{32}P]lecithin.

initial rate was 1·5 μmoles of substrate/mg. of acid-precipitable nitrogen/hr. The breakdown of [^{32}P]lecithin by different concentrations of the large-granule fraction is also shown in Fig. 4. The degree of breakdown is only linear at very low concentrations of the large-granule fraction. The maximum amount of [^{32}P]lecithin that could be broken down by the large-granule fraction was 2·3 μg. (10% of that present in the incubation mixture). Whereas the enzymic activity was stable to freezing (−10°) for several weeks, it could be completely destroyed by boiling the lysosomal fraction at pH 4·2 for 15 min. The effect of Ca^{2+} on the activity of the enzyme was studied by adding calcium chloride to the incubation mixture. In concentrations ranging from 0·01 mM to 10 mM, Ca^{2+} did not activate the hydrolysis of either [^{32}P]lecithin or of [^{32}P]phosphatidylethanolamine. Indeed, the higher concentrations of Ca^{2+} inhibited the enzyme: 10 mM-calcium chloride inhibited the breakdown of both substrates by about 70%.

Properties of lysosomal phospholipase A_2. This enzyme had a pH optimum at 6·5 in glycylglycine–sodium hydroxide buffers and in tris–sodium succinate buffers. The rate of breakdown of [^{32}P]phosphatidylethanolamine was the same in both buffers, but lecithin was hydrolysed in the latter buffer at half the rate that it was hydrolysed in glycylglycine–sodium hydroxide buffer. About three-quarters of the total enzymic activity remained in the supernatant when either the dialysed lysosomal fraction or the dialysed large-granule fraction was centrifuged at 110000*g* for 60 min. The effect of enzyme concentration on the breakdown of the two substrates by the lysosomal fraction is shown in Fig. 5: [^{32}P]phosphatidylethanolamine was hydrolysed about twice as rapidly as was [^{32}P]lecithin.

The rate of breakdown catalysed by the lysosomal fraction from the sucrose density gradient was 0·68 μmole of [^{32}P]phosphatidylethanolamine/mg. of acid-precipitable nitrogen/hr. Boiling this fraction for 15 min. at pH 6·5 destroyed 97% of the enzymic activity, but the enzymic activity was unchanged when the fraction was kept at −10° for several weeks. Triton X-100 (150 μg./ml.) inhibited the enzyme by 50% and cetylpyridinium bromide (100 μg./ml.) inhibited it completely. Calcium chloride (0·01–10 mM) did not activate the enzymic hydrolysis of [^{32}P]lecithin, and at a concentration of 8 mM the hydrolysis was inhibited by 60%. However, with [^{32}P]phosphatidylethanolamine as a substrate, low concentrations of Ca^{2+} activated the hydrolysis catalysed by the lysosomal fraction, but at higher concentrations inhibition again occurred (see Fig. 6).

Studies on other cell fractions. The microsomal fraction and the final supernatant were each dialysed and examined for phospholipase activity with buffers ranging from pH 3 to pH 9. No enzymic activity could be detected in these fractions

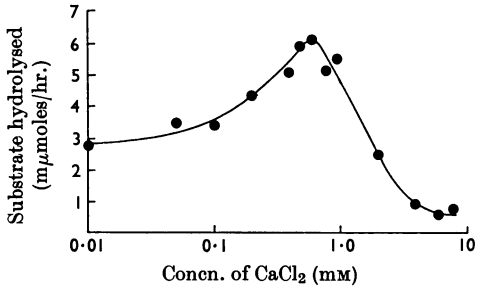

Fig. 6. Effect of Ca^{2+} on the hydrolysis of [^{32}P]phosphatidylethanolamine by phospholipase A_2. The lysosomal fraction (4·1 μg. of acid-precipitable N) was incubated with 22 μg. of [^{32}P]phosphatidylethanolamine in glycylglycine–NaOH buffer containing $CaCl_2$. The rate of hydrolysis at 0·01 mM-Ca^{2+} was the same as that found in the absence of added $CaCl_2$.

that could not have been explained by the presence of the lysosomal enzymes.

The experiments reported above, as well as those on the lysosomal phospholipases, were carried out with dialysed fractions of the homogenate. Some experiments were also carried out on undialysed fractions and evidence was found for the presence of low-molecular-weight heat-stable material that catalysed the breakdown of ^{32}P-labelled phospholipids. Thus when [^{32}P]lecithin was incubated with the undialysed final supernatant it was hydrolysed to [^{32}P]lysolecithin. The hydrolysis was optimum between pH 3·0 and 6·5. Most of the catalytic activity in the undialysed supernatant fraction was not affected by boiling for 20 min., and could be recovered quantitatively in the diffusate after dialysis. The undialysed large-granule fraction also contained heat-stable diffusible material that catalysed the breakdown of [^{32}P]lecithin at low pH. However, the non-diffusible activity in the large-granule fraction could be destroyed by heat and was due to the presence of the two lysosomal phospholipases.

DISCUSSION

Measurement of phospholipase A activity. To study the subcellular distribution of phospholipase A activity we have used ^{32}P-labelled phospholipids of high specific radioactivity since, with these substrates, it is possible to measure the breakdown of very small amounts of phospholipids (van den Bosch & van Deenen, 1964; Scherphof & van Deenen, 1965; Elsbach, van den Berg, van den Bosch & van Deenen, 1965). With this method all the radioactive breakdown products of the substrate could be recovered and their radioactivity measured. This made it possible to determine the breakdown of as little as 5% of the substrate, i.e. 1·4 mμmoles. However, probably because of the use of such small amounts of substrate, it was difficult to obtain breakdown proportional both to time and to enzyme concentration. This difficulty was particularly evident with tissue fractions relatively rich in phospholipids, such as the large-granule fraction (see Fig. 4), and might therefore have been due to a gradual dilution of the small amounts of ^{32}P-labelled substrate with unlabelled endogenous phospholipids. Accordingly, conclusions about the subcellular localization of phospholipase A activity can only be made if it can be demonstrated for each fraction that the breakdown of radioactive substrate is proportional to enzyme concentration. A further problem associated with the incubation of small amounts of ^{32}P-labelled phospholipids with tissue fractions was encountered: this was the hydrolysis, presumably non-enzymic, of phospholipids catalysed by diffusible heat-stable material. That the catalytic activities present in lysosomes are due to enzymes is shown by their heat-lability and by the fact that they were non-diffusible on dialysis.

It should also be pointed out that assays of the rate of breakdown of ^{32}P-labelled phospholipids by means of radioactivity measurements do not necessarily give the rates of breakdown of unlabelled substrate. It can be calculated from the present results that, after incubation of rat liver with [^{32}P]phosphate, at the most only about 5% of the lecithin molecules contain ^{32}P. Since it has been shown by Collins (1963) that the different molecular species of rat liver lecithin are not uniformly labelled by ^{32}P *in vivo*, it is likely that the ^{32}P introduced *in vitro* would not be uniformly distributed. Further, it is known that snake-venom phospholipases act preferentially on some molecular species of lecithin (Moore & Williams, 1964; Nutter & Privett, 1966). The rates of breakdown of ^{32}P-labelled phospholipids given in the present paper may not therefore represent the true catalytic activity of the enzymes.

It can be seen that the method used in this work has certain limitations, but that these limitations are derived from the requirement for a sensitive method of assay for phospholipase activity.

Presence of two phospholipases in lysosomes. The use of radioactively labelled substrates has made it possible to demonstrate and characterize two enzymes of the phospholipase A type in lysosomes of bovine adrenal medulla. Though present in the same type of cell particle, the enzymes differed in their pH optima and in their positional specificities. They also differed in their substrate specificities: [^{32}P]lecithin and [^{32}P]phosphatidylethanolamine were hydrolysed by phospholipase A_1 at the same rate, whereas the former substrate was hydrolysed

by phospholipase A_2 half as rapidly as the latter substrate was.

The lysosomal phospholipase A_1 has optimum activity at pH 4·2 and it is noteworthy that similar enzymes have already been described in other tissues. A similar pH optimum and the same positional specificity were found for enzymes in calf spleen (Lloveras, Douste-Blazy & Valdiguié, 1963) and in rat brain (Gatt, Barenholz & Roitman, 1966). Elsbach (1966) found phospholipase A activity with an optimum at pH 4·0 in rabbit alveolar macrophages; it is known that these cells contain lysosomes (Cohn & Wiener, 1963). Mellors & Tappel (1967) have shown that rat liver lysosomes can hydrolyse phospholipids and that this activity is optimum at pH 4·5. These results suggest that a phospholipase A with an acid pH optimum may be present in lysosomes of several tissues.

The lysosomal phospholipase A_2 of the bovine adrenal medulla has optimum activity at pH 6·5. There are a few reports of enzymes with a similar pH optimum in other tissues. Epstein & Shapiro (1959) described phospholipase activity in the rat intestinal mucosa that had a pH optimum at pH 6·5 and that was found both in the crude mitochondrial and microsomal fractions. In rabbit polymorphonuclear leucocytes, phospholipase A activity with a pH optimum at 6·0 has been demonstrated (Elsbach & Rizack, 1963; Elsbach *et al.* 1965); part of this activity was present in a particulate fraction that contained the lysosomes (Elsbach & Rizack, 1963). Stoffel & Greten (1967) reported that lysosomes from rat liver contained a phospholipase with optimum activity at pH 6·5.

A phospholipase A_2 with an alkaline pH optimum has been found in the mitochondrial fraction from rat liver (Scherphof & van Deenen, 1965; Scherphof, Waite & van Deenen, 1966; Rossi, Sartorelli, Tatò, Baretta & Siliprandi, 1965; Bjørnstad, 1966*a*; Waite & van Deenen, 1967), and there is some evidence that this enzyme is present in mitochondria and not in lysosomes (Rossi *et al.* 1965; Bjørnstad, 1966*a*). In the present work an alkaline phospholipase A was not detected in the mitochondria from bovine adrenal medulla, but this may have been because the incubation mixture did not contain Ca^{2+}, which has been shown to activate the mitochondrial enzyme from rat liver (Bjørnstad, 1966*a*). A similar explanation may account for the fact that in preliminary experiments we could not demonstrate alkaline phospholipase activity in the microsomal fraction of bovine adrenal medulla, since this enzyme, at least in rat liver, also requires Ca^{2+} (Bjørnstad, 1966*b*).

Functional significance of lysosomal phospholipases. de Duve & Wattiaux (1966), in their excellent review on lysosomes, discussed 'the widely held belief that these particles may lack the ability to degrade lipids'. If this would be so, they argued, a much greater rate of lipid accumulation should occur in lysosomes, owing to autophagy, than is actually observed. They postulated therefore that with sensitive assay methods lipid-degrading enzymes would be discovered in lysosomes. The present work, together with the earlier report (Blaschko *et al.* 1967*a*) and the work of others (Mellors & Tappel, 1967; Stoffel & Greten, 1967), confirms this speculation. These findings make it likely that the phospholipids of membranes taken up into lysosomes by endocytosis or by autophagic processes can be metabolized.

The question arises whether the lysosomal phospholipases have any special function in chromaffin cells. The problem that initiated this study was the origin of the lysolecithin in the chromaffin granules. If further studies fail to detect phospholipase A activity in the chromaffin granules, this will raise the question whether the lysosomal phospholipases are responsible for the formation of the lysolecithin. It is, perhaps, relevant to this question that the lysosomes and the chromaffin granules both originate from the same part of the cell, namely the Golgi region. The lysosomes and their enzymes might also be involved in the final stages of the life of the chromaffin granule. It is now known that the secretion of the hormones is accompanied by the release of all the other soluble constituents of the granules and that the granule membrane remains behind in the cell (see Schneider, Smith & Winkler, 1967). The empty granule membranes may be removed by autophagic processes, which would require, besides other lysosomal enzymes, the phospholipases.

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