3. The 'active sulphate' acts as donor in the formation of phenolic sulphates by liver or mucosal enzymes. No evidence for the sulphation of simple mono- and di-saccharides could be obtained.

4. Mucosal enzymes catalyse breakdown of adenosine triphosphate to inosine, inosine monophosphate and hypoxanthine.

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The Structure of the Naturally Occurring Phosphoglycerides

4. ACTION OF CABBAGE-LEAF PHOSPHOLIPASE *D* ON OVOLECITHIN AND RELATED SUBSTANCES*

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The enzyme phospholipase *D*, which liberates choline from the naturally occurring lecithins, has been found in several plants, including carrot root, cabbage, spinach and sugar-beet leaves, the latex of *Hevea brasiliensis* and defatted cottonseed meal (Hanahan & Chaikoff, 1947*a*, *b*, 1948; Smith, 1954; Kates, 1954; Tookey & Balls, 1956). In those plants investigated by Kates (1954), the enzyme was reported to reside exclusively in the plastids, and its activity was greatly increased by the addition of ether.

In the work to be described, an examination of several plant species for phospholipase *D* activity revealed that the leaves of Savoy cabbage and Brussels sprouts were much the richest sources. Furthermore, the enzyme was present in the cell sap as well as in the plastids. The enzyme from the cell sap has been partially purified and found to exhibit an absolute requirement for calcium ions. This enzyme preparation, which removes all the choline from ovolecithin and from synthetic L-α- and DL-α-lecithins, also acts upon egg phosphatidylethanolamine.

Evidence has been obtained for the presence of another enzyme in the supernatant from a Savoy-cabbage homogenate; this appears to be a phosphodiesterase, different from phospholipase *D*, which hydrolyses water-soluble phosphodiesters.

* Part 3: Long & Penny (1957).

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Some of the results obtained with Savoy-cabbage phospholipase *D* have already been reported briefly (Davidson, Long & Penny, 1956; Davidson & Long, 1957).

EXPERIMENTAL

Materials

Ovolecithin. This was obtained from egg yolk and purified either through the cadmium complex (Pangborn, 1951) or by chromatography on silicic acid (Lea, Rhodes & Stoll, 1955). Some samples were hydrogenated catalytically as described by Long & Maguire (1954).

Dipalmitoyl-L-α-lecithin. This was a gift from Dr D. J. Hanahan.

Lysolecithin. A solution containing 600 mg. of ovolecithin in 400 ml. of dry peroxide-free ether was treated with 4 mg. of dried cottonmouth-moccasin venom (Ross Allen's Reptile Institute, Florida, U.S.A.) dissolved in 4 ml. of 0·005 M-$CaCl_2$ (Long & Penny, 1957). The mixture was stirred mechanically at 18°. After 3 hr., the precipitated lysolecithin was separated by centrifuging, washed with 100 ml. of acetone and dissolved in 10 ml. of $CHCl_3$. After filtering off a trace of insoluble material, the filtrate was treated with 100 ml. of acetone. The precipitate of lysolecithin was finally dissolved in 20 ml. of $CHCl_3$ and stored at 2°. The ratio, equivalents of fatty acid ester: atoms of P, was 1·02.

Egg phosphatidylethanolamine. This was prepared from egg yolk by chromatography on silicic acid (Lea *et al.* 1955).

Ox-brain 'kephalin'. Material corresponding with fractions III–V of ox-brain kephalin was prepared by the method of Folch (1942). This consists largely of phosphatidylethanolamine and phosphatidylserine, together with the corresponding plasmalogens.

Sphingomyelin. A sample was given by Drs J. A. Lovern and J. Olley.

Synthetic phosphoglycerides. Dipalmitoyl-DL-α-lecithin, distearoyl-DL-α-lecithin, distearoyl-β-lecithin, dimyristoyl-DL-α-phosphatidylethanolamine and dimyristoyl-β-phosphatidylethanolamine were gifts from Drs T. Malkin and T. H. Bevan. A sample of dimyristoyl-L-α-lecithin was given by Dr E. Baer.

Glycerylphosphorylcholine. This was prepared from ovolecithin by the mild alkaline-hydrolysis procedure of Dawson (1954).

Sodium diphenyl phosphate. A specimen of pure diphenyl hydrogen phosphate was given by Dr T. Hofman. Another sample, given by L. Light and Co. Ltd., Colnbrook, Bucks, was converted into the sodium salt as follows. A solution of the material (10 g.) in ethanol (50 ml.) was neutralized with 40% (w/v) NaOH. Ether (250 ml.) was then added and the colourless crystalline ppt. of the sodium salt was filtered off. It was used without further purification.

Di-p-nitrophenyl hydrogen phosphate. A pure specimen was given by Dr T. Hofman.

Phosphorylcholine. A sample of the calcium salt was given by Dr M. G. Macfarlane.

Choline iodide. This was prepared from methyl iodide and dimethylaminoethanol, and was used as a choline standard, since it is non-deliquescent (personal communication from Dr T. H. Bevan).

Calcium phosphate gel. This was prepared by the procedure of Keilin & Hartree (1951). The suspension had a dry weight of 15 mg./ml.

Buffers. For experiments carried out at pH 5·6, a sodium acetate–acetic acid buffer was used. For other pH values, equal volumes of 0·25 M-solutions of acetic acid and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) were mixed, the pH was then adjusted to the desired value by the addition of HCl or NaOH, and the buffer solution was diluted so that the final concentrations of acetate and tris were each 0·1 M.

All other materials were the best commercial preparations.

Sources of enzyme. Most of the vegetables were purchased locally. Onion roots were grown by placing onion sets on the necks of 100 ml. conical flasks completely filled with tap water, and leaving for one month.

Methods

Analytical procedures. Dry weights were obtained by drying overnight at 105°. Protein N was determined on precipitates insoluble in 5% (w/v) trichloroacetic acid by a micro-Kjeldahl method. Choline was determined by the enneaiodide procedure described by Shapiro (1953), ethanolamine by the 1-fluoro-2:4-dinitrobenzene method of Axelrod, Reichenthal & Brodie (1953), or by the periodate procedure of Burmaster (1946), and serine by the method of Axelrod *et al.* (1953). Phenol was determined by means of the reagent of Folin & Ciocalteu (1927), and phosphate by the method of Berenblum & Chain (1938) as modified by Long (1943). Fatty acid ester was determined by the hydroxamic acid procedure of Shapiro (1953). After ashing at 650° and solution of the residue in HCl, followed by neutralizing, calcium was determined according to Clark & Collip (1925).

Enzyme preparations. The plant material, sometimes after a preliminary mincing, was homogenized with 2·5–10 vol. of water, usually for 5 min. in a top-drive macerator (Townson and Mercer Ltd., Croydon), though other machines were also used with similar results. The fibrous material was removed by filtering through silk. The suspension was then centrifuged for 30 min. at 13 000 *g* in a refrigerated centrifuge (Angle 13; Measuring and Scientific Equipment Co. Ltd.). Occasionally the supernatant and sediment were tested separately for phospholipase *D* activity, but normally only the supernatant was examined. In most experiments Savoy cabbage (*Brassica oleracea* var. *Bullata*) was used; when this was not available, other cabbages or Brussels sprouts were used according to the season.

Reaction system for measuring phospholipase D *activity.* The enzymic degradation was carried out in 15 ml. glass-stoppered test tubes. To 1·25 ml. of an emulsion of ovolecithin (16 μmoles) in 0·1 M-acetate buffer, pH 5·6, 0·25 ml. of M-$CaCl_2$ and 1 ml. of enzyme preparation were added. The tubes were warmed to 26°. At zero time 1 ml. of ether was added, the contents of the tubes were shaken vigorously and the reaction mixtures were incubated at 26° for 10 or 12 min. The enzymic reaction was stopped by addition of 1 ml. of 30% (w/v) trichloroacetic acid. After adding 1·5 ml. of water, the mixtures were again shaken and the tubes were centrifuged. The ethereal phase, containing most of the phosphatidic acid and unchanged ovolecithin, was removed with a Pasteur pipette and discarded. The

aqueous phase was filtered and the clear filtrate was warmed on a water bath at 50° to remove dissolved ether; this last step was essential since ether interferes with the subsequent estimation of choline. Choline was determined on 2 ml. of the ether-free filtrate.

With substrates other than ovolecithin, the general procedure was similar. However, 0·1 ml. of $CHCl_3$ was often added to the reaction mixture, as well as 1 ml. of ether, especially with those substrates which were sparingly soluble in ether. Parallel experiments with ovolecithin as substrate showed that $CHCl_3$ had no inhibitory effect on phospholipase *D* activity, but rather produced a slight activation.

Relative chlorophyll content. Samples (1 ml.) of the Savoy-cabbage-leaf fractions were extracted with 85% (v/v) acetone. The combined filtrates and washings were diluted to 10 ml. and the optical densities were determined in a photo-electric colorimeter (Evans Electroselenium Ltd.) with a red filter. This is essentially the procedure of Clendenning & Gorham (1950).

RESULTS

Distribution of phospholipase D *activity in plants*

Phospholipase *D* activity was assayed by determining the quantity of choline liberated from ovolecithin by an homogenate of the plant tissue. The experimental conditions were as described in the Methods section. As shown in Table 1, of the limited number of species examined, Savoy cabbage had the highest activity and onion the lowest. None of the plant tissues tested was completely lacking in phospholipase *D* activity. Reliable results could not be obtained with potato tuber, since the presence of starch interfered with the determination of choline by the enneaiodide method.

A small amount of acid-soluble organic phosphate was liberated from ovolecithin by homogenates of the leaves and tubers of potatoes (0·04 and 0·31 μg.atoms of P/mg. dry wt./hr. respectively); a trace of inorganic phosphate was formed by the action of an onion homogenate. Other plant homogenates did not liberate acid-soluble phosphorus from ovolecithin under the conditions used for determining phospholipase *D* activity.

Enzymic degradation of ovolecithin by Savoy-cabbage-leaf supernatant

Savoy-cabbage homogenate showed an average phospholipase *D* activity of 2·51 μmoles of choline/mg. dry wt./hr. (Table 1), which corresponds to a $Q_{choline}$ value (μl. of choline/mg. dry wt./hr.) of about 56. Control experiments showed that when the homogenate was incubated with choline chloride, none of the latter was destroyed. With the supernatant from a Savoy-cabbage homogenate, the average rate of liberation of choline from ovolecithin was 4·2 μmoles/mg. dry wt./hr. (average $Q_{choline}$, 94). In these experiments, there was no liberation of choline in the absence of ovolecithin, but the Savoy cabbage contained a small amount of material which was estimated as choline; this blank value has been deducted in all cases.

In the absence of ether, the rate of enzymic degradation during a 10 min. incubation period was too low to be measurable, so that it was permissible, and very convenient, to take zero time as the moment of adding the ether. Variations in the volume of ether used affected somewhat the rate of liberation of choline. In one experiment, 5·5 and 8·2 μmoles of choline were liberated in the presence of 0·5 and 3·0 ml. of ether respectively.

The rate of hydrolysis was practically constant during the first 20 min. of incubation, provided that less than 50% of the 16 μmoles of ovolecithin originally present was hydrolysed; thereafter, the rate declined owing to depletion of substrate. With

Table 1. *Distribution of phospholipase* D *activity in plants*

Conditions for determining phospholipase *D* activity are described in the Methods section; the less active plant tissues were incubated for longer periods. Whole homogenates were used.

Plant	Portion used	No. of observations	Fresh tissue (mg.)	Duration (min.)	Average phospholipase *D* activity (μmoles of choline/mg. dry wt./hr.)
Savoy cabbage	Leaves	3	100	10	2·51
Other cabbages	Leaves	2	100	10	0·80
Brussels sprouts	Sprouts	1	100	10	2·18
Turnip	Leaves	1	400	10	0·58
Turnip	Midribs of leaves	1	400	10	0·34
Lettuce	Leaves	2	400	10	0·54
Carrot	Root	1	400	10	0·44
Potato	Leaves	1	400	10	0·19
Beetroot	Globe	2	400	10	0·11
Pea	Dried seeds	1	100	10	0·09
Parsley	Leaves	1	333	60	0·05
Onion	Roots	1	200	180	0·03
Onion	Bulb	1	800	120	0·01

4·5 μmoles of ovolecithin, enzymic hydrolysis was complete within 2 hr.

Kates (1954) reported an optimum pH between 5 and 6 for phospholipase *D* in a suspension of cabbage-leaf chloroplasts. A similar value has been found with the supernatant from a Savoy-cabbage homogenate.

Activation by calcium ions. In the absence of added Ca^{2+} ions, the rate of enzymic hydrolysis of ovolecithin by Savoy-cabbage supernatant was very low. In the system containing 16 μmoles of ovolecithin, the maximum rate was observed in the presence of 0·1 M-$CaCl_2$ (Davidson *et al.* 1956), and this was about seven times the rate observed in the absence of added Ca^{2+} ions. The optimum concentration of Ca^{2+} ions was independent of the enzyme concentration, but was roughly proportional to the amount of ovolecithin used as substrate; a similar relationship had been found for snake-venom phospholipase *A* by Long & Penny (1957). For phospholipase *D*, the optimum ratio was 15 μmoles of Ca^{2+} ions/μmole of ovolecithin, whereas for phospholipase *A* Long & Penny (1957) found a value of about 0·03.

Strontium and Ba^{2+} ions also activated phospholipase *D*. At a final concentration of 0·1 M, $SrCl_2$ and $BaCl_2$ showed 67 and 34 % respectively of the activity of 0·1 M-$CaCl_2$ (Davidson *et al.* 1956). A smaller activation (12 % of the Ca^{2+} ion effect) was shown by 0·1 M-$ZnCl_2$. The following metal chlorides (0·2 M with respect to Cl^- ion) were either without effect or slightly inhibitory: NaCl, KCl, NH_4Cl, $MgCl_2$, $MnCl_2$ and $FeCl_3$.

Action of Savoy-cabbage supernatant on other substrates

The Savoy-cabbage supernatant liberated all the choline from the following lecithins: ovolecithin, hydrogenated ovolecithin, dipalmitoyl-L-α-lecithin, lysolecithin, synthetic dimyristoyl-L-α-lecithin, dipalmitoyl-DL-α-lecithin and distearoyl-DL-α-lecithin. Synthetic distearoyl-β-lecithin was only slowly attacked, as was sphingomyelin. In those instances where the effects were examined, enzymic degradation was stimulated by Ca^{2+} ions and by ether, with the exception that the hydrolysis of lysolecithin was inhibited by ether. This observation may be associated with the well-known water-solubility and ether-insolubility of lysolecithin. The rate of hydrolysis of lysolecithin was 0·05–0·12 of that of ovolecithin.

Ethanolamine was readily liberated from egg phosphatidylethanolamine and from synthetic dimyristoyl-DL-α-phosphatidylethanolamine, but dimyristoyl-β-phosphatidylethanolamine was acted upon less rapidly. Some of these results have been summarized by Davidson *et al.* (1956). As with ovolecithin, Ca^{2+} ions were necessary for the enzymic degradation of phosphatidylethanolamine. The optimum pH with this substrate was between 5·6 and 6·0. The supernatant from Savoy cabbage attacked egg phosphatidylethanolamine at about 60 % of the rate with ovolecithin; with the supernatant from Brussels sprouts, the two substrates were hydrolysed at approximately equal rates.

Savoy-cabbage-leaf supernatant slowly hydrolysed the water-soluble substrates sodium diphenyl phosphate and glycerylphosphorylcholine, but the rates were increased only about 20–40 % in the presence of Ca^{2+} ions. The rate of hydrolysis of neither substrate was increased by the presence of ether. The optimum pH was significantly higher for diphenyl phosphate (about 7) and for glycerylphosphorylcholine (about 8) than for ovolecithin or egg phosphatidylethanolamine. Under optimum conditions, the rates of hydrolysis of diphenyl phosphate and glycerylphosphorylcholine by a Savoy-cabbage supernatant were about 2 and 1 % respectively of the rate for ovolecithin.

Phosphorylcholine was hydrolysed even more slowly than was glycerylphosphorylcholine.

Distribution of phospholipase D *activity between soluble and insoluble cell components*

Kates (1954) has reported that phospholipase *D* activity is present in leaf chloroplasts. The distribution of the enzyme has now been determined in (*a*) the outer dark-green leaves, (*b*) the inner light-green leaves, and (*c*) the yellow heart of a Savoy cabbage. Weighed samples of leaves (15 g.) were ground in a mechanical mortar and then homogenized with 35 ml. of water for 5 min. in a top-drive macerator (Measuring and Scientific Equipment Co. Ltd.). Each sample was then treated in the following way. Phospholipase *D* activity and relative chlorophyll content were determined on measured volumes of the homogenate. The rest was filtered through silk and the enzymic activity of the fibre-free suspension was determined. The remainder of the suspension was centrifuged at 13 000 *g* for 30 min. at 10°, and the residue was suspended in a volume of water equal to that of the supernatant removed. Both the suspended residue and the almost clear supernatant were tested for phospholipase *D* activity. The results are shown in Table 2.

It will be seen that, whereas the insoluble fraction from each type of leaf had approximately the same phospholipase *D* activity when measured in the presence of 0·1 M-$CaCl_2$, the soluble fraction from the yellow heart had a much higher activity than the corresponding fraction from the outer green leaves. The activity of the supernatant from the inner green leaves was intermediate in magnitude. In the insoluble fraction from the outer green leaves, further investigation showed that the

Table 2. *Phospholipase* D *activity of soluble and insoluble cell components of aqueous Savoy-cabbage homogenates*

Experimental details are given in the text. Conditions for determining enzymic activity and relative chlorophyll content are described in the Methods section. Parallel determinations of enzymic activity were also made in the absence of added $CaCl_2$. Volume of suspension or supernatant used was 1 ml. Incubation was for 12 min. at 26°.

Type of leaf	Relative chlorophyll content of homogenate (galvanometer reading)	$CaCl_2$ concn. (M)	Phospholipase *D* activity (μmoles of choline liberated/12 min.) Whole homogenate	Fibre-free suspension	Soluble fraction	Insoluble fraction
(*a*) Dark-green outer	46	0	5·0	5·1	0·8	1·7
		0·1	8·8	9·8	4·1	4·3
(*b*) Light-green inner	19	0	2·5	2·0	0·4	0·5
		0·1	11·3	10·4	7·1	4·3
(*c*) Yellow 'heart'	0	0	2·7	2·8	0·5	0·5
		0·1	10·2	10·5	11·7	4·1

enzyme was very tightly bound to the particles. Repeated washing with water did not diminish the phospholipase *D* activity. The insoluble fraction, consisting of both intact and damaged chloroplasts, was further comminuted in a Potter–Elvehjem (1936) homogenizer, but the enzyme remained attached to the particles, which were composed of partly disintegrated grana.

It will also be noted that the insoluble fraction of the outer green leaves possessed an appreciable phospholipase *D* activity in the absence of added Ca^{2+} ions; this activity is equivalent to the liberation of 1·6 μmoles of choline/mg. dry wt./hr. and is consistent with the findings of Kates (1954), who observed the liberation of choline from ovolecithin by cabbage-leaf chloroplasts in the absence of added Ca^{2+} ions. His published data, when converted into μmoles of choline/mg. dry wt./hr., correspond with 2·2, 1·9 and 0·8 for sugar-beet, spinach and cabbage leaves respectively, values which are very similar to that found in the present work. These calculations suggest that, on the basis of dry weight, the plastid enzyme of Kates is only about 40% as active as the soluble phospholipase *D* studied here; however, the activity of the plastids can be increased considerably by addition of Ca^{2+} ions.

In the experiment shown in Table 2, the outer dark-green leaves gave rise to an insoluble particle fraction, which on analysis contained 9·8 μg. of calcium/mg. dry wt.; on the assumption that this calcium is ionized and is contained within the particles in a non-diffusible form, this amount would correspond with an internal Ca^{2+} ion concentration of about 0·04 M, taking the dry weight of the particles as 13% of the wet weight. At this Ca^{2+} ion concentration, the phospholipase *D* activity would be expected to be submaximum (Davidson *et al.* 1956), which is consistent with the observation that, in the presence of added Ca^{2+} ions, the activity is increased about 2·5-fold.

The requirement of phospholipase *D* for Ca^{2+} ions is supported by two further pieces of evidence, namely the finding by Kates (1954) that the plastid phospholipase *D* activity was inhibited by fluoride, and our own observation that 0·01 M-ethylenediaminetetra-acetate completely suppressed the phospholipase *D* activity of a whole homogenate of Savoy cabbage in the absence of added Ca^{2+} ions.

Partial purification of phospholipase D *from Savoy-cabbage supernatant*

The following procedure was found to result in a 45-fold purification of the enzyme, on the basis of protein N, with a recovery of 50% of the total activity. The inner light-green leaves of a Savoy cabbage (200 g.) were homogenized with 300 ml. of water for 5 min. The homogenate was largely freed from fibre by squeezing through silk and then centrifuged at 13 000 *g* for 30 min., giving about 380 ml. of almost clear supernatant (stage 1). The supernatant was maintained at 55° for 5 min. and then rapidly cooled. The bulky precipitate which had formed was centrifuged down as before and discarded; the supernatant amounted to 340 ml. (stage 2). This supernatant was cooled to 0° and then treated with 2 vol. of acetone at −15°; after 10 min., the precipitate, which contained most of the enzyme, was centrifuged down (stage 3). The precipitate was dissolved in 80 ml. of water, and a slight amount of insoluble material was removed by centrifuging at 13 000 *g*. A sample (12 ml.) of the clear solution was then treated with 7·2 ml. of calcium phosphate gel (15 mg. dry wt./ml.). The enzyme was completely adsorbed. (The amount of gel required to adsorb the enzyme varied between individual experiments, and had to be determined in each case.) The gel was centrifuged down at 0° at 5000 *g* for 20 min. The enzyme was eluted from the gel with 12 ml. of 0·05 M-phosphate buffer (Sørensen), pH 7·5, and the eluate was dialysed against water for 2 hr. at 2°. The enzyme was again

Table 3. *Partial purification of Savoy-cabbage phospholipase* D

The fractionation procedure is described in the text. Determinations of dry weight, protein N and phospholipase *D* activity (with ovolecithin as substrate) are as given in the Methods section. All concentrations and activities are calculated as if the various fractions had been diluted to the original volume of the crude supernatant.

Stage	Treatment	Dry wt. (mg./ml.)	Protein N (mg./ml.)	Phospholipase *D* activity (μmoles of choline/ml./10 min.)	Phospholipase *D* activity (μmoles of choline/mg. of protein N/10 min.)	Purification	Yield (%)
1	Crude supernatant	21·5	0·378	7·4	19·6	1	100
2	Supernatant after heat-coagulation	19·6	0·153	7·4	48·4	2·5	100
3	Pptd. with 2 vol. of acetone	1·3	0·048	5·7	119	6·1	77
4	Eluate from $Ca_3(PO_4)_2$ gel	—	0·0042	3·8	905	46·2	51

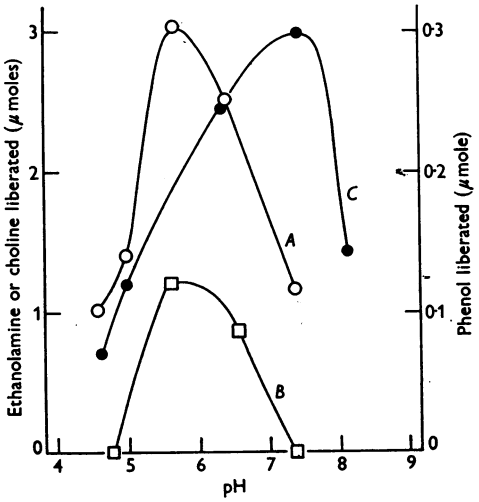

Fig. 1. pH–Activity curves for enzyme preparation from Savoy cabbage. Reaction mixtures contained: partially purified (stage 3) enzyme (1 ml.), 0·1 M-acetate–tris buffer (1 ml.), M-$CaCl_2$ (0·25 ml.) and substrate; total aqueous volume 2·5 ml. Substrates: curve *A*, ovolecithin (16 μmoles); curve *B*, egg phosphatidylethanolamine (6·2 μmoles); curve *C*, sodium diphenyl phosphate (15 μmoles). Curve *A*, ether (1 ml.) added at zero time; curve *B*, ether (1 ml.) + $CHCl_3$ (0·1 ml.) added at zero time. Incubation at 26° for 10 min. (curve *A*), 12 min. (curve *B*) or 30 min. (curve *C*).

precipitated with 2 vol. of acetone at 0°. It was finally dissolved in 12 ml. of water and stored at −15° (stage 4). At each stage, dry weight, protein N and phospholipase *D* activity were determined. The results are summarized in Table 3.

Properties of partially purified phospholipase D

The phospholipase *D* preparation of highest purity (Table 3; stage 4) proved to be rather unstable even when stored as a frozen solution at −15°. For this reason routine purification has not been continued beyond the acetone-precipitation step (Table 3; stage 3). This partially purified enzyme acted readily upon ovolecithin and egg phosphatidylethanolamine. The optimum pH was the same as for the crude Savoy-cabbage supernatant, namely about 5·6 (Fig. 1). Little or no activity was observed in the absence of added Ca^{2+} ions (Table 4) or of ether.

Table 4. *Effect of calcium ions on the degradation of ovolecithin and egg phosphatidylethanolamine by partially purified phospholipase* D

Enzyme: partially purified (Table 3; stage 3) preparation from Savoy-cabbage supernatant. Substrates: ovolecithin, 4·8 μmoles in Expt. 1; egg phosphatidylethanolamine, 6·2 μmoles in Expt. 2. Other conditions were similar to those in Fig. 1, except that acetate buffer, pH 5·6, was present in all reaction mixtures, and the concentration of $CaCl_2$ was varied.

$CaCl_2$ concn. (M)	Expt. 1 Choline formed (μmoles/12 min.)	Expt. 2 Ethanolamine formed (μmoles/30 min.)
0	0	0·21
0·01	1·55	—
0·10	1·75	1·69
1·00	0·75	—

A comparison was made of the rates of hydrolysis of some phosphoglycerides of known structure by the partially purified enzyme. Taking the rate of hydrolysis of ovolecithin as 100, the relative rates were: dimyristoyl-L-α-lecithin, 100; dipalmitoyl-L-α-lecithin, 85; dipalmitoyl-DL-α-lecithin, 15; distearoyl-DL-α-lecithin, 2; dipalmitoyl-β-lecithin, 2; distearoyl-β-lecithin, 1; egg phosphatidylethanolamine, 60; dimyristoyl-DL-α-phosphatidylethanolamine, 4; dimyristoyl-β-phosphatidylethanolamine, 0·3. Prolonged incubation of all these substrates, except dimyristoyl-β-phosphatidylethanolamine, resulted in virtually complete liberation of the base.

When fractions III–V of ox-brain kephalin Folch, 1942), which mainly consist of phosphatidylethanolamine and phosphatidylserine to-

gether with the corresponding plasmalogens, were acted upon by the partially purified enzyme, the ethanolamine was released much more rapidly than the serine. The ethanolamine was ultimately released completely, but only 63 % of the serine was released even after incubation for 24 hr.

The partially purified enzyme preparation still acted upon diphenyl phosphate at about 2 % of the rate found with ovolecithin, but had very little effect on glycerylphosphorylcholine (about 0·2 % of the rate with ovolecithin). That the hydrolysis of diphenyl phosphate is catalysed by an enzyme different from phospholipase *D* is clearly indicated by the experiments shown in Table 5. Expt. 1 shows the extent of adsorption of each enzyme when treated with different amounts of calcium phosphate gel. In Expt. 2 a partial separation of the two enzymes was achieved by treatment with successive amounts of gel followed by elution with phosphate.

Enzymic degradation of ovolecithin in the presence of water-soluble phosphodiesters

Diphenyl phosphate. Since the results obtained in Table 5 clearly showed that the enzymic hydrolyses of ovolecithin and of diphenyl phosphate were catalysed by separate enzymes, it was to be expected that mixed-substrate experiments would show that ovolecithin and diphenyl phosphate were degraded independently of one another. However, as shown in Table 6, diphenyl phosphate was found to stimulate the enzymic hydrolysis of ovolecithin. The effect increased with increasing concentrations of diphenyl phosphate up to about 0·16 M. Di-*p*-nitrophenyl phosphate had a similar though quantitatively smaller effect.

The possibility that the stimulation of phospholipase *D* activity might be due to the products of enzymic hydrolysis of diphenyl phosphate, rather than to diphenyl phosphate itself, was investigated by examining the action of monophenyl phosphate, phenol and inorganic phosphate on the system. As shown by the experiment in Fig. 2, in which a crude supernatant from a Savoy-cabbage homogenate was used, all these compounds had a slight stimulating effect at low concentrations, the effect always being much less than that produced by the corresponding concentration of diphenyl phosphate. Phenol was the most active; monophenyl phosphate and inorganic phosphate inhibited at higher concentrations. It is quite impossible therefore that diphenyl phosphate exerted its stimulatory effect after being enzymically hydrolysed. This conclusion was supported by the observation that diphenyl phosphate exerted an almost identical effect on the hydrolysis of ovolecithin by a phospholipase *D* preparation from which the phosphodiesterase activity had been removed by

Table 5. *Separation of phospholipase* D *and phosphodiesterase activities*

Expt. 1. Enzyme solution (Table 3, stage 3; 2 ml.) was treated with different volumes of $Ca_3(PO_4)_2$ gel; water was added to final volume 3·5 ml. After centrifuging, the supernatants were tested for phospholipase *D* and phosphodiesterase activities. Phospholipase *D* activity was determined after incubation for 12 min. with ovolecithin (16 μmoles) as substrate, at pH 5·6 and in the presence of 0·1 M-$CaCl_2$ and ether (1 ml.). Phosphodiesterase activity was determined after incubation with 15 μmoles of diphenyl phosphate at pH 6·6, in the presence of 0·1 M-$CaCl_2$ and in the absence of ether.

Vol. of $Ca_3(PO_4)_2$ gel (ml.)	Phospholipase *D* activity (μmoles of choline/ml./hr.)	Phosphodiesterase activity (μmoles of phenol/ml./hr.)
0	44·4	0·718
0·3	46·6	0·434
0·6	40·0	0
0·9	28·6	0
1·2	26·6	0
1·5	24·0	0

Expt. 2. Same enzyme solution was treated with 0·6 ml. of $Ca_3(PO_4)_2$ gel and after 15 min. the suspension was centrifuged. The ppt. was eluted with 2 ml. of 0·1 M-phosphate buffer, pH 7·5, and the eluate was dialysed (fraction 1). The supernatant was treated with 1·4 ml. of gel; after centrifuging, this ppt. was also eluted with phosphate and the eluate was dialysed (fraction 2). All activities relate to 0·5 ml. of the untreated enzyme or to fractions derived therefrom. Activities were determined as in Expt. 1.

	Phospholipase *D* activity (μmoles of choline/hr.) (*a*)	Phosphodiesterase activity (μmoles of phenol/hr.) (*b*)	Ratio (*a*/*b*)
Untreated enzyme	22·2	0·36	62
Fraction 1	3·7	0·17	21
Fraction 2	15·6	0·015	~1000

Table 6. *Effect of diphenyl phosphate on the activity of partially purified phospholipase* D

Reaction mixtures contained the following components: ovolecithin, 16 μmoles; partially purified enzyme (stage 3) from Savoy cabbage, 1 ml.; acetate buffer (pH 5·6), 1·25 ml.; M-$CaCl_2$, 0·25 ml.; total aqueous volume 3 ml. Ether (1 ml.) was added at zero time. Incubation was for 10 min. at 26°. Under these conditions, 3·8 μmoles of choline were liberated in the absence of diphenyl phosphate.

Diphenyl phosphate concn. (M)	Phospholipase *D* activity (% of control)	
	Expt. 1	Expt. 2
0	100	100
0·004	157	—
0·012	187	—
0·040	236	197
0·080	—	208
0·160	—	230
0·240	—	210
0·400	—	29

adsorption on calcium phosphate gel. At a concentration of 0·02 M-diphenyl phosphate, activations of the order of 170–250% were obtained at values between pH 5·0 and 7·4 and with $CaCl_2$ concentrations between 0·025 and 0·25 M. The percentage activation was not affected by the degree of purification of the enzyme used. At the highest concentration used (0·4 M), diphenyl phosphate had an inhibitory effect (Table 6), which was probably due to its calcium-binding activity.

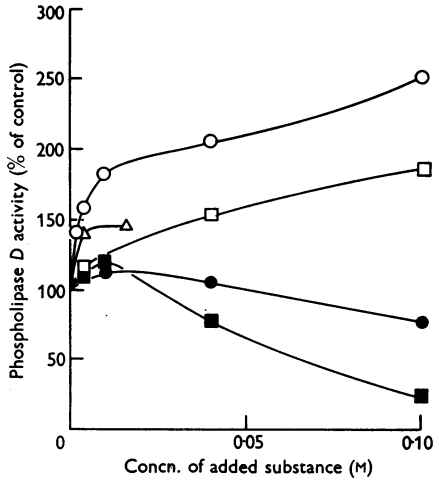

Fig. 2. Effects of diphenyl phosphate (○), monophenyl phosphate (●), di-*p*-nitrophenyl phosphate (△), phenol (□) and inorganic phosphate (■) on the rate of hydrolysis of ovolecithin by phospholipase *D* of Savoy-cabbage supernatant. The reaction mixtures contained: ovolecithin, 16 μmoles; M-$CaCl_2$, 0·25 ml.; 0·1 M-acetate buffer (pH 5·7), 1·25 ml.; additions, as indicated; total aqueous volume 2·5 ml. Ether (1 ml.) was added at zero time. Incubation was for 12 min. at 26°.

Table 7. *Activation of partially purified phospholipase* D *by glycerylphosphorylcholine*

Partially purified enzyme (stage 3) from Brussels sprouts was used. Experimental conditions were as described in the Methods section. Substrates: Expt. 1, ovolecithin (15 μmoles); Expt. 2, egg phosphatidylethanolamine (6·4 μmoles). Amount of glycerylphosphorylcholine (GPC) as indicated. Incubation for 10 min. at 26°.

	Expt. 1 (μmoles of choline liberated)	Expt. 2 (μmoles of ethanolamine liberated)
GPC (7·8 μmoles)	0	—
Ovolecithin	3·86	—
Ovolecithin + GPC (7·8 μmoles)	5·50	—
GPC (13·2 μmoles)	—	0
Egg phosphatidylethanolamine	—	2·93
Egg phosphatidylethanolamine + GPC (13·2 μmoles)	—	3·48

The rates of enzymic degradation of phosphatidylethanolamine, dipalmitoyl-β-lecithin and lysolecithin were also found to be stimulated by diphenyl phosphate.

Glycerylphosphorylcholine. Glycerylphosphorylcholine was also found to stimulate the enzymic breakdown of both ovolecithin and phosphatidylethanolamine. Results of an experiment with the partially purified enzyme from Brussels sprouts are shown in Table 7. After the incubation of the enzyme with glycerylphosphorylcholine both in the presence and absence of ovolecithin, the filtrates, free from protein and lipid, were hydrolysed by heating with N-HCl on a boiling-water bath for 20 min., in order to liberate choline from the added glycerylphosphorylcholine. In both cases, the amount of extra choline formed corresponded closely with the amount expected on the assumption that no glycerylphosphorylcholine had undergone enzymic hydrolysis. This result shows that the glycerylphosphorylcholine caused activation without being hydrolysed.

When egg phosphatidylethanolamine was used as substrate, no choline was liberated from the added glycerylphosphorylcholine.

DISCUSSION

Since phospholipase *D* is particularly active in the leaves of Savoy cabbage, this plant has been used for most of the work described. It has not been determined whether the properties of the enzyme from other less potent sources are similar to those from Savoy cabbage, although from observations made with the limited number of plants investigated we have no reason to suspect any important differences.

Although Kates (1954) concluded that phospholipase *D* was present exclusively in the plastids, we have readily obtained it in solution, especially from the 'heart' of Savoy cabbage. The probable explanation for the discrepancy between our results and those of Kates (1954) is that the plastids contain sufficient calcium to show a measurable activity, whereas the concentration of Ca^{2+} ions of the supernatants is so low that activity can only be demonstrated in the presence of added Ca^{2+} ions.

There are several reasons for believing that the soluble enzyme did not arise in our experiments from the plastids during the homogenizing procedure. (1) The plastids of Savoy cabbage are known to be very resistant to disruption. (2) Even after the plastids had been ground in a Potter–Elvehjem homogenizer, the enzyme remained firmly bound to the grana, none passing into solution. (3) The enzymic activity of the insoluble particles derived from the outer green leaves of Savoy cabbage, when measured in the absence of

added Ca^{2+} ions, was found to be similar to those reported by Kates (1954); this would not have been the case if enzyme had been leached from the particles during the preparation of the homogenate. (4) The activity of whole homogenates (particles plus supernatant), when measured in the presence of Ca^{2+} ions, greatly exceeded that found in plastids alone by Kates (1954).

Like the plastid enzyme, the soluble enzyme was also found to require the addition of ether for activity to become measurable.

The partial purification of the enzyme followed conventional lines. However, the fraction of highest purity proved to be rather labile, so that the product obtained after selective heat-denaturation and acetone-precipitation, which proved fairly stable when stored as a frozen solution, was normally used. This enzyme preparation was almost inactive in the absence of added Ca^{2+} ions, a fact which had earlier been found with the crude soluble enzyme (Davidson *et al.* 1956).

This partially purified enzyme acted readily upon both ovolecithin and egg phosphatidylethanolamine. Its action upon DL-α- and -β-lecithins showed clearly that this enzyme was not specific for substrates possessing the L-α-configuration; in this respect it differs from the phospholipase *A* of snake venom (Long & Penny, 1957). The partially purified enzyme also had a slight action upon diphenyl phosphate. However, it was possible, by selective adsorption upon calcium phosphate gel, to separate the enzymes acting upon phosphoglycerides from those acting upon water-soluble phosphodiesters.

The activation of phospholipase *D* by water-soluble phosphodiesters such as diphenyl phosphate and glycerylphosphorylcholine was unexpected. These activators exerted their effects with all those substrates of phospholipase *D* which were tested, and there was good evidence to show that the activation process was not accompanied by the enzymic hydrolysis of the activators. We are unable to offer any explanation for this activation.

SUMMARY

1. Evidence has been obtained that a soluble phospholipase *D* is present in the leaves of Savoy cabbage and in several other plants, in addition to the enzyme of plastids studied by Kates (1954).

2. The soluble enzyme, which is found in highest amount in the yellow 'heart' of Savoy cabbage, has been partially purified. It acts readily upon ovolecithin, egg phosphatidylethanolamine and other phosphoglycerides possessing the L-α structure; it has a smaller activity towards synthetic substrates with the DL-α or -β structure. In all cases the enzyme required the presence of both ether and calcium ions. Lysolecithin also underwent slow enzymic hydrolysis, a process which required calcium ions but was inhibited by ether.

3. Diphenyl phosphate and other water-soluble phosphodiesters were slightly hydrolysed both by a crude Savoy-cabbage supernatant and by the partially purified enzyme. It was possible to separate the enzyme acting upon phosphoglycerides (phospholipase *D*) from that acting on water-soluble phosphodiesters (phosphodiesterase) by selective adsorption upon calcium phosphate gel.

4. The partially purified phospholipase *D* was stimulated by diphenyl phosphate, di-*p*-nitrophenyl phosphate and glycerylphosphorylcholine.

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