The Distribution of Phospholipase D in Developing and Mature Plants

By R. H. QUARLES AND R. M. C. DAWSON

*Department of Biochemistry, Agricultural Research Council Institute of Animal Physiology,
Babraham, Cambridge*

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1. The distribution of phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) was examined in the tissues of a number of plants and seeds. 2. The highest activities were found in various swollen storage tissues of certain plants: cabbage, central stalk; cauliflower, flower; celery, swollen leaf stalk; Kohl rabi, swollen stem; carrot, root; pea and marrow, seed. 3. Appreciable activity was retained in pea seeds for at least 1 year after drying. After germination and growth in the dark the total activity present in the cotyledons and also in the whole seedling decreased. 4. In the growing pea seedling (7 days old), about 3% of the total activity was in the plumule, 9% in the root and the remainder in the cotyledons. However, the activity in the root on a dry-weight basis was higher than that in the cotyledons. In both the root and the plumule the activity on a wet- or a dry-weight basis was highest in the growing tip. 5. The activity per dry weight in the roots and aerial parts of pea plants declined to low values as growth continued, but roots struck from cuttings of mature plants showed the same high activity as found in roots from young seedlings with cotyledons attached. 6. The total phospholipids present in the cotyledons of pea seeds were depleted on germination and growth. Of the individual phospholipids, phosphatidylcholine and phosphatidylethanolamine showed the same loss in 11 days as the whole phospholipid fraction, whereas phosphatidylinositol was decreased to a greater extent and cardiolipin and phosphatidylserine were not decreased. There was no increase of phosphatidic acid, as might have been expected if the phospholipids had disappeared through phospholipase D hydrolysis. 7. It is concluded that phospholipase D in plant storage tissues and seeds may be related to the rapid growth involved in their formation rather than being necessary for the utilization of their food reserve substances.

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) is found in the tissues of a wide variety of higher plants (Hanahan & Chaikoff, 1947; Kates, 1954; Acker & Ernst, 1954; Smith, 1954; Tookey & Balls, 1956; Davidson & Long, 1958; Einset & Clark, 1958; Yang, Freer & Benson, 1967), but little is known about its physiological role. The soluble form of the enzyme can be stimulated by adding certain anionic amphipathic substances such as dodecyl sulphate and by ultrasonic treatment of the phosphatidylcholine substrate (Dawson & Hemington, 1967). The use of both dodecyl sulphate and ultrasonic treatment of the substrate provides a very sensitive assay system in which only a very small volume of tissue extract need be used relative to the amount of substrate, and which therefore should be less susceptible than the ether assay previously used (Kates, 1954; Davidson & Long, 1958) to inhibitory substances in the tissue extracts. Both Kates (1954) and Tookey & Balls (1956) reported that leaf

cytoplasm contains a macromolecular inhibitor of the enzyme, which might be a basic protein, since these are known to be highly inhibitory to the enzyme (Dawson & Hemington, 1967). Using the very sensitive assay system we have made a study of the distribution of phospholipase D in a variety of plant tissues. Particular emphasis has been placed on a comparison of enzyme activities in different tissues of the same species, and on changes in enzyme activities during the development of the plant. Also, phospholipid analyses were performed on the cotyledons of dry and germinating pea seeds to see whether evidence could be obtained for phospholipase D activity during germination.

METHODS

Seeds and plants. Plant tissues were obtained from local markets or the gardens of laboratory workers. Mung beans and Jerusalem-artichoke tubers were given by Dr W. D. Bonner. Soya beans were provided by Soya Foods Ltd., London E.C.3. Pea seeds were an early dwarf marrow-fat

variety (Kelvedon Wonder) and were over 1 year old when used. Pea seeds were germinated at room temperature in vermiculite moistened with tap water. When pea cotyledons were analysed for enzyme or lipids, the testa and also the embryo (dry pea) or the growing root and plumule (seedling) were removed. Cuttings from mature pea plants were rooted in moist sand under conditions of high humidity to minimize water loss.

Preparation of phospholipase D extracts. The plant tissues or seeds were ground at 4° with an equal weight of acid-washed sand and a little water in a mortar. After a paste had been produced, more water was gradually added to give a final volume equivalent to five to ten times the original weight of tissue. The dispersion was centrifuged at 13000g_{av.} for 30 min., and the supernatant containing the soluble enzyme was collected.

Phospholipase D assay. A suitable portion of the extract was incubated (in duplicate) with a mixture of phosphatidylcholine (ovolecithin) (5 μ moles) and sodium dodecyl sulphate (5 μ moles). The mixture was prepared by adding a solution of sodium dodecyl sulphate to a suspension of phosphatidylcholine in water and treating this ultrasonically until it became clear (15 min. at 0° in the MSE-Mullard 60 w instrument). The incubation medium contained 40 mM-CaCl₂ and 50 mM- β '-dimethylglutaric acid-NaOH buffer, pH 6.8, and the total volume was 1.2 ml. After incubation for 10–30 min. at 27°, the reaction was stopped and the choline release determined as described by Dawson & Hemington (1967). To improve the reproducibility of the assay it was found essential to soak glassware in K₂Cr₂O₇-H₂SO₄ cleaning fluid, rinse it thoroughly and finally boil it in glass-distilled water. The incubation medium containing the substrate was made up in bulk and distributed to individual incubation tubes.

Under the conditions given, the assay of phospholipase D in a tissue showed acceptable reproducibility within a given run. However, when the same enzyme extract was examined in subsequent runs the results fluctuated to some extent. Thus, for example, the same enzyme preparation gave values of choline release that varied randomly in the range 35–59 μ g./min. on eight occasions over a 5-week period. No cause could be ascertained for this fluctuation. Therefore it was arranged in comparative experiments to conduct the assays in the same run. Thus, in the experiments to investigate the change of phospholipase D activity during growth, germination of the different groups of peas was arranged so that seedlings in various stages of development were examined for activity in a single run. In measuring the distribution in the tissues of individual plants (Table 1) the assays were also conducted on a single run; however, small absolute differences between various species must be viewed with some reservation.

Extraction and analysis of phospholipids. The pea cotyledons were homogenized in a blender with chloroform-methanol (2:1, v/v) at 55° to which water had been added to compensate for the difference in water content of dry and germinated cotyledons; water was added to bring the total water to four times the dry weight, and chloroform-methanol (2:1, v/v) to 100 times the dry weight. The homogenate was maintained at 55° for 1 hr., cooled and centrifuged, and the residue was re-extracted for 30 min. with chloroform-methanol (2:1, v/v) at 55°. After centrifugation, the residue was washed with chloroform-methanol (2:1, v/v) and the combined extracts and washings were

shaken with 0.2 vol. of 0.9% NaCl (Expt. 2 in Table 3) or 0.04% CaCl₂ (Expts. 1 and 3 in Table 3). The lower phase was washed again with an equal volume of theoretical upper phase (Folch, Lees & Sloane-Stanley, 1957). The distribution of individual phosphoglycerides was estimated by chromatography and ionophoresis of the partial degradation products (Dawson, Hemington & Davenport, 1962). In some experiments a suitable portion of the phospholipid extract was chromatographed on a thin-layer plate (silica gel H; E. Merck A.-G., Darmstadt, Germany) in a saturation chamber with chloroform-methanol-water (19:13:1, by vol.) or chloroform-methanol-6.5 M-NH₃ solution (65:30:4.7, by vol.). The phosphorus-containing spots were located by the Dittmer & Lester (1964) spray.

Phosphatidylmethanol was prepared by the method of Dawson (1967).

Other analyses. Nitrogen was determined by the method of Lang (1958). Dry weights were determined on analogous tissue samples or seeds by drying to constant weight at 110°.

RESULTS

Phospholipase D assay. With the extraction method employed, little of the phospholipase D was left in the pellet after centrifugation at 13000g_{av.}. For instance, with cabbage leaves 4% remained in the pellet, and with pea plumules the corresponding value was 7% (cf. Davidson & Long, 1958). All the activities reported below refer to the soluble enzyme, which presumably represents the major fraction present in the tissue. Under the incubation conditions used, maximal activity with the enzymes extracted from pea roots and plumules was found between pH 6 and 7 with 40 mM-calcium chloride. Both extracts of pea roots and plumules and dry peas gave maximal activity with the phosphatidylcholine and dodecyl sulphate in equimolar proportions. Ultrasonic treatment of the substrate particles increased the activity about fivefold. In general, no evidence of inhibitory substances could be detected on dilution of the extracts, and the same enzymic specific activity was obtained at various concentrations of extract in the incubation medium. The single exception was found in the basal stem region of the celery plant, where the activity increased appreciably on dilution of the extract.

Distribution of phospholipase D in various vegetables and their parts. Table 1 shows the phospholipase D activities present in the tissues of various vegetables and seeds. The specific activity varied markedly both between different plant species and between various tissues of the same species. All parts of the cabbage were appreciably active, although the maximum enzyme activity on a dry-weight basis was present in the central stalk. In the cauliflower very high activities were found in the flower tips and less in the flower stalks, with very low activity in the green leaves. The swollen stem of the Kohl rabi, the swollen leaf stalk of

Table 1. *Distribution of phospholipase D activity in extracts of various plant tissues*

Plant	Tissue	Specific activity of phospholipase D (μ g. of choline/min./mg. dry wt.)	Plant	Tissue	Specific activity of phospholipase D (μ g. of choline/min./mg. dry wt.)
Apple (<i>Pyrus malus</i> L.)	Fruit		Marrow (<i>continued</i>)	Mature	
	Peel	0		Outer peel	0.5
	Pulp	0		Inner flesh	30.0
	Seeds	0		Seeds	37.0
Artichoke (Jerusalem) (<i>Helianthus tuberosus</i>)	Tuber	18.0		Seeds (dry, 10 months old)	0.3
Cabbage (<i>Brassica oleracea</i> var. <i>capitata</i> L.)	Leaves		Mung bean (<i>Phaseolus mungo</i> L.)	Seed (dry)	1.7, 1.4
	Outer green	21.8			
	Inner light green	32.3	Oats (<i>Avena sativa</i> L.)	Seed (dry)	0.6
	Inner yellow	27.0		Root (6 days in the dark)	10.0
	Central stalk	64.5		Coleoptile (6 days in the dark)	8.9
	Buds	29.5		Endosperm (6 days in the dark)	1.6
	Seed (dry)	0			
Cauliflower (<i>Brassica oleracea</i> var. <i>botrytis</i>)	Flower		Onion (<i>Allium cepa</i> L.)	Outer leaf of bulb	0
	Tips	72.0		Inner leaf of bulb	0.3
	Stalks	42.0		Basal stem	4.1
	Basal stalk	23.4	Pea (<i>Pisum sativum</i> L.)	Leaf	0.8, 0.3
	Leaf (green)	6.7		Stem	0.8
	Leaf stalk	1.5		Root	0.7
Carrot (<i>Daucus carota</i> L.)	Root (swollen)	14.3		Pod	0.7
	Leaf	0.1		Seed (immature)	20.0
Celery (<i>Apium graveoleus</i> L.)	Leaf (green)	0		Seed (normal)	22.5, 21.2
	Leaf stalk (green)	0.4		Seed (dry)	17.2, 21.0
	Leaf stalk (white edible)	80.0	Potato (<i>Solanum tuberosum</i> L.)	Tuber	
	Basal region (stem)	66.0		Outer	0.1, 0
Horse chestnut (<i>Aesculus hippocastanum</i> L.)	First leaf	5.5		Inner	0.3
	Cotyledon	1.0		Shoot from sprouted	0.1
	Fruit coat (green spiny)	0		Young root (5 days)	0, 0
Corn (maize) (dried) (<i>Zea mays</i> L.)	Germ	1.0	Rose hips (<i>Rosa</i> sp.)	Outer tissue (flesh)	0
	Endosperm	0.3		Inner seeds	0
Kohl rabi (<i>Brassica caulorapa</i> Pasq.)	Swollen stem		Runner bean (<i>Phaseolus multiflorus</i> Willd.)	Bean seed (young, hydrated)	7.6
	Outer	27.2		Pod (green)	0.3
	Inner	47.2	Soya bean (<i>Glycine soja</i> Sieb. et. Zucc.)	Seed (dry)	13.1
	Normal stem	4.1			
	Leaf	2.1	Spinach (<i>Spinacia oleracea</i> L.)	Seed (dry)	0.1
	Leaf stalk	0.6		Root (approx. 10 days in the dark)	1.4
	Root	1.7		Plumule (approx. 10 days in the dark)	7.1
Leek (<i>Allium porrum</i> L.)	Inner leaf	8.3			
	Inner stem	8.8	Tomato (<i>Solanum lycopersicum</i> L.)	Immature green (whole)	0.1
Lemon (<i>Citrus medica</i> L.)	Peel and pith	0.2		Mature	
	Juicy centre	0		Pulp	0
	Seed	0.1		Seed	0.1
Lettuce (<i>Lactuca sativa</i> L.)	Inner leaf	25.6	Yeast (baker's) (<i>Saccharomyces cerevisiae</i>)	Pressed	0
	Leaf stalk	8.4			
Marrow (<i>Cucurbita pepo</i> L. var.)	Very young (whole)	2.5			
	Young				
	Flesh	9.1			
	Seeds (immature)	14.9			

celery and the swollen root of the carrot had high specific activity, whereas in the other tissues of the same plants there was comparatively little. The enzyme was also present at high concentrations in the tuber of the Jerusalem artichoke; in contrast the potato tuber contained negligible amounts, and this was true for all parts of the potato plant. In the mature pea grown under garden conditions by far the highest specific activity was present in the seed, and this was retained in dry seeds kept for more than 1 year. All the other parts of the plant including the pod, leaf, stem and root had very low activity. Among the legume seeds examined apart from the pea, soya bean and runner bean also contained appreciable activity, whereas the mung bean contained little. In the mature marrow the

seed had very high activity, the inner flesh (containing very small rudimentary seeds) somewhat less, and the outer flesh practically none. This activity in marrow seed was, unlike that of the pea, not retained after drying and storage. The activity in corn (maize seeds) was rather low, the specific activity of the germ being higher than that of the endosperm. However, since the total dry weight of the endosperm is about three times that of the germ, the total enzyme activity was about equally divided between the two parts. Other seeds such as those of the cabbage, tomato, apple, spinach, lemon and rose hip had low or negligible activity.

Distribution of phospholipase D during growth of the pea plant. When pea seeds were germinated and grown in the dark, examination of the whole

Table 2. *Distribution of phospholipase D in tissues of the young pea seedling*

Expt. no.	Days of growth and conditions	Tissue	Segment no.	Dry wt. (% of wet wt.)	N (% of dry wt.)	Phospholipase D activity (μ g. of choline liberated/min.)			
						Per total tissue (one seedling)	Per mg. wet wt.	Per mg. dry wt.	Per mg. of N
1	0 dark	Pea seed				6350	26.8	30.0	—
	2 dark	Seedling				5950	29.0	29.1	
	5 dark	Seedling				3900	6.0	19.8	
	7 dark	Seedling				3460	5.0	20.0	
2	7 dark	Plumule		6.5	9.1	133	0.95	14.8	162
		Root		6.5	—	354	1.69	25.2	—
		Cotyledon		30.0	4.0	3250	6.35	21.1	525
3	8 dark	Plumule [5.5–7 cm. long cut into five equal segments from tip (1) to cotyledon (5) end]	1	10.5	11.0	60.5	1.12	11.2	100
			2	4.6	10.0	16.8	0.31	6.8	68
			3	4.2	9.0	7.6	0.15	3.6	40
			4	5.3	7.5	17.0	0.32	6.1	78
			5	5.7	7.4	25.9	0.41	7.3	97
4	8 dark	Root [4–6 cm. long cut into five equal segments from tip (1) to cotyledon end (5)]	1	6.4	7.9	95	5.2	81.3	1020
			2	6.3	6.6	72	2.7	42.8	618
			3	5.7	9.1	64	2.3	40.3	435
			4	6.5	7.1	68	2.4	36.9	520
			5	6.8	6.8	92	2.1	30.9	458
5	4 dark	Root				72.5	3.0		
	6 dark	Root				128.0	2.7		
	8 dark	Root				172.0	1.1		
	4 dark	Plumule				30.5	2.1		
	6 dark	Plumule				48.5	1.1		
	8 dark	Plumule				161.0	0.76		
6	9 dark	Plumule (1 cm. growing tip)			N (% of fresh wt.)				
					1.3	45	2.4		186
					1.1	35	1.9		174
7	7 light 18 light 7 light 18 light	Plumule			0.52	230	0.92		186
		Plumule			0.80	290	0.35		44
		Root			0.31	318	1.62		525
		Root			0.25	412	0.45		180
8	12 days	Root from mature pea plant cutting		6.3			1.2	19

Fig. 1. Loss of phospholipase D from cotyledons during growth of the pea plant. ●, Total phospholipase D activity in the cotyledons of one pea seed or seedling; ○, phospholipase D activity/mg. of total N in the cotyledons.

seedling including the cotyledons showed that there had been considerable depletion of phospholipase D compared with the original seed (Expt. 1 in Table 2). This was markedly reflected in the activity of enzyme on a wet-weight basis, owing to the far greater hydration of the seedling, but even on a dry-weight basis there was a decrease in the activity. Examination of the cotyledons in a similar experiment showed a corresponding depletion of total phospholipase D, but when the activity was expressed on a nitrogen basis the decrease was far less (Fig. 1). Clearly phospholipase D was being lost at a rate that almost corresponded to that of the nitrogen reserves in the cotyledons.

In the pea seedling after 7 days' growth, most of the activity (87%) was present in the cotyledons (Expt. 2 in Table 2); of the remainder present in the growing tissues the root contained more enzyme than the plumule. This was consistently found in all experiments, and the specific activity of the enzyme was greater in the root on a wet-weight, dry-weight and nitrogen basis. The activity in the root on a dry-weight basis was even higher than that present in the cotyledon. The total enzyme activity increased in both the root and the plumule during growth in the dark, but the activity per mg. wet wt. decreased (Expt. 5 in Table 2). The same increase in total content was observed over a longer period of growth (18 days) in the light, but the activity on a nitrogen basis fell markedly (Expt. 7 in Table 2), although at the 18-day stage it was still higher than that found in the mature pea plant. Freshly developed roots from a mature pea plant cutting (2–5 cm. long) showed a phospholipase D activity that was approximately the same as that found in roots at a similar state of development taken from a young pea seedling (Expt. 8 in Table 2).

In two experiments (Expts. 3 and 4, Table 2) the growing plumules and roots of pea seedlings grown

for 8 days in the dark were cut into five equal segments. In both, the growing tip end showed the highest activity of enzyme per mg. wet wt. or dry wt. In terms of nitrogen, the tip end of the root again had the highest activity, but in the plumule the cotyledon end had about the same activity as the growing tip with a decrease towards the middle of the plumule. When a group of pea seedlings were grown in the dark for 7 days and then half were kept in the light for an additional 2 days (Expt. 6 in Table 2), there was only a slight decrease in the total amount and specific activity of the enzyme in the growing tip of the plumule exposed to light, even though considerable chlorophyll synthesis had occurred.

Phospholipids of the pea cotyledon during growth. When pea seeds were germinated the cotyledons of the developing plant lost a considerable part of their total phospholipid complement over an 11-day period. When they were grown in the dark, the loss was 60% in two experiments, and in the light it was 48% in one experiment (Table 3). Consideration of the individual phospholipids showed that the losses were by no means uniform. The zwitterionic nitrogen-containing phosphoglycerides (phosphatidylcholine and phosphatidylethanolamine), which comprise the bulk of the phospholipids, were decreased to about the same extent as the total phospholipid fraction. On the other hand, there was a greater percentage depletion of phosphatidylinositol than of total phospholipid in all experiments, whereas the small amounts of cardiolipin and phosphatidylserine present in the original seed were not lost from the cotyledon during growth. The concentrations of phosphatidic acid present in the cotyledons showed no evidence of an increase, as might have been expected if the phosphoglycerides disappearing were broken down by phospholipase D. In fact the total amount of phosphatidic acid present in the cotyledons was depleted to differing extents in each experiment, but to approximately the same extent as the total phospholipid fraction. In all three experiments there was a marked increase in a small phospholipid fraction that we have provisionally identified as phosphatidylmethanol. Its phosphorus-containing deacylation product chromatographed similarly to methyl L-3-glycerophosphate, and on thin-layer chromatograms (silica gel H) of pea lipid extracts it ran to the same position as phosphatidylmethanol with neutral and ammoniacal chloroform-methanol solvent systems (see the Methods section). Although barely detectable in the dry pea, it increased to 2–5% of the total phospholipid in pea cotyledons taken for analysis after 11 days of growth. It seems likely that the phosphatidylmethanol present may be an artifact formed through the transferase activity of phospholipase D

Table 3. *Disappearance of phospholipids from pea cotyledons after germination and growth*

The values express the content of individual phospholipids as percentages of the total lipid phosphorus analysed. Phosphatidyl-'methanol' has been only tentatively identified as such.

	Expt. 1			Expt. 2			Expt. 3		
	Content (%)		% of original dry-pea phospholipid remaining after 11 days' growth	Content (%)		% of original dry-pea phospholipid remaining after 11 days' growth	Content (%)		% of original dry-pea phospholipid remaining after 11 days' growth
	Dry peas	Peas grown for 11 days in the dark		Dry peas	Peas grown for 11 days in the dark		Dry peas	Peas grown for 11 days in the light	
Total phospholipid content ($\mu\text{g. of P/pea}$)	125	50	40	140	56	40	118	60	52
Phosphatidylcholine	53.3	54.0	41	49.0	53.0	41	55.0	62.0	58
Phosphatidylethanolamine	13.0	14.2	42	13.5	13.8	43	16.8	16.4	50
Phosphatidylinositol	15.6	10.6	27	17.5	10.8	25	17.0	11.0	33
Phosphatidylserine	0.3	1.2	150	0.4	0.9	90	0.4	0.9	133
Phosphatidic acid	3.6	6.0	67	2.9	1.9	42	3.6	2.8	39
Cardiolipin	0.6	1.6	100	0.7	1.6	91	0.8	1.5	100
Phosphatidylglycerol			310	1.0	0.8	32			—
Phosphatidyl-'methanol'	0.6	5.0		0.3	2.6	350	0	2.1	

(Yang *et al.* 1967; Dawson, 1967) and the methanol used in the extraction solvent. The higher amount in the germinated cotyledon would then be due to the enzyme initially reacting in a more hydrated environment, which would delay its eventual denaturation.

In all analyses the pea cotyledons were found to be virtually devoid of plasmalogens and sphingomyelin. However, another phospholipid that was partially alkali-stable was present to the extent of about 5% of the total phospholipids in dry pea cotyledons. This rapidly disappeared when the seeds were hydrated in the initial stages of germination, so that within 17 hr. only about 20% of the original remained. On t.l.c. it ran more rapidly than any other phospholipid; its structure is not yet known. It was not broken down by phospholipase D preparations.

DISCUSSION

The present results, showing that after extraction of pea plumules and cabbage leaves by grinding with sand the phospholipase D is present almost exclusively in the supernatant fraction, are at variance with the results of Kates (1954), who found that the enzyme was associated with particulate matter, in particular the chloroplasts. Davidson & Long (1958) reported that in the cabbage the enzyme was present in both a soluble and a particle-bound form, and soluble forms of the enzyme have been reported in cotton seed (Tookey & Balls, 1956) and carrot (Einset & Clark, 1958). Although the present method of enzymic assay is likely to be less sensitive to inhibiting substances present in the cytoplasm (Kates, 1954; Tookey & Balls, 1956), it seems that this cannot explain the discrepancy. We have examined cabbage leaves after homogenization in a Waring Blender and found the enzyme to be virtually all present in the supernatant fraction by using the less sensitive ether activation assay (R. H. Quarles & R. M. C. Dawson, unpublished work). The present distribution survey has also given evidence that the enzyme is not particularly associated with functioning chloroplasts. The distribution of the enzyme in cabbage plants and cauliflowers is the reverse of that expected if the enzyme was mainly present in chloroplasts. Again, Jerusalem-artichoke tubers, soya beans and marrow seeds are rich in the enzyme yet practically devoid of chloroplasts. In addition, the enzyme in the pea seedling has a higher specific activity in the root than in the plumule and does not increase in concentration in the plumule when this is exposed to light.

The distribution of the soluble enzyme in the tissues of various plants suggests that it is often, but not invariably, associated with storage organs

or with parts of the plant that are undergoing or about to undergo intense growth. Both the cabbage and cauliflower can be regarded as swollen apical buds full of food-reserve materials that are available for the final flowering and seeding of the plant in maturity. Again, phospholipase D is greatly enriched in the Kohl rabi in the swollen stem, rather than in the ordinary stem, and in celery in the swollen leaf stalk; in the carrot it is present in the swollen root rather than in the leaves. In these species, as in the pea seed, it seems that the enzyme is only present in those parts of the plant that contain reserve materials for eventual maturation or development of new plants. However, these conclusions are by no means absolute, since, although the tubers of the Jerusalem artichoke contain appreciable enzyme, there is none in the potato tuber. Again, although pea, soya-bean and marrow seeds contain high enzymic activities, rather low activities are present in many other seeds.

Although seeds and swollen storage tissues containing high activities are more or less dormant at the time of analysis, they are formed after a period of intense growth and metabolic activity by the plant. The question arises therefore whether the enzyme has a function in the subsequent germination or growth (or both) of the plant, or whether it has been formed during the stage of intense growth and has then just remained in the seed or storage organ. Certainly in the experiments with the recently germinated pea the enzyme in the plumule and root is enriched in the actively growing tip region: further back from the tip the activity declines, and generally the older the pea tissue becomes the less enzyme on a dry-weight basis is found. This distribution of enzyme is typical of that found for many enzymes in the growing root and plumule (see, e.g., Robinson & Brown, 1952), and suggests that the phospholipase D is formed in parts of the plant undergoing active growth. If the phospholipase D was remaining in the storage organ or seed after accumulating during the intense growth responsible for the tissue's formation it would have to be assumed that this differs from normal plant tissue in, for example, the lack of proteolytic activity to degrade the enzyme after its formation during the storage process.

Phospholipase D in the pea cotyledons is unlike most of the hydrolytic and transferase enzymes present in seeds in that it does not increase in activity during germination and growth, but in fact decreases. However, such increases in enzymic activity are not universally seen during growth and development and can vary from species to species (Tazawa & Hirokawa, 1956; Young & Varner, 1959; Brown & Wray, 1968). The results in Fig. 1 indicate that pea seed phospholipase D, rather than increas-

ing in activity to break down storage substances, disappears from the cotyledons at approximately the same rate as the storage proteins. Thus its specific activity in the cotyledon on a nitrogen basis remains approximately constant even though protein is being rapidly lost from the tissue. Nor do the results on the concentration of phospholipids in pea seeds give any conclusive evidence of the enzyme being functionally operative during germination of the seed. Certainly, phospholipids disappear from the pea cotyledons during growth, as was found for bean cotyledons in the early work of Jordan & Chibnall (1933), but there is no evidence of an elevated concentration of phosphatidic acid, as might be expected if phospholipase D was active. In fact the phosphatidic acid concentration present in the cotyledons is decreased after 11 days to the same extent as the total phospholipid fraction. Although it is possible that the phosphatidic acid formed is rapidly metabolized further, we were unable to detect any rapid metabolism of phosphatidic acid on incubation of this with pea cotyledon homogenates (R. H. Quarles & R. M. C. Dawson, unpublished work). It is also possible that the enzyme functions as a transferase (Dawson, 1967; Yang *et al.* 1967) so that phosphatidic acid is not produced. In any case, from the small amount of phospholipids stored in pea seeds it seems unlikely that they function to any significant extent as reserve substances, and perhaps they are merely present as constituents of the storage-cell membranes that eventually disappear during the catabolism of the cotyledon. Earlier work indeed suggested that the small amounts of choline-containing phospholipids present in soya-bean seeds are not lost to any appreciable extent during early germination in the dark and that often a small increase in concentration occurs initially (Ducet, 1949), before an eventual fall. The phospholipase D activity is so high in the pea seed that there is sufficient to metabolize phospholipid at a rate tens of thousands of times greater than the rate of disappearance of phospholipid observed during development of the pea seedling.

It seems unlikely that whole protein molecules of phospholipase D could be transported from the seed to the growing tip of the plumule and root to participate in the growth processes; thus in the young roots of cuttings from mature pea plants, specific activity of the phospholipase D is as high as that of similar roots from seedlings, in spite of the parent tissues from which they are formed (stem and leaf) showing a 20-fold lower specific activity compared with the seed. This suggests that the enzyme is synthesized *in situ* in the rapidly growing root cells. This conclusion is supported by the finding that much higher activities of phospholipase D are present in the

young plumules and roots of oat and spinach seedlings than in the original seeds. The present results do not suggest any reason why the enzyme is needed in these actively growing cells, and the actively growing root of a potato tuber is devoid of phospholipase D activity, so the enzyme cannot be an essential prerequisite of the growth process.

Although the present results are far from conclusive they favour the idea that phospholipase D is formed in some types of higher-plant cells during a period of active growth, e.g., in the rapidly growing tip of a root or plumule. Usually this activity is then lost so that the mature plant stem or leaf is comparatively devoid of activity. However, when this active growth is associated with the formation of a storage tissue and the deposition of reserve substances it is possible that the phospholipase D formed is sometimes not lost, but remains in the seed or tissue until development occurs and the proteins including the enzyme are lost by proteolytic activity. In this connexion the deposition of inhibitors of proteolytic activity that often occurs in seeds (Pusztai, 1968) may protect the enzyme from decomposition before germination. It is noteworthy that in those seeds containing an abundant reserve of proteins appreciable phospholipase D is found in the seed on drying.

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