of this investigation. We are grateful also to Dr A. N. Davison for a specimen of SKF 525-A, and to the directors of Fisons Pest Control Ltd. for permission to publish this paper.

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The Phosphatase and Metaphosphatase Activities of Pea Extracts

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Metaphosphates and inorganic polyphosphates are widespread in living organisms (see reviews by Ingelman, 1950; Schmidt, 1951; Winkler, 1953). Apart from the ubiquitous formation of pyrophosphate by various enzyme reactions, large amounts of these polyphosphates have been detected in moulds, bacteria, algae, the seeds of cotton plants and some insect tissues, although not so far in the higher animals or the leaves of higher plants. Enzymes which degrade the inorganic polyphosphates, with or without the liberation of orthophosphate, have also been detected in extracts of a wide range of organisms (see Ingelman, 1950; Schmidt, 1951). There have, however, been few studies on the purification and properties of these enzymes.

In the course of previous work on the purification of plant ribonuclease (Holden & Pirie, 1955*a*; Pierpoint, 1956), it was observed that pea-leaf extracts would liberate orthophosphate from commercial 'hexameta-phosphate'. In view of the increasing interest in the occurrence and metabolism of poly- and meta-phosphates, this metaphosphatase has been characterized further. In particular, some attention has been paid to its specificity, since very few of the metaphosphatases described have been tested against more than one or two inorganic polyphosphates.

Evidence is presented here that the metaphosphatase activity of the pea extracts is associated with an unspecific phosphoesterase, which liberates orthophosphate from trimetaphosphate and tripolyphosphate as well as a number of organic phosphates. During the purification of this enzyme by ion-exchange chromatography, it was resolved into two distinct components with similar but not identical properties.

EXPERIMENTAL

Enzyme preparations. Extracts of pea seedlings that had been grown in a glasshouse for two or three weeks were fractionated by the method of Holden & Pirie (1955*a*). The fractions are referred to by the letters that these workers used. Many of the experiments were performed with fraction E, since it contained most phosphatase activity on a protein-nitrogen basis. It was prepared by precipitation with ammonium sulphate between 50 and 85% saturation, from a citrate extract of pea seedlings that had been clarified

at pH 4·5. The precipitate was dissolved in citrate buffer at pH 5, dialysed and then centrifuged.

Enzyme estimations. The various phosphatase activities were measured in terms of the inorganic orthophosphate liberated from the substrates.

Glycerophosphatase activity was measured by incubating samples of the enzyme for 1 hr. at 37° in a solution containing β-glycerophosphate at a concentration of 0·4 mg. of P/ml., and 25 mM citrate buffer (pH 5) in a total volume of 4 ml. Samples were taken before and after incubation and usually pipetted directly into the sulphuric acid used in the phosphate assay. However, when the activity of unfractionated sap was being measured, or shorter periods of incubation were used, the reaction was stopped and protein removed by the addition of 2 ml. of 15% trichloroacetic acid. Samples for phosphate assay were then taken after the precipitate had been removed by centrifuging for 10 min. at 3000 rev./min.

Phosphatase activity against the polyphosphates adenosine diphosphate (ADP) and adenosine triphosphate (ATP) was measured in the same way with the substrates present at a concentration of 0·4 mg. of P/ml. The final pH was 6 instead of 5, since this was nearer the pH optimum for these substrates with fraction E. In more purified enzyme preparations the pH optimum for glycerophosphate, as well as these other substances, was near 6, and some of the glycerophosphatase measurements involving very small amounts of enzyme were done at this pH. These measurements are indicated in the appropriate places.

Phosphodiesterase activity was looked for by the liberation of phenol from diphenyl phosphate. The enzyme was incubated for 1 hr. at 37° in a solution containing diphenyl phosphate at a concentration of 0·4 mg. of P/ml. and citrate buffer 50 mM, in a total volume of 4 ml. at pH 5. Samples were taken before and after incubation and the phenol in them estimated with the Folin–Ciocalteu (1927) reagent.

The unit of phosphatase activity was defined as the activity that would liberate 31 mg. of P as orthophosphate/l. of solution, when incubated for 1 hr. under the conditions described. Since the liberation of phosphate did not vary in a linear manner with amount of enzyme added (Fig. 1), the assay was performed with various amounts of enzyme, and the amount containing one unit of activity measured graphically.

Chemical estimations. Orthophosphate was estimated as described by Holden & Pirie (1955*b*). The samples were pipetted into 5 ml. of 2N sulphuric acid; 1 ml. of 7·5% (w/v) ammonium molybdate was added, and water to 9 ml. The solution was shaken well before and during the addition of 1 ml. of freshly prepared stannous chloride (0·2%), and after a few minutes the colour was compared with a suitable standard on a visual colorimeter. Total phosphate was estimated in a similar way after digestion with a mixture of sulphuric and perchloric acids (Holden & Pirie, 1955*b*).

Nitrogen was determined by a micro-Kjeldahl method, and the ammonia measured either by titration with hydrochloric acid or by nesslerization. To determine the amount of protein nitrogen in enzyme solutions, an equal volume of 10% (w/v) trichloroacetic acid was added, and the precipitate centrifuged down at 5000 *g*, washed with 5% (w/v) trichloroacetic acid, and then dissolved in a small volume of NaOH solution before the Kjeldahl digestion.

Protein was also estimated in terms of its ultraviolet absorption at 280 mμ. in the 1 cm. cells of a Unicam SP. 500 spectrophotometer.

Chemicals. Tripolyphosphate ($Na_5P_3O_{10},6H_2O$) and the cyclic metaphosphates, trimetaphosphate [$(NaPO_3)_3,6H_2O$] and tetrametaphosphate [$(NaPO_3)_4,10H_2O$] were gifts from Albright and Wilson Ltd. The trimetaphosphate and tripolyphosphate contained 6 and 0·5% respectively of a phosphate that reacted as orthophosphate in the phosphate assay. The number of acid groups that could be titrated between pH 5 and 9 was consistent with these amounts of impurity, and paper chromatography in a *tert.*-butyl alcohol system (Crowther, 1954) indicated that the contaminant in the tripolyphosphate was orthophosphate, whereas that in the trimetaphosphate was probably pyrophosphate. No impurity could be detected in the tetrapolyphosphate.

The three phosphates, unlike the small amounts of contaminants, did not react in the phosphate assay, nor were they hydrolysed under the conditions of the assay.

Calcium phosphate was a three-year-old specimen that had been prepared by the method of Singer & Kearney (1950).

All the other chemicals were obtained commercially and were AnalaR as far as possible. The metaphosphoric acid (Hopkins and Williams Ltd.) contained 4–5% of orthophosphate.

Chromatographic methods. The procedure used for the chromatography of the enzymes was a pH–gradient elution method in which the sulphonated-polystyrene cation-exchange resin Dowex 50 (200–400 mesh, × 12) was used. The Dowex was converted into its sodium salt as described by Moore & Stein (1951) and packed into a glass column approximately 2 cm. × 35 cm. After each run it was regenerated by washing with 2N sodium hydroxide solution.

The eluting gradient was produced by a system essentially the same as that described by Boman (1955), and was almost linear over the effective range. The eluting buffer was 0·2M citrate (pH 6·7), which siphoned over from a 500 ml. conical flask into an open mixing vessel containing the

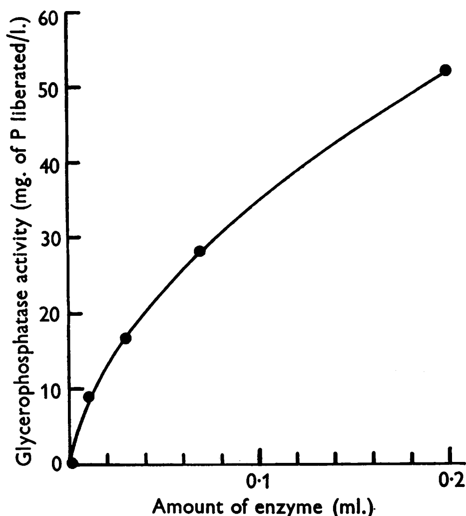

Fig. 1. Variation of glycerophosphatase activity with amount of enzyme. The enzyme used was a preparation of fraction E containing 0·16 mg. of protein nitrogen/ml. Enzyme activity was measured as described in the text.

0·2 M starting citrate buffer (pH 4·5). This mixing vessel was simply a piece of glass tubing of internal diameter 4 cm., with a rubber stopper as bottom. It is connected through this stopper to the flask of eluting buffer and the top of the column. The mixing of buffers in this vessel is promoted by a bubbler attached through a gas-washing bottle to an air pump.

At the beginning of each experiment the resin was washed well with 0·2 M citrate (pH 4·5) and then the enzyme solution, fraction E, containing 1000–2000 units of glycerophosphatase in 0·1 M citrate (pH 4·5) was added and allowed to sink into the resin. The column was then washed for 2–3 hr. with more buffer at pH 4·5. Elution was started by adjusting the level of citrate (pH 4·5) in the mixing vessel to that of the citrate (pH 6·7) in the conical flask, and opening the connexion between the two vessels.

Elution was continued for 24 hr. in the cold room at 2–4°. During this time the average flow rate was 22 ml./hr. Fractions of the eluate (10·1 ± 1·5 ml.) were collected in an automatic fraction collector of the second type described by James, Martin & Randall (1951).

All pH measurements were made with a glass electrode at room temperature.

RESULTS

Preliminary observations suggested that in a large number of pea extracts the ratio of glycerophosphatase to metaphosphatase was constant within the limits of the accuracy of the estimations. Table 1 shows some of these results obtained with fractions

Table 1. *Ratio of glycerophosphatase to metaphosphatase in pea-leaf extracts*

The fractions were obtained by the method of Holden & Pirie (1955*a*), and the enzyme activities measured as described in the Experimental section.

Expt.	Fraction	Further treatment	Glycerophosphatase (units/ml.)	Metaphosphatase (units/ml.)	Glycerophosphatase / Metaphosphatase
1	B	—	54	46	1·17
	E	—	94	92	1·02
	F	—	44	35·5	1·24
	J	—	42	34·4	1·26
	F	Ppt. at 0–50% saturation with $(NH_4)_2SO_4$	29	21	1·38
2	E	—	15·6	18·8	0·83
3	E	—	18·8	16·3	1·16
4	E	—	12·5	13·4	0·93
5	E	—	19·2	22·0	0·87
	E	Heated at 37° for 180 min.	18·3	18·8	0·97
	E	Heated at 56° for 45 min.	4·1	4·7	0·88

Table 2. *Phosphatase activities in fractions of pea extracts*

Solution E was fractionated either with cold ethanol (−10°), or, after its pH had been adjusted to 7, with ammonium sulphate. The precipitates were collected by centrifuging in the cold, dissolved in 0·1 M citrate buffer (pH 5), and dialysed overnight against water before being assayed for enzymic activity.

		Glycerophosphatase		Metaphosphatase		
Expt.	Fraction	(units/mg. of protein N)	Recovery (%)	(units/mg. of protein N)	Recovery (%)	Glycerophosphatase / Metaphosphatase
1	E	81	100	91	100	0·89
	Precipitate obtained on addition of 0–0·55 vol. of ethanol	51	11·7	58	11·4	0·88
	Precipitate obtained on addition of 0·55–1·66 vol. of ethanol	159	40	152	35	1·05
	Supernatants after addition of 3·0 vol. of ethanol	50	7·1	45	6	1·11
2	E	156	100	100	100	1·56
	Ppt. at 0–50% saturation with $(NH_4)_2SO_4$	116	6·3	120	10·2	0·97
	Ppt. at 50–60% saturation with $(NH_4)_2SO_4$	127	13	132	21	0·97
	Ppt. at 60–70% saturation with $(NH_4)_2SO_4$	200	19·2	152	23	1·30
	Ppt. at 70–80% saturation with $(NH_4)_2SO_4$	435	15	150	7·1	2·90

Table 3. *Adsorption of phosphatase and metaphosphatase on calcium phosphate gel*

Various amounts of phosphate gel were added to 4 ml. samples of fraction E containing 67 units of glycerophosphatase, and water to 6·5 ml. After standing at 0° for 10 min. the gel was removed by centrifuging, and enzyme activity, protein and dry-weight contents were measured in the supernatant solutions. Protein was estimated from the optical density at 280 and 260 mμ. (Warburg & Christian, 1941).

Phosphate gel added (mg. dry wt.)	Metaphosphatase remaining (%)	Glycerophosphatase remaining (%)	Dry wt. remaining (%)	Protein remaining (%)
0	100	100	100	100
1·77	88	91	89	89
5·3	52	51	—	73
11·6	13	19	68	61
21	1·6	3·0	61	51
45	<1	<1	58	45

Table 4. *Adsorption of phosphatase and metaphosphatase on Dowex* 50

Samples of solution E containing 1·8 units of glycerophosphatase in 80 mM citrate (pH 4·5), total volume 3·6 ml., were agitated for 15 min. at 0° with various amounts of the sodium salt of Dowex 50. The resin was removed by centrifuging and the enzymes were estimated in the supernatant solutions.

Amount of Dowex added (mg. dry wt.)	Metaphosphatase remaining (%)	Glycerophosphatase remaining (%)
0	100	100
40	60	55
120	5	0
200	0	0

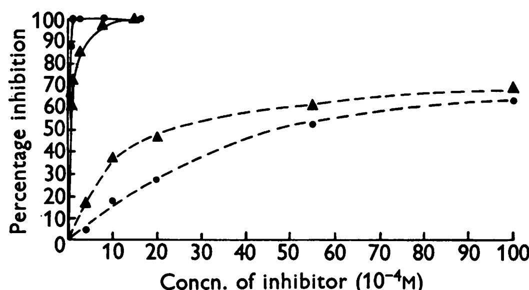

Fig. 2. Inhibition of glycerophosphatase and metaphosphatase activities. Samples of fraction E (0·05 ml.) were incubated at 37° with glycerophosphate (●) or metaphosphate (▲) at concentrations of 0·4 mg. of P/ml.; citrate, 50 mM (pH 6); sodium fluoride (broken lines) or ammonium molybdate; water to 4 ml. Samples were taken for orthophosphate estimations after 1 hr.

of the extracts prepared by the method of Holden & Pirie (1955*a*). Similar ratios were observed with solutions obtained during the chromatography of leaf ribonuclease (Pierpoint, 1956). Fractionation of E with ethanol did not materially affect this ratio (Table 2). Nor did ammonium sulphate fractionation normally, although in some experiments, as in the one illustrated, there was a twofold increase in the proportion of glycerophosphatase activity in the small fraction precipitated between 70 and 80 % saturation. The erratic nature of this effect makes it difficult to interpret. Total enzyme recoveries were low (50–60 %) in both fractionations (Table 2).

Phosphatase and metaphosphatase activities disappeared in similar manner when fraction E was allowed to stand for 10 min. in the presence of calcium phosphate gel. Both had completely disappeared from solution before 55 % of the total protein, measured spectrophotometrically, had been adsorbed (Table 3). Much smaller amounts of both enzyme activities were also adsorbed from citrate buffer (pH 4·5) on the sodium salt of Dowex 50 (Table 4).

The behaviour of the enzyme solution in the presence of both glycerophosphate and metaphosphate is consistent with the view that both activities belong to the same enzyme. Thus in the presence of optimum amounts of both substrates, at either pH 5 or 6, the amount of orthophosphate liberated and its rate of liberation are approximately equal to those from the most active substrate alone. Also consistent with this view is the effect of molybdate and fluoride on these two activities. Both are affected to approximately the same degree by these substances at pH 6 (Fig. 2). Moreover at pH 5 the effect of fluoride on both activities was slightly greater, and that of molybdate slightly less, than at pH 6. In these experiments, unlike those with purified preparations of phosphatase from human prostate (Reiner, Tsuboi & Hudson, 1955), there was no reversal of the fluoride inhibition at higher concentrations of fluoride.

London & Hudson (1955) have shown that the heat-denaturation properties of phosphatases are specific and can be used in demonstrating the association of different enzyme activities with the same protein. Consequently a few observations have been made on the inactivation by heat of

glycerophosphatase and metaphosphatase under different conditions of pH and temperature. In these experiments, suitable amounts of enzyme were incubated in the required conditions, and samples taken at various times. Enzyme activities in these samples were measured using an incubation time of 15 min., all the other conditions being the same as in the usual estimations. At pH 6 and 51·8°, both activities were destroyed in a manner that approximated to first-order kinetics. The heat-denaturation constants, expressed in $hr.^{-1}$, were 2·5 for glycerophosphatase and 2·1 for metaphosphatase. At pH 5 and 44·5° no loss in either enzyme could be detected in periods up to 100 min. In view of the great differences that were observed between different phosphomonoesterases by London & Hudson (1955), these results are compatible with the idea that the two enzyme activities are associated with the same protein.

Experiments already described indicate that the material responsible for both enzyme activities could be absorbed from buffer at pH 4·5 on the sodium form of Dowex 50. Other experiments indicated that it could be eluted at higher pH values. Attempts were therefore made to chromatograph the enzymes on a Dowex column, with buffer of gradually increasing pH as eluting agent. Under these conditions the enzyme was eluted from the column as two distinct components. Fig. 3 illustrates a typical result. In this experiment, 10 ml. of fraction E, containing 1000 units of glycerophosphatase, and a total of 1·4 mg. of protein nitrogen was added to the column in 0·1 M citrate (pH 4·5). In their distribution in the fractions of eluate both enzyme activities follow the same pattern. There is a small peak of activity, hereafter referred to as peak *A*, which appears when the eluate has reached a pH of 5·0–5·1, and a second larger peak, peak *B*, eluted at a pH of 5·6–5·7. Three different preparations of E have been examined and all show these two components. The proportions of the two components in different preparations, estimated as the areas under the curves, vary somewhat, but the pH values of the eluate at which they first appear seem constant. In nine experiments performed on the three samples of solution E, the averages and standard deviations of these pH values were 5·0 ± 0·24 and 5·7 ± 0·18 respectively.

The way in which metaphosphatase and glycerophosphatase follow the same elution pattern strongly indicates that they are both associated with the same protein. As described in the legend of

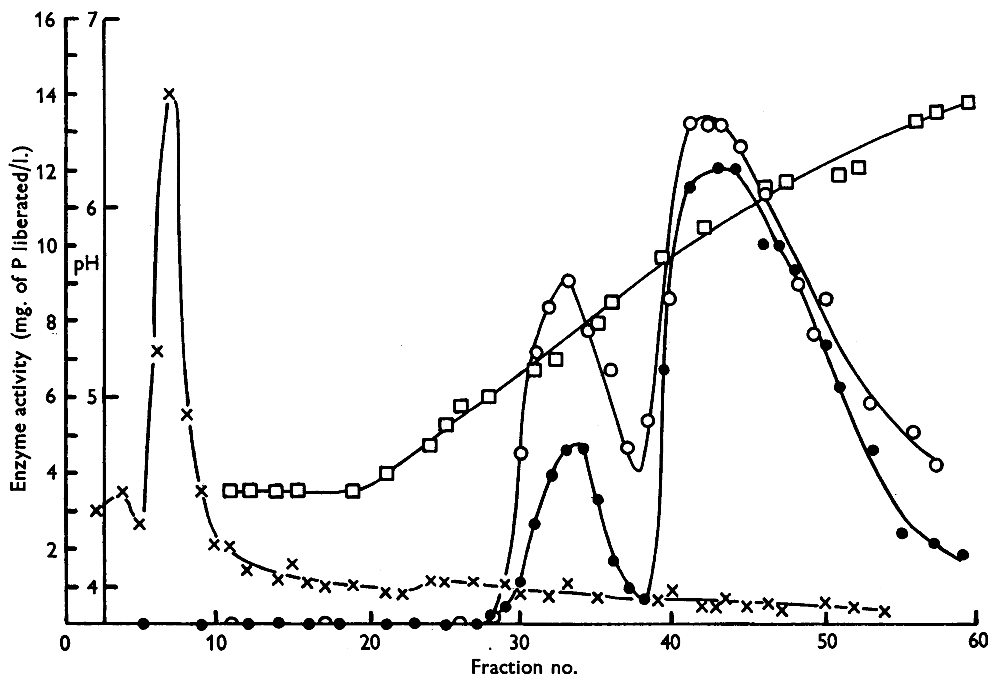

Fig. 3. Chromatography of pea phosphatase. A portion (10 ml.) of fraction E (1000 units of glycerophosphate, 1·4 mg. of protein) was chromatographed as described in the text. The pH values (□), optical density at 280 mμ.(×), glycerophosphatase (●) and metaphosphatase (○) activities were determined in each fraction. Enzyme activities were determined on 0·2 ml. samples by the method described, except that the concentration of the buffers was increased to 60 mM. The ordinate with optical density units is not shown, but the maximum value on the optical density curve is 1·4.

Fig. 3 the enzyme activities are estimated in single samples (0·2 ml.) of the fractions. Since the activity of the enzymes does not vary in a linear manner with the amount of enzyme present (Fig. 1), this estimation is not a strictly quantitative measure of the enzyme in the fractions. Consequently both enzyme activities were measured in the usual quantitative manner in eight selected fractions. If the fractions contained any activity at all, then the ratio of glycerophosphatase to metaphosphatase was constant, being independent of the peak of activity to which they belonged and of their position on that peak.

Further observations on the chromatography of the enzyme

It is evident from the curve of optical density at 280 mμ. in Fig. 3 that both components represent a considerable enrichment of enzyme activity. The bulk of the material in fraction E which absorbs at this wavelength is not adsorbed on the Dowex, but is washed straight through the column. It is not possible to measure accurately the amount of protein associated with the two components in terms of absorption at this wavelength, as the citrate buffers always had a small absorption, and there was the possibility of interference from resin material of low molecular weight (Boman, 1954). Estimations of protein N, however, give values for the specific activity of pooled *A* and *B* of 1440 and 1250 units of glycerophosphatase/mg. of protein N. These represent enrichments of the order of 7–10 times over the original fraction E. Because of the small amounts of enzyme used in these chromatographic runs, it has not proved possible to estimate the nitrogen associated with each enzyme component. Consequently it is not known if they have similar specific activities. The total amount of enzyme recovered was measured in a number of experiments and found to be between 50 and 60 % of that added to the column.

Although the formation of the two enzymic components could have been brought about by the chromatographic procedure itself, a number of experiments argue against this. Thus it has been possible to rechromatograph the components separately. Figs. 4 and 5 show the results of two experiments in which the fractions of eluate containing either component *A* or *B* were pooled and rechromatographed. Both experiments were performed on a sample of solution E containing the two components in a similar ratio to the one used in Fig. 3. The separated components behaved in a homogeneous manner and were eluted at the same pH as in the initial chromatograms. They were contaminated with 5 % or less of each other.

Another possible cause of the apparent inhomogeneity of an enzyme is the presence of traces of metal ions in the enzyme preparations (see e.g. Hirs, Moore & Stein, 1953). This is unlikely here because the incorporation of 8-hydroxyquinoline (2 mM) and disodium ethylenediaminetetraacetic acid (mM) into both the buffers used in the chromatography did not affect the elution pattern of the enzyme. These compounds had no effect on the estimation of the enzyme.

The relative amounts of the two components in a sample of E varied with the age of E. As a preparation aged the relative amount of component *A* in the total recovered enzyme increased. Fig. 6 illustrates this phenomenon and shows that it is due not to an increase in the amount of *A* present but rather to a decrease in the amount of *B*. Judged by the areas under the curve the proportion of *A* in this preparation increased from about 15 to 40 % during the

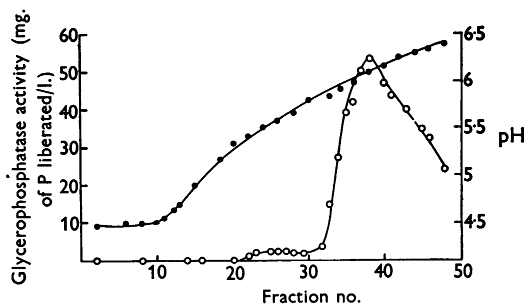

Fig. 4. Isolation of component *B*. The fractions containing *B*, derived from an experiment similar to that of Fig. 3, were pooled and chromatographed after adjustment of their pH to 4·5. Samples of the eluate were taken for pH (●) and enzyme (○) estimation. Glycerophosphatase was measured on 0·4 ml. samples by the method described, except that the buffer used was 100 mM citrate (pH 6), and that the incubation period was 2 hr.

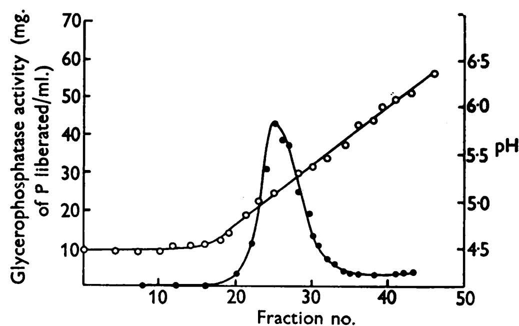

Fig. 5. Isolation of component *A*. The fractions containing *A*, derived from an experiment similar to that of Fig. 3, were pooled and chromatographed after adjustment of their pH to 4·5. The pH (○) and glycerophosphatase content (●) were measured on samples of the eluate. Glycerophosphatase was measured on 0·4 ml. samples by the method described, except that the buffer used was 100 mM citrate (pH 6), and the incubation time was 2 hr.

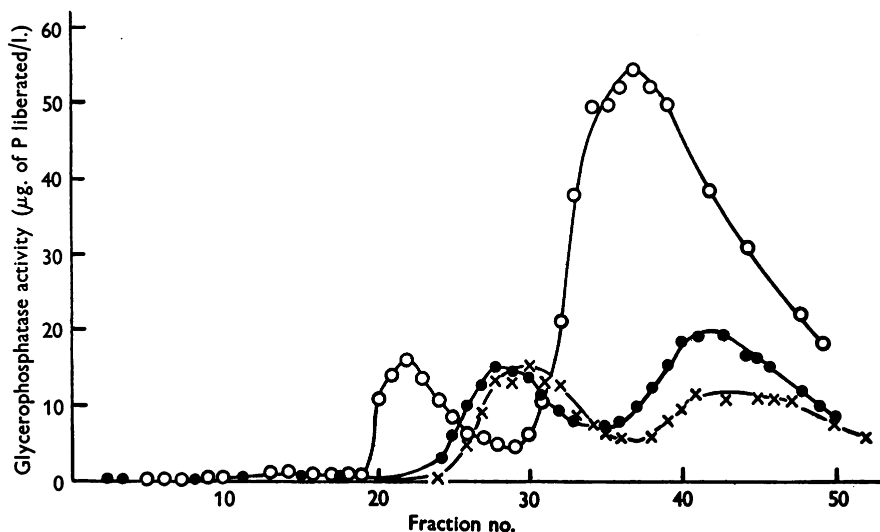

Fig. 6. Effect of age on the amounts of the enzyme components. Samples (10 ml.) of fraction E, initially containing 168 units of glycerophosphatase/ml., were chromatographed 1 day (○), 49 days (●), and 53 days (×) after preparation. Between the experiments the enzyme solution was stored at about 2°, and by the 49th day had lost 66% of its activity. Glycerophosphatase was estimated on samples (0·2 ml.) of the eluate as previously described, except that the buffer was 56 mM citrate (pH 6), and the incubation period was 2 hr. The peaks from the three different chromatographic runs do not coincide because of differences in the shape of the pH gradients in the three runs.

Table 5. *Heat stability of the two components of pea phosphatase*

Solutions of *A* and *B*, containing 2·8 and 6·7 units of phosphatase respectively, were adjusted to the required pH with molar citrate or citric acid, and incubated at the temperatures stated below. Samples were taken for enzyme estimations at appropriate times.

Time of incubation (min.)	Enzyme activity remaining after incubation at pH 5·9 and 48·5°		Enzyme activity remaining after incubation at pH 5·0 and 48·5°	
	A	*B*	*A*	*B*
0	100	100	100	100
27	55·5	28·5	—	—
40	—	—	35·5	29
44	36	10·8	—	—
60	26·5	6·6	—	—
70	—	—	18	14
107	—	—	10	6·5

53 day storage in the cold room. These values are only approximate, as they assume similar total recoveries and similar degrees of peak overlapping in the different chromatographic runs.

In the few properties that have been observed, *A* and *B* seem to resemble each other closely. Their pH optima, measured against glycerophosphate in citrate buffers, both lie within the region 5·5–6·0, and, like the enzyme in solution E, neither component is affected by the presence of Mg^{2+} ions. In Table 5 the heat stabilities of *A* and *B* at two pH values are compared. Although they are quite similar, the greater stability of *A* at pH 5·9 may partly explain the changes in the relative amounts of the two which takes place as E is stored.

In order to characterize further the phosphatase and its components a number of observations on their specificity have been made. The criterion used in assigning a given enzyme activity to the phosphatase under investigation is that it shows the same distribution in fractions from the chromatogram as the glycerophosphatase activity. For this purpose the action of fractions obtained from a typical chromatogram have been tested against some phosphates. Fig. 7 shows the distribution of tripolyphosphate and trimetaphosphatase. Both show two peaks corresponding to the glycerophosphatase peaks; but although *A* and *B* seem to have a similar ratio of activity against trimetaphosphate and against glycerophosphate, *A* seems to be relatively more active against tripolyphosphate. Since the measurement of enzyme activities with single samples from each fraction is not strictly quantitative, this conclusion has been confirmed by the more exact method of measuring enzyme activity. Table 6 shows that, relative to their action on glycerophosphate, the enzyme of peak *A* is 2–3 times as active against tripolyphosphate as is that of peak *B*.

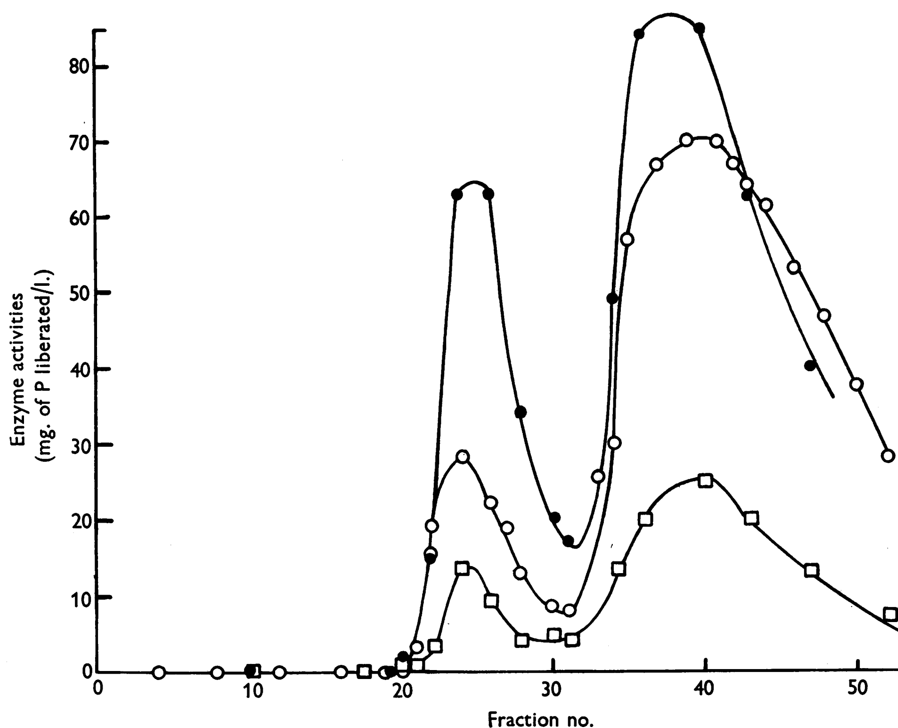

Fig. 7. Polyphosphatase activity in the eluate from a chromatogram. A portion (10 ml.) of fraction E (1750 units of glycerophosphatase) was chromatographed and samples of the eluate fractions (0·2 ml.) were tested for glycerophosphatase (○), tripolyphosphatase (●) and trimetaphosphatase (□). Enzyme activities were estimated as described, except that the buffer was 50 mM citrate (pH 6), and the incubation period was 2 hr.

Table 6. *Ratio of polyphosphatase activities in the chromatographed enzyme components*

Glycerophosphatase, tripolyphosphatase and trimetaphosphatase were estimated in the enzyme components *A* and *B* by the method described, except that the buffer used was 75 mM citrate (pH 6) throughout.

Expt.	Enzyme peak	Tripolyphosphatase / Glycerophosphatase	Trimetaphosphatase / Glycerophosphatase
1	*A*	3·16	0·35
	B	1·44	0·31
2	*A*	3·5	—
	B	1·05	—
3	*A*	4·0	—
	B	1·26	—

The distribution of ATP-ase and ADP-ase among the fractions from the chromatographic column also shows two peaks which correspond to those of glycerophosphatase. Although the ratio of the ATP-ase activity of the two components is similar to the ratio of their glycerophosphatase activity, the second is about half as active as the first towards ADP. None of the fractions had any perceptible activity in liberating orthophosphate from tetrametaphosphate, or phenol from diphenyl phosphate.

It has been suggested (Ledoux, 1953, 1954*a*, *b*) that the multiple nature of pancreatic ribonuclease, as revealed by chromatographic methods, is due to different oxidation–reduction states of four –SH groups in the ribonuclease molecule. Although the hypothesis that ribonuclease has any –SH groups which can affect either its enzymic activity on its chromatographic properties has by no means been universally accepted (e.g. Anfinsen, Redfield, Choate, Page & Carrol, 1954; Rabinovitch & Barron, 1955; Gawron, Keil & Glaid, 1956; Holden & Pirie 1955*b*; Craddock & Dalgliesh, 1955) it seemed worth while to ascertain whether the pea phosphatase is an –SH enzyme. Consequently the action of a number of compounds recommended by Barron (1951) has been investigated on the glycerophosphatase activity. Iodoacetic acid, iodosobenzoic acid and *p*-chloromercuribenzoate had no inhibitory action in concentrations up to $0·25 \times 10^{-2}$ M when tested in citrate, maleate or veronal–acetate buffers either with fraction E or with the chromatographed enzyme. Nor was any inhibition brought about by incubating the enzyme with the

–SH reagents for periods up to 30 min. before adding the glycerophosphate. It is concluded therefore that the enzyme does not contain any –SH groups that are necessary for its phosphatase activity.

DISCUSSION

Although the hydrolysis of the poly- and metaphosphates containing three or more phosphate groups has been reported a number of times with extracts of higher plants, in only a few of these have there been any observations on the specificity of the enzymes involved. Thus Yoshida (1953) has produced some evidence that the metaphosphatase of rice bran, estimated by its action on Graham's salt (sodium hexametaphosphate), can be partially separated from the glycerophosphatase activity of his preparations, and Rothenbach & Hinkelman (1954) have prepared extracts of germinated and ungerminated barley which will liberate orthophosphate from hexametaphosphate and trimetaphosphate. The metaphosphatase activity of pea extracts described in the present paper is almost certainly associated with an unspecific phosphatase. Although it does not attack diphenyl phosphate, the enzyme cannot be strictly regarded as a phosphomonoesterase, as it liberates orthophosphate from trimetaphosphate, a reaction involving the splitting of diphosphoester as well as monophosphoester bonds.

Trimetaphosphatase activity has been reported in moulds and animal tissues (Malmgren, 1952), in yeast extracts (Meyerhof, Shatas & Kaplan, 1953; Mattenheimer, 1953, 1956*a*) and in extracts of germinated and ungerminated barley (Rothenbach & Hinkelman, 1954). Only the yeast enzyme has been characterized (Kornberg, 1956; Mattenheimer, 1956*b*, *c*) and shown to be able to convert the cyclic trimetaphosphate into the linear tripolyphosphate. This enzyme is therefore a cyclophosphatase, in the terminology of Mattenheimer (1956*c*), having phosphodiesterase but not phosphomonoesterase activity. In view of this, and of the usual specificity of phosphatases for either phosphodiesters or phosphomonoesters (see Roche, 1950), the splitting of trimetaphosphate by the pea enzyme has been given a little consideration. One possible alternative explanation is that two enzymes are involved, although the distribution of the activity in the eluate from the chromatogram argues against this. It also seems unlikely that the trimetaphosphate is converted non-enzymically into tripolyphosphate on incubation and that this is the compound broken down by the enzyme, since incubation of trimetaphosphate at 37° and at any pH between 3·5 and 7·8 does not cause a detectable increase in the amount of acid groups which dissociate between pH 5 and 7. We intend to study this apparent association of phosphomonoesterase and phosphodiesterase activity, and to ascertain whether this ability to degrade trimetaphosphate distinguishes the pea enzyme from other unspecific phosphomonoesterases.

None of the pea extracts examined had any power to liberate orthophosphate from tetrapolyphosphate, or to convert the latter into a compound which could be degraded to orthophosphate by the enzyme described in this paper. Tetrametaphosphatase activity has been detected in yeast extracts (Mattenheimer, 1956*a*, *b*, *c*), where it converts tetrametaphosphate into orthophosphate by way of tetrapoly-, tripoly- and pyro-phosphate.

The appearance of two components in the chromatography of pea phosphatase is not an artifact caused by the chromatographic process or the presence of trace amounts of metal ions in the solution. There are left, therefore, two main explanations of their appearance. In the first place they may represent completely different enzymes with distinct chemical and physical structures, and perhaps different physiological functions and cellular distributions. An argument against this is the similarity of the component enzymes. They have the same pH optimum against glycerophosphate, are not affected by Mg^{2+} ions or chelating agents, have similar heat stabilities, and the differences between the specificities of the two are only quantitative ones. The alternative explanation, more in keeping with these similarities, and which has been used to explain the chromatographic heterogeneity of other biologically active proteins, is that the two components represent different forms of the same protein molecule.

The other proteins that have been resolved chromatographically into two or more active components are bovine pancreatic ribonuclease (Martin & Porter, 1951), lysozyme (Tallan & Stein, 1953) and insulin (Boardman, 1955). Recently Tanford & Hauenstein (1956) have shown by titrimetric methods that the main, if not the only, difference between the two forms of ribonuclease is the presence of an extra free carboxyl group in one of them. It is suggested that this difference is due, not to the incorporation of an extra amino acid into the ribonuclease molecule, but to the hydrolysis of an amide grouping. The two main components of insulin also differ by the presence of an extra amide group (Harfenist, 1953) and, although it has not been proved, the same suggestion has been made for lysozyme (Tallan & Stein, 1953).

It may well be that a similar explanation holds for the two compounds of pea phosphatase and that they are both derived from the same protein molecule, and differ only in one chemical group. On the other hand, one may represent a much more drastically altered form of the parent protein. There

is evidence from the enzymic digestion of pepsin (Perlmann, 1954), mercuripapain (Hill & Smith, 1956), ribonuclease (Kalnitsky & Rogers, 1955) and the hypophyseal growth hormone (Li, Papkoff, Fønss-Bech & Condliffe, 1956) that proteins may undergo extensive degradations without there being corresponding alterations in their biological activities. Whether the two components of the pea phosphatase represent different states of the same protein, and, if they do, whether the differences exist *in vivo* or are produced during the extraction and purification procedures, are questions for further work.

SUMMARY

1. Pea extracts have a hexametaphosphatase activity which is closely associated with an unspecific phosphatase. A variety of procedures, including protein precipitations, heat denaturation, adsorption and ion-exchange chromatography do not separate these activities or appreciably alter their ratio.

2. The phosphatase liberates orthophosphate from tripolyphosphate, trimetaphosphate, β-glycerophosphate, and adenosine di- and tri-phosphate, but not from tetrametaphosphate. It does not liberate phenol from diphenyl phosphate.

3. The phosphatase is inhibited by fluoride and molybdate but not by a variety of compounds that combine with –SH groups.

4. Ion-exchange chromatography of the phosphatase, with a buffer of higher pH as eluting agent, reveals two distinct enzymically active components. Each component can be separated from the other and rechromatographed as a single peak.

5. The two components are very similar in properties. They have the same pH optimum, are not affected by Mg^{2+} ions or chelating agents, have similar heat stabilities and differ only quantitatively in their specificities.

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