sitol is drained away from the cotyledons and translocated to the growing parts of the plant. Whether inositol is translocated in the free state, or in some combined form, is not known at present, but the metabolic fate of inositol during the early development of the bean seedling seems to depend on the site to which translocation takes place.

SUMMARY

1. The inositol content of each anatomical part of the bean seedling has been determined, by microbiological assay, at each of six stages during the early development of the plant.

2. The total inositol content of the bean seedling decreases by 40 % during the first 2 weeks of growth in the dark.

3. Inositol is liberated from its bound forms (phytin) in the cotyledons and translocated to the growing parts of the plant.

4. The inositol content of the plumules increases 20-fold during early development of the plant, the greater part of the increase being due to free inositol.

5. The increase in content of the roots (about eightfold) is due mainly to bound forms of inositol.

6. The testas do not appear to play any part in the metabolism of inositol during germination.

7. The hypocotyls show an increase in total inositol content, mainly due to an influx of free inositol. The epicotyls behave similarly.

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Phytase and Acid Phosphatase in the Dwarf Bean, *Phaseolus vulgaris*

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A comprehensive review of phytase has appeared (Sloane-Stanley, 1961) and the acid phosphatases have also been discussed (Morton, 1961). In the present paper some properties of phytase and a purified acid phosphatase in the dwarf French bean are presented, together with some observations on these activities in the early stages of metabolism of the developing plant.

EXPERIMENTAL

Materials

The plant used was the dwarf French bean, *Phaseolus vulgaris* var. Improved Canadian Wonder.

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Enzyme substrates. A commercial preparation of sodium phytate was recrystallized from water. The crystalline product was dried *in vacuo* for several days and ground to a white powder, which was moisture-free and contained no free phosphate. Chromatography revealed the presence of only one P-containing compound. This preparation was used as a substrate (1 mM) for phytase assays.

Disodium phenyl phosphate was used as substrate for acid-phosphatase assays.

Diethylaminoethyl(DEAE)-cellulose was prepared according to the method of Peterson & Sober (1956) and stored as a dry powder until required.

Calcium phosphate gel was prepared according to Dixon & Webb (1958) and stored for at least a month before use.

Buffer systems. Stock buffer solutions were prepared according to the data of Gomori (1955) except that concentrations were increased to 1·0 M. The following systems were used, over the pH ranges indicated: pH 2·0–3·5, glycine–

HCl; pH 4·0–5·5, acetate–acetic acid; pH 5·5–6·5, maleate; pH 5·5–8·5, tris–maleate; pH 7·2–8·5, tris–HCl; pH 8·6–10·0, glycine–NaOH. Measured portions of these solutions were taken to give appropriate final concentrations of buffer.

Methods

Germination. The seeds were covered with moist vermiculite contained in large dishes of depth 2 in., and allowed to germinate in the dark. The plant seedlings were removed when 10–12 days old.

Preparation of phytase. All operations were carried out at 2° unless otherwise stated. Cotyledons were homogenized with 5 vol. of 0·01 M-maleate buffer at pH 6·4 in an Ato-Mix blender for three half-minute periods at intervals of 5 min. The homogenate was then centrifuged at 26 500*g* in a refrigerated centrifuge for 30 min. The supernatant was adjusted to pH 4·8 with dilute acetic acid and centrifuged at 26 500*g* for 45 min. The residue was rejected and the supernatant subjected to $(NH_4)_2SO_4$ fractionation at pH 4·8 and room temperature. The fraction precipitating between 30 and 50% saturation (the '30–50% fraction') contained most of the phytase activity, and was used as the enzyme source in these experiments.

DEAE-cellulose chromatography. DEAE-cellulose (3–4 g.) was dispersed in water and poured into a glass column (2·5 cm. diam.) fitted with a sintered-glass filter at one end. The DEAE-cellulose bed was usually about 10 cm. in height and would accommodate about 300 'E_{280} units' of protein (1 unit is the amount of protein which in a 1 cm. tube has E at 280 mμ 1·0). The column was equilibrated with the buffer to be used during the fractionation, usually 0·01 M-tris–HCl, pH 7·4, until the pH of the effluent was identical with that of the applied buffer.

A portion of '30–50% fraction' containing 100 'E_{280} units' (28·7 mg. of N) was dialysed against the buffer at pH 7·4 for 24 hr., and then applied to the column. The concentrations of NaCl solutions used for elution of protein from the column are indicated in Fig. 1, which shows part of a typical elution pattern relating phytase and acid-phosphatase activities to total protein in the eluate fractions. The extinctions of the fractions (9–10 ml.) were determined in 1 cm. silica cells at 280 mμ with a Unicam SP. 500 spectrophotometer. Enzyme assays were performed as described below.

Ultrafiltration. This was used to concentrate dilute enzyme solutions. Narrow dialysis tubing (Visking; Hudes Merchandise Co.) was secured to the outlet of a small separating funnel. This was then fitted to a rubber bung, and the whole inserted into the mouth of a Buchner filter flask. The solution to be concentrated was placed in the funnel and reduced pressure applied to the flask. Water and small molecules pass through the membrane and the rate of filtration can be adjusted by altering the length of dialysis tubing or the vacuum applied, or both.

Determination of phosphate. Total phosphorus and free orthophosphate were determined by the colorimetric method of Allen (1940). Extinctions were read in an EEL colorimeter with red (OR 1) filters.

Deproteinization. Samples withdrawn from enzyme digests for phosphate determinations were added to 0·5 vol. of 20% (w/v) trichloroacetic acid contained in 10 ml. tapered centrifuge tubes, well mixed and allowed to stand for 1 hr. The precipitated protein was removed by centrifuging and the supernatant used for phosphate determination.

Total nitrogen. This was determined by the micro-Kjeldahl method.

Assay of enzyme activities. Phytase activities were assayed by incubating the enzyme with sodium phytate at pH 5·2 (acetate buffer) in a total volume of 5 ml. Final concentrations of phytate and buffer were 1 mM and 0·1 M respectively. After incubation for 3 hr. at 40° samples were withdrawn from the digest, deproteinized, centrifuged and orthophosphate determinations carried out on the supernatant.

Acid-phosphatase activities were assayed by incubation for 10 min. at 25° with disodium phenyl phosphate at pH 6·0 (for crude extracts) or pH 5·5 (for purified preparations) in a total volume of 1 ml. Final concentrations of substrate and acetate buffer were 0·01 and 0·1 M respectively. Usually the amounts of enzyme preparation were so small that deproteinization was unnecessary, no interference occurring in phosphate determination.

The unit of activity of the enzyme was defined as the amount of enzyme bringing about the release of 1 μg. of orthophosphate P under the conditions of assay described for the respective enzymes.

Separation of acid phosphatase. (*a*) Further fractionation of the '30–50% fraction' with $(NH_4)_2SO_4$ was carried out at pH 4·8 and room temperature. Protein fractions were precipitated at intervals of 5% saturation between 30 and 50% and the phytase and acid-phosphatase activities of these fractions are shown in Table 1.

(*b*) A sample of '30–50% fraction' containing 100 'E_{280} units' (28·7 mg. of N) was dialysed against 0·01 M-tris–HCl

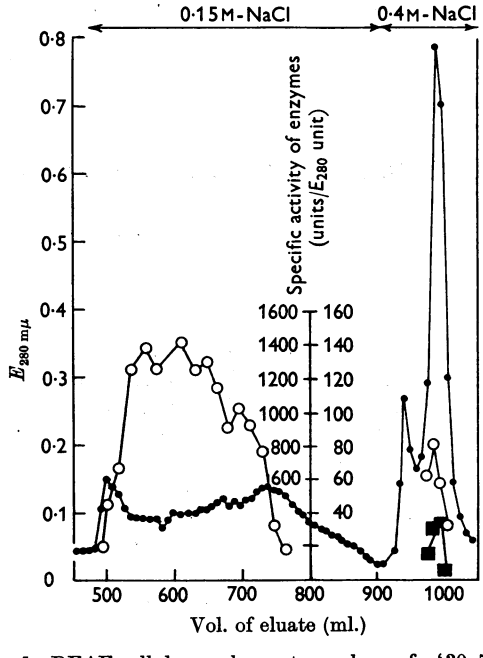

Fig. 1. DEAE-cellulose chromatography of '30–50% fraction' phytase preparation. Protein was eluted from column with NaCl solutions in 0·01 M-tris–HCl buffer, pH 7·2. ●, E at 280 mμ; ○, acid phosphatase; ■, phytase.

buffer, pH 7·4, for 24 hr., and then applied to the DEAE-cellulose column as already described. The acid phosphatase eluted with 0·15 M-NaCl was combined and concentrated by ultrafiltration to 17·5 ml. No phytase activity could be demonstrated in the concentrate. Further purification of the acid phosphatase was effected by calcium phosphate-gel adsorption at pH 6·8. The gel was added at the rate of 1 mg. (dry wt.)/ml. of enzyme solution. After standing for 5 min. the gel was removed by centrifuging and the supernatant assayed for enzyme activity, *E* at 280 mμ being measured. The complete scheme of purification is shown in Table 2.

RESULTS

Some properties of phytase in the '30–50 % fraction'

Effect of pH. Activity determinations were carried out at various pH values under the assay conditions described, '30–50 % fraction' (1·0 ml.) being used as enzyme source. Fig. 2 shows the pH–activity curve for phytase, with an optimum at pH 5·2.

Effect of substrate concentration on phytase activity. The substrate was sodium phytate (0·1 M), buffered at pH 5·2 with acetate buffer. Suitable amounts of this stock solution, diluted where necessary, were pipetted into test tubes, such that the final concentration covered the range 0·1–10·0 mM. The digestions were carried out under the assay conditions described, except that the final volume was 2·0 ml. and the incubation time was reduced to 2 hr. The enzyme source was '30–50 % fraction' (1·0 ml.).

Table 1. *Fractionation of '30–50% fraction' by ammonium sulphate precipitation at* pH 4·8 *and* 18°

(A) Phytase was assayed by incubation with 1 mM-sodium phytate for 3 hr. at pH 5·2 and 40°. (B) Acid phosphatase was assayed by incubation with 0·01 M-phenyl phosphate for 10 min. at pH 6·0 and 25°.

Fraction (% saturation)	Enzyme units/mg. of N		Enzyme units/fraction	
	(A)	(B)	(A)	(B)
35–40	606	4 138	1 305	8 904
40–45	798	8 136	823	8 387
45–50	807	18 180	507	14 160

The initial velocity of the reaction, in terms of μmoles of phytate converted/hr., was calculated and plotted against substrate concentration (Fig. 3). From a plot of $1/v$ against $1/s$ the Michaelis constant was found to be 0·15 mM.

Phytase is shown to be inhibited by higher concentrations of substrate. A similar finding has been reported for grass phytase (Perlès, 1955), which was shown to behave normally at substrate concentrations of 0·2 mM, but was completely inhibited at 4 mM. With bean phytase, inhibition is first obvious at about 2 mM and virtually absolute at about 10 mM.

Effect of calcium and magnesium ions on phytase activity. It has been reported that Ca^{2+} and Mg^{2+} ions exhibit a stimulatory effect on the phytases of wheat (Peers, 1953; Barré, Courtois & Wormser, 1956) and of *Escherichia coli* (Courtois & Manet, 1952). Results for bean phytase are given in Table 3. The phytase was incubated under the usual assay conditions for 2 hr. in the presence of various concentrations of Ca^{2+} ions added as calcium chloride. A parallel experiment was done with Mg^{2+} ions.

Both ions have a stimulating effect at concentrations of 1 μM and above. At 10 mM-Ca^{2+} ions,

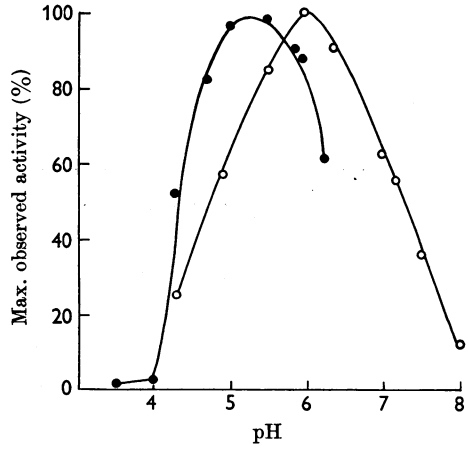

Fig. 2. pH-optimum curves for phytase and acid phosphatase. Phytase (●) was incubated for 3 hr. at 40° with 1 mM-sodium phytate. Acid phosphatase (○) was incubated for 10 min. at 25° with 0·01 M-phenyl phosphate.

Table 2. *Purification of acid phosphatase from bean cotyledons*

Fractionation stage	Enzyme units			Purification		Percentage recovery	
	Units/ml.	Units/mg. of N	Units/E_{280} unit	For stage	Overall	For stage	Overall
Crude extract	226	235	—	—	—	100	100
'30–50 % fraction'	753	1901	352	8·1	8·1	97	97
DEAE-cellulose eluate	1035	—	1238	3·5	28·3	51·2	49·6
Gel supernatant	342	—	2265	1·8	50·9	34·9	17·3

the insoluble calcium phytate is precipitated, with a corresponding decrease of phytate concentration in solution. At the same concentration the stimulatory action of Mg^{2+} ions is eliminated and slight inhibition is observed.

Effect of inhibitors on phytase activity. The effect of iodoacetamide, *p*-chloromercuribenzoate and F^- ions on phytase activity was studied, the reactions being carried out under usual assay conditions in a total volume of 2·5 ml. Inhibitors were added over the range of final concentrations indicated in Fig. 4.

Iodoacetamide appears to have no effect at these concentrations. *p*-Chloromercuribenzoate caused almost complete inhibition at 0·1 mM; F^- ions produced less inhibition at comparable concentrations.

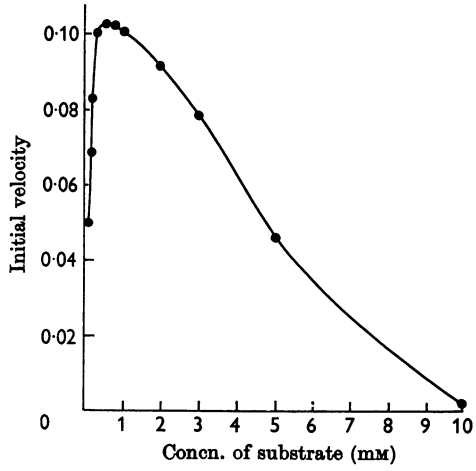

Fig. 3. Effect of substrate concentration on phytase activity. Enzyme was incubated for 2 hr. at 40° and pH 5·2 with indicated concentrations of substrate. Initial velocity (μmole of phytate converted/ml. of enzyme soln./hr.) is plotted against concentration (mM) of phytate.

Table 3. *Effect of calcium and magnesium ions on phytase activity*

Enzyme was incubated with 1 mM-sodium phytate at pH 5·2 (0·1 M-acetate buffer). Orthophosphate was determined colorimetrically after 2 hr. at 40°.

Concn. of added Ca^{2+} ions (M)	Activity as % of maximum rate	Concn. of added Mg^{2+} ions (M)	Activity as % of maximum rate
0	85	0	88
10^{-6}	91	10^{-6}	95
10^{-5}	100	10^{-5}	100
10^{-4}	99	10^{-4}	97
10^{-3}	98	10^{-3}	99
10^{-2}	Ppt. calcium phytate	10^{-2}	83

Some properties of acid phosphatase

Effect of pH. By similar methods to those described for phytase, the pH optimum of crude extracts of acid phosphatase appeared to be about pH 6·0 (Fig. 2). The preparation, purified by DEAE-cellulose chromatography and calcium phosphate-gel adsorption as described, had an optimum at pH 5·5.

Determination of K_m. The initial velocities of reaction were measured over the range 0·5–50 mM-phenyl phosphate at pH 5·5, and maximum velocity was attained at 20 mM and remained constant to 50 mM. The Michaelis constant, calculated as previously, was 1·2 mM. The acid phosphatase is thus quite distinct from the phytase.

Assay of phytase and acid phosphatase in cotyledons during germination

Samples of 20 cotyledons were taken at 3-day intervals, homogenized with 0·01 M-maleate buffer, pH 6·4, and dialysed at 2° against the buffer for 48 hr. The homogenate was adjusted to 100 ml. and portions (1 ml.) were used for assay of phytase and acid phosphatase. The results are shown in Fig. 5. Both enzymes increase in activity during the first 9 days of growth, but later show a marked decrease.

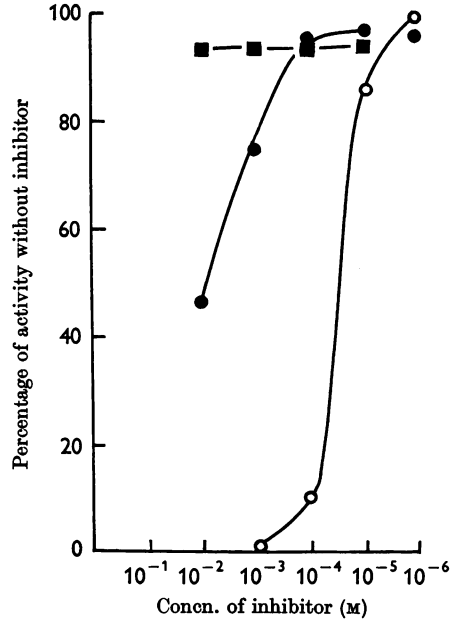

Fig. 4. Effect of inhibitors on phytase activity. Enzyme was incubated for 3 hr. at 40° and pH 5·2 with 1 mM-sodium phytate and inhibitors at indicated concentrations. ○, *p*-Chloromercuribenzoate; ●, fluoride; ■, iodoacetamide.

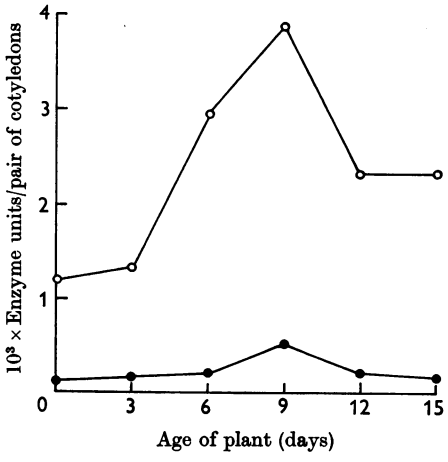

Fig. 5. Assay of phytase (●) and acid phosphatase (○) in cotyledons during germination. Whole homogenates of material were used as enzyme source. Phytase was assayed at pH 5·2 and 40° in 1 mM-sodium phytate incubated for 3 hr. Acid phosphatase was assayed at pH 6·0 and 25° in 0·01 M-phenyl phosphate incubated for 10 min.

DISCUSSION

Many attempts have been made to establish the distinction or otherwise of phytase from other phosphatases, and the existence of a specific phytase has sometimes been considered doubtful (Luers & Silbereisen, 1927; Horiuchi, 1931; Carandante, 1944; Courtois, 1947*a*, *b*; Yoshida, 1950). Courtois (1945) introduced the term 'phytophosphatase' to indicate an enzyme activity towards several orthophosphate esters, including phytate. The phytase preparation reported in the present paper was active towards phytate and phenyl phosphate, whereas the acid phosphatase was active only towards the latter compound, and showed no phytase activity even at high concentration. The pH optima of the two activities were distinct: pH 5·2 for phytase and pH 6·0 for acid phosphatase in the '30–50% fraction', although the latter shifted to pH 5·5 on purification.

The inhibition of phytase by high substrate concentrations indicates that a two-point attachment of the phytate to the enzyme may be operating and it is interesting to speculate to what extent this inhibition exerts a controlling influence on the phosphate metabolism of the young plant. Acid phosphatase did not show substrate inhibition up to 50 mM-phenyl phosphate.

The increase in phytase and acid-phosphatase activities in the cotyledons during the early stages of growth reflects the increase in metabolic activity of the plant at this stage. Later, when the cotyledonary reserves are becoming exhausted, the activities fall off, and probably internal disintegration accounts for some of this decrease.

SUMMARY

1. Some properties of a phytase preparation from *Phaseolus vulgaris* are reported. The enzyme has a pH optimum of 5·2, is inhibited by high substrate concentrations, and by *p*-chloromercuribenzoate (95% at 0·1 mM) and F^- ions (55% at 10 mM) but not by iodoacetamide up to a concentration of 10 mM. It has K_m 0·15 mM.

2. The acid phosphatase, present in the phytase preparations, has been separated from the latter enzyme by diethylaminoethylcellulose chromatography and purified 50-fold. This enzyme has a pH optimum at 6·0 in crude preparation, and at 5·5 after purification. The K_m is 1·2 mM with phenyl phosphates substrate, and the enzyme does not have phytase activity.

3. Phytase and acid phosphatase have been assayed in the cotyledons of *P. vulgaris* during germination. Both enzymes increase in activity during the early stages, but later decrease.

L.N.G. thanks the Department of Scientific and Industrial Research for a Research Studentship.

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