The Hydrolysis of Glucose Monophosphates by a Phosphatase Preparation from Pea Seeds

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Although many phosphatase preparations have been obtained from plant tissues there are few data available on the breakdown of glucose monophosphates, and of glucose 1-phosphate in particular. In the work of Porter (1953), Spencer (1954) and Roberts (1956), it was found that glucose 1-phosphate was hydrolysed by extracts from potato tubers, tomato leaves and wheat leaves respectively; however, more information is desirable on whether glucose 1-phosphate was hydrolysed directly, or through the intermediate formation of glucose 6-phosphate by phosphoglucomutase and the subsequent hydrolysis of the 6-ester. Axelrod (1947) found that glucose 1-phosphate was not hydrolysed by the acid phosphatase of citrus fruit although other phosphoric compounds such as fructose 1:6-diphosphate were hydrolysed.

With extracts from some animal tissues it has been shown that the hydrolysis of glucose 1-phosphate may take place via glucose 6-phosphate. Broh-Kahn & Mirsky (1948) could find no evidence for the existence of a glucose 1-phosphatase in liver extracts, and Goodlad & Mills (1957) concluded that the main route of glucose 1-phosphate hydrolysis in rat liver is through a preliminary conversion into glucose 6-phosphate. The direct hydrolysis of glucose 1-phosphate by a hexose 1-phosphatase from silkworm blood was reported by Faulkner (1955); this extract did not attack glucose 6-phosphate. Morton (1955) found that purified alkaline phosphatases from cow's milk and calf intestinal mucosa hydrolysed both glucose 1-phosphate and glucose 6-phosphate.

Previous investigations (Turner & Turner, 1957) had indicated the presence of glucose monophosphatase activity in extracts from pea seeds. When glucose 1-phosphate was incubated with these extracts, glucose and inorganic phosphate were formed. However, further work was needed to establish that this hydrolysis did not proceed by way of glucose 6-phosphate.

In the present investigation the acid phosphatase from pea seeds was purified 20-fold and a number of its properties studied. Both glucose 1-phosphate and glucose 6-phosphate were hydrolysed directly although the rate of hydrolysis of glucose 6-phos-

phate was higher than that of glucose 1-phosphate. Inorganic phosphate acted as a competitive inhibitor of the phosphatase action.

MATERIALS AND METHODS

Substrates

Glucose 1-phosphate (G 1-P) was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill. Fructose 6-phosphate (Neuberg, Lustig & Rothenberg, 1943) and fructose 1:6-diphosphate (Neuberg & Lustig, 1942) were also prepared in the laboratory. Glucose 6-phosphate (G 6-P) and 3-phosphoglycerate were obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., galactose 1-phosphate and *p*-nitrophenyl phosphate from Sigma Chemical Co., St Louis, Mo., U.S.A., and adenosine monophosphate and adenosine triphosphate from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Preparation of enzyme extracts

Pea-seed acid phosphatase. Immature pea seeds (*Pisum sativum* L., var. 99L) (40 g.) were blended with 40 ml. of glass-distilled water at 4°. The mixture was centrifuged at 1000 g for 10 min. and the supernatant centrifuged at 20 000 g for 20 min. at room temperature. Saturated (NH₄)₂SO₄, pH 6.0, was added to the clear supernatant at 4°, and the fraction precipitating between 55 and 75% saturation was suspended in 7 ml. of water and dialysed with rocking against glass-distilled water for 2.5 hr. at 4°.

The slightly turbid phosphatase extract could be stored at -15° for 2 months with little loss of activity. The preparation contained approx. 4.9 mg. of protein/ml. and was used for all the studies reported except for those on the substrate specificity.

Purified pea-seed acid phosphatase. The dialysed extract (pH 5.8) was brought to pH 3.6 by the addition of 0.1 N-HCl, centrifuged and the clear supernatant adjusted to pH 5.8 with 0.1 N-NaOH. This was then stirred with 0.1 vol. of calcium phosphate gel (Keilin & Hartree, 1938) containing 10 mg. of Ca₃(PO₄)₂/ml. at room temperature for 15 min. and centrifuged. The precipitate was stirred with 0.15 M-phosphate buffer (KH₂PO₄-NaOH), pH 7.0, for 15 min., centrifuged and the precipitate again stirred with phosphate buffer. The combined supernatants were dialysed against glass-distilled water for 3.5 hr. at 4°. The preparation contained 0.89 mg. of protein/ml. and was used for the study of the substrate specificity of the preparation.

The purification effected by these treatments is shown in Table 1. There was no significant change in the ratio of glucose 6-phosphatase activity to glucose 1-phosphatase

Table 1. *Purification of pea-seed acid phosphatase*

Reaction mixtures were of the composition described for the assay of phosphatase activity. The amount of enzyme fraction added was adjusted so that not more than 10% hydrolysis of the substrate occurred in the reaction time.

Fraction no.	Treatment	Total protein content of fraction (mg.)	Phosphatase activity (μ m-moles of phosphate liberated/mg. of protein/min.)		Ratio: glucose 6-phosphatase / glucose 1-phosphatase
			G 1-P	G 6-P	
I	Crude extract dialysed	429	5.4	31.5	5.8
II	(NH ₄) ₂ SO ₄ precipitation, dialysis	40.3	52.6	324.0	6.2
III	Fraction II adjusted to pH 3.6, supernatant adjusted to pH 5.8	23.4	72.6	464.5	6.4
IV	Fraction III adsorbed and eluted from Ca ₃ (PO ₄) ₂ gel, dialysed	8.98	100.0	637.0	6.4

activity during the course of the purification procedure. The glucose 1-phosphatase was purified 18-fold and the glucose 6-phosphatase 20-fold.

Pea-seed phosphoglucomutase. A crude extract from dried pea seeds was prepared by the method of Turner (1957) and centrifuged at 20 000 *g* for 20 min. at room temperature. The supernatant was fractionated by addition of saturated (NH₄)₂SO₄, pH 7.0; the precipitate was collected between 55 and 80% saturation, dissolved in water and dialysed against 25 mm-phosphate buffer (KH₂PO₄-NaOH), pH 7.0, at 4° for 2.5 hr.

Enzyme digests

The reaction mixtures were maintained at 30°.

Acid phosphatase activity. For the assay of phosphatase, the enzyme (containing approx. 0.25 mg. of protein) was incubated with 2-amino-2-hydroxymethylpropane-1:3-diol (tris; 0.75 m-mole)-acetic acid buffer, pH 5.4, in a volume of 14.5 ml., and the reaction was started by the addition of 7.5 μ moles of substrate in 0.5 ml. of water. The reaction time was 60 min. with G 1-P as substrate and 10 min. with G 6-P. The amount of enzyme preparation added was adjusted to give not more than 10% hydrolysis of the substrate in these times; under these conditions the rate of reaction was approximately constant during the incubation period. For the study of the effect of various inhibitors, the enzyme was incubated in 14.5 ml. of buffer containing the inhibitor for 15 min. at 30°, and the reaction started by the addition of substrate.

In the determination of phosphatase activity the reaction mixtures were inactivated by the addition of 0.1 vol. of 40% (w/v) trichloroacetic acid, and samples were used for the estimation of inorganic phosphate. Centrifuging was not necessary except where crude extracts were used. In the study of phosphoglucomutase, 6 vol. of 6% (w/v) trichloroacetic acid was added to the reaction mixtures, which were centrifuged and samples taken for the estimation of acid-labile and inorganic phosphate. Trichloroacetic acid extracts of the reaction mixtures were kept at 0° and manipulations carried out without delay; under these conditions there was no detectable hydrolysis of G 1-P.

Analytical methods

Determination of inorganic orthophosphate. In preliminary studies and in some experiments with inhibitors,

Table 2. *Phosphoglucomutase activity in extract from dried pea seeds*

Reaction mixtures contained 30 μ moles of G 1-P, tris (75 μ moles)-acetic acid buffer of required pH, MgCl₂ added as indicated and 0.5 ml. of pea-seed phosphoglucomutase preparation (containing 13.9 mg. of protein); total volume, 0.9 ml. Incubation time, 60 min. Temp. 30°.

pH	Final concn. of MgCl ₂ (mm)	Decrease in acid-labile phosphate (μ moles/reaction mixture)
5.4	0	0.5
5.4	1	1.3
7.5	0	5.3
7.5	1	14.7

inorganic phosphate was estimated by the method of Allen (1940) as modified by Turner (1957). For other determinations, the method of Lowry & Lopez (1946) was used.

Acid-labile phosphate. This was determined by measuring the inorganic phosphate released in 7 min. in *N*-HCl at 100°, by the method of Allen (1940).

Detection of glucose. Glucose in the reaction mixtures was demonstrated on paper chromatograms by the methods of Turner, Turner & Lee (1957).

Determination of protein. The protein content of the enzyme preparations was estimated by the spectrophotometric method of Warburg & Christian (1941).

RESULTS

Some properties of the phosphoglucomutase from dried pea seeds

When G 1-P was incubated with the phosphoglucomutase preparation at pH 5.4 and 7.5 in reaction mixtures of the composition described in Table 2, in the presence and absence of soluble starch (final concn. 0.13%), there was no change in inorganic phosphate. This indicated that the preparation contained no starch phosphorylase and showed little phosphatase activity. It is probable

that starch phosphorylase was eliminated in the $(\text{NH}_4)_2\text{SO}_4$ fractionation and that phosphatase activity was suppressed by the high inorganic phosphate level in the reaction mixtures because of dialysis of the enzyme preparation against phosphate buffer.

Phosphoglucumutase activity, as measured by decrease in acid-labile phosphate, is shown in Table 2. The activity at pH 7.5 was over ten times that at pH 5.4. The addition of MgCl_2 (final concn. mM) increased the phosphoglucumutase activity approximately 2.5-fold.

Some properties of the phosphatase from pea seeds

The formation of inorganic phosphate from G 1-P and G 6-P on incubation with pea-seed phosphatase is shown in Fig. 1. G 6-P was hydrolysed at a considerably faster rate than G 1-P.

When trichloroacetic acid extracts of the reaction mixtures were chromatographed on paper, visual examination showed that glucose was the only free sugar present. There was no decrease in the sum of acid-labile phosphate plus inorganic phosphate during the hydrolysis of G 1-P, and there was no increase in inorganic phosphate in the presence of ammonium molybdate (mM) and starch (0.13%). This concentration of molybdate completely inhibited phosphatase activity and thus would allow the detection of starch phosphorylase activity.

Effect of pH on phosphatase activity. The effect of pH on the hydrolysis of both G 1-P and G 6-P is shown in Fig. 2. The curves are similar, but G 6-P had optimum pH 5.4–5.7, whereas G 1-P had a more marked optimum at pH 5.4. With increase in

pH the rate of reaction decreased rapidly until, at pH 7.6, the rate was negligible. The addition of MgCl_2 (final concn. mM) to the reaction mixtures at both pH 5.4 and 7.5 had no effect on the rate of hydrolysis of either G 1-P or G 6-P. There was no

Fig. 2. Effect of pH on the rate of hydrolysis of G 1-P and G 6-P. Composition of the reaction mixtures was that described for the assay of phosphatase. Reactions were studied in tris (0.75 m-mole)-acetic acid buffers of pH indicated. Temp. 30°. ○, G 1-P, incubation time 60 min.; ●, G 6-P, incubation time 10 min.

Fig. 1. Hydrolysis of G 1-P (○) and G 6-P (●) by pea-seed phosphatase. Reaction mixtures (total vol. 45 ml.) contained 22.5 μmoles of G 1-P (or G 6-P), tris (2.25 m-moles)-acetic acid buffer, pH 5.4, and 1.5 ml. of enzyme (containing 1.5 mg. of protein). Temp. 30°.

Fig. 3. Lineweaver-Burk plots [reciprocal of the initial rate of hydrolysis, $1/V$ (μmoles of inorganic phosphate/reaction mixture), against the reciprocal of the substrate concentration, $1/[S]$ (m-moles/l.)] for pea-seed phosphatase with G 1-P at two inorganic phosphate concentrations. The reaction mixtures (total vol. 15 ml.) contained G 1-P, tris (0.75 m-mole)-acetic acid buffer, pH 5.4, inorganic phosphate in concentrations given below and 0.5 ml. of enzyme (containing 0.25 mg. of protein). Temp. 30°. Incubation time 60 min. ○, No orthophosphate; Δ, 75 μM- KH_2PO_4 ; ▲, 150 μM- KH_2PO_4 .

Table 3. *Effect of inhibitors on phosphatase activity*

Reaction mixtures were of the composition described for the assay of phosphatase with the addition of inhibitors as shown. Temp. 30°.

Inhibitor	Final concn. (mm)	Inhibition (%)	
		Substrate G 1-P (incubation time 60 min.)	Substrate G 6-P (incubation time 10 min.)
Glucose	2.0	0	0
Inorganic phosphate	0.075	36	19
Inorganic phosphate	0.15	52	29
Iodoacetate	10.0	69	75
HgCl ₂	0.2	74	73
Fluoride	2.0	75	78
Fluoride	20.0	100	100
Molybdate	1.0	100	100

Fig. 4. Lineweaver-Burk plots [reciprocal of the initial rate of hydrolysis, V (μ moles of inorganic phosphate/reaction mixture), against the reciprocal of the substrate concentration, $[S]$ (m-moles/l.)] for pea-seed phosphatase with G 6-P at two inorganic phosphate concentrations. The reaction mixtures (total vol. 15 ml.) contained G 6-P, tris (0.75 m-mole)-acetic acid buffer, pH 5.4, inorganic phosphate in concentrations given below and 0.5 ml. of enzyme (containing 0.038 mg. of protein). Temp. 30°. Incubation time 60 min. ●, No orthophosphate; Δ , 75 μ M-KH₂PO₄; $\blacktriangle$, 150 μ M-KH₂PO₄.

Table 4. *Hydrolysis of various substrates by purified pea-seed phosphatase*

Reaction mixtures contained 7.5 μ moles of substrate, tris (0.75 m-mole)-acetic acid buffer, pH 5.4, and purified pea-seed phosphatase (containing 0.06 mg. of protein); total volume, 15 ml. Incubation time was from 3 to 120 min., so that not more than 10% of substrate was hydrolysed. Temp. 30°.

Substrate	Relative rate of hydrolysis
G 1-P	1
G 6-P	6
Galactose 1-phosphate	1
Fructose 6-phosphate	8
<i>p</i> -Nitrophenyl phosphate	38
3-Phosphoglycerate	32
Adenosine monophosphate	2
Adenosine triphosphate	35
Fructose 1:6-diphosphate	15

proceed for longer periods suggested that the decline in rate was not predominantly due to substrate depletion. These observations indicated that products of the reaction may have been inhibiting phosphatase activity. The effects of addition of glucose and inorganic phosphate to the reaction mixtures are shown in Table 3. Glucose had no effect on phosphatase action whereas inorganic phosphate in low concentration inhibited the breakdown of both G 1-P and G 6-P.

The effects of two concentrations of inorganic phosphate on the rates of reaction with varying concentrations of G 1-P and G 6-P were examined, and in Figs. 3 and 4 the data are plotted according to the method of Lineweaver & Burk (1934). Inorganic phosphate was a competitive inhibitor of both glucose 1-phosphatase and glucose 6-phosphatase activities.

Effect of other inhibitors on phosphatase activity. The effects of addition of several other inhibitors are also shown in Table 3. Molybdate, iodoacetate, HgCl₂ and fluoride all inhibited the phosphatase activity. In separate experiments it was found that ethylenediaminetetra-acetate (final concn.

detectable decrease in the sum of acid-labile phosphate plus inorganic phosphate in any of the reaction mixtures.

Effect of concentration of substrates. The effect of concentration of G 1-P and G 6-P on the initial reaction velocity is shown in Figs. 3 and 4. The relative rate of hydrolysis of G 1-P to that of G 6-P increased with increasing substrate concentration.

Inhibition of phosphatase by inorganic phosphate. Preliminary experiments showed that the rate of reaction fell markedly as the reaction proceeded. Indications of this may be seen in Fig. 1, and experiments in which the reaction was allowed to

10 mM) stimulated the phosphatase activity by approx. 20%.

Specificity of the phosphatase preparation. The action of the purified pea-seed phosphatase preparation on a number of phosphoric compounds is shown in Table 4. The enzyme preparation attacked a wide range of substrates and the hydrolysis of adenosine triphosphate, *p*-nitrophenyl phosphate, 3-phosphoglycerate and fructose 1:6-diphosphate was more rapid than that of the glucose monophosphates.

DISCUSSION

The present investigation has shown that both G 1-P and G 6-P are hydrolysed directly by the pea-seed phosphatase preparation. The lack of accumulation of acid-stable phosphate during the hydrolysis of G 1-P indicated that, if hydrolysis took place through G 6-P, phosphoglucomutase rather than phosphatase must be the rate-limiting step. The observations that the optimum pH for the hydrolysis of G 1-P (like that for G 6-P) was 5.4 whereas the activity of pea-seed phosphoglucomutase at pH 7.5 was approximately ten times that at pH 5.4 eliminated the possibility of hydrolysis via G 6-P. Additional evidence was provided by the addition of magnesium chloride, which markedly stimulated the action of pea-seed phosphoglucomutase but had no effect on the phosphatase. Cardini (1952) found that the phosphoglucomutase of the jack-bean had an optimum pH of 7.5 and also required Mg^{2+} ions. There was no inhibition of phosphatase activity by ethylenediaminetetra-acetate.

It is not known if two separate enzymes were responsible for the hydrolysis of G 1-P and G 6-P. There was no indication of any separation of the two activities during the purification procedures. The effects of various inhibitors on the hydrolysis of the two glucose monophosphates by the pea-seed preparations were similar.

Inorganic orthophosphate was a competitive inhibitor for the hydrolysis of G 1-P and G 6-P by the pea-seed phosphatase. An inhibition of this type may have been partly responsible for observations of low phosphatase activity in the presence of high starch-phosphorylase activity (Porter, 1953; Turner & Turner, 1957). Inhibition of phosphatase action by inorganic phosphate was observed by Jacobsen (1932, 1933), who concluded that the rate of hydrolysis of glycerophosphate by alkaline kidney phosphatase depended on the ratio of substrate to inorganic phosphate. Pfankuch (1936) found that the hydrolysis of α - and β -glycerophosphate, fructose 1:6-diphosphate, phosphoglycerate and phytate by potato phosphatase was inhibited by inorganic phosphate. Inhibition of calf intestinal mucosa alkaline phosphatase by

disodium phosphate was observed by Morton (1955).

It is possible that inhibition of phosphatase by inorganic phosphate may have some metabolic significance because of the important role of inorganic phosphate in a number of processes. It has been suggested that a lowered concentration of inorganic phosphate may cause the inhibition of glycolysis by oxygen through the glyceraldehyde 3-phosphate dehydrogenase reaction (Johnson, 1941; Lynen, 1941); Lardy & Wellman (1952) have shown that inorganic phosphate may limit the rate of oxidation of respiratory substrates by mitochondria. If the concentration of inorganic phosphate fell to a low level (which might, for example, inhibit glycolysis) there would be a tendency for the glucose monophosphates (and possibly the other phosphoric compounds) to be hydrolysed and so increase the inorganic phosphate concentration. Conversely, if the inorganic phosphate concentration were increased (through anaerobiosis for example) there may tend to be a sparing effect on the phosphoric compounds due to inhibition of phosphatase action by the higher concentration of inorganic phosphate. The phosphatase may exert a balancing effect and so tend to stabilize the concentration of inorganic phosphate. An effect such as this would be analogous to the 'primitive' control mechanisms (i.e. mechanisms not controlled by hormones or a central nervous system) described by Krebs (1957).

SUMMARY

1. A phosphatase from pea seeds that hydrolysed both glucose 1-phosphate and glucose 6-phosphate was purified approximately 20-fold.
2. Glucose 1-phosphate was hydrolysed directly and not by way of glucose 6-phosphate.
3. The effects of pH, substrate concentration and inhibitors on the hydrolysis of the glucose monophosphates were studied.
4. The hydrolysis of other phosphoric compounds by the enzyme preparation was examined.
5. Low concentrations of inorganic phosphate competitively inhibited the hydrolysis of both glucose 1-phosphate and glucose 6-phosphate. The possible metabolic significance of this inhibition has been discussed.

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The Preparation of 2-Guanidinoethyl Phosphate

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During the isolation of lombricine from *Lumbricus terrestris* L. (Pant, 1959), it was observed that when the concentrated ammoniacal eluate from the $C_{50}D$ cation column was kept in the refrigerator for 4–5 days, a white crystalline solid separated out. This compound on being purified was identified chromatographically as guanidinoethyl phosphate, which is one of the products of hydrolysis of lombricine (Van Thoai & Robin, 1954). Our repeated attempts to prepare this compound by the only available method described by Van Thoai & Robin (1954) proved unsuccessful. However, the recent synthesis of monophosphotaurocyamine by Morrison, Ennor & Griffiths (1958) suggested to us the possibility of its synthesis by a similar procedure.

This paper deals with the details of the preparation of guanidinoethyl phosphate and the study of some of its properties.

EXPERIMENTAL

Paper chromatography. Paper partition chromatography of Consden, Gordon & Martin (1944) was employed. Whatman no. 1 filter-paper sheets were used. The spots were developed at room temperature (22°) for about 7–8 hr. and the solvent was allowed to ascend a distance of about 23–25 cm. The operation was conducted in an air-tight glass-aquarium tank fitted with a lid. The atmosphere of the tank was kept saturated with the vapours of the solvent.

Spray reagents. The following were used: ninhydrin solution (0.1%, w/v) in butanol; Sakaguchi reagents (Pant, 1959) and molybdate reagents (Hanes & Isherwood, 1949).

Preparation of 2-aminoethanol 1-phosphate. This ester was prepared by treating phosphoryl chloride with aq. 2-aminoethanol as described by Outhouse (1937). To phosphoryl chloride (15 ml.) placed in a 500 ml. (Büchner) flask and cooled by immersion in a salt-ice mixture (–5°) an ice-cold mixture of 2-aminoethanol (10 ml., 1 mol.) and water (5 ml., 2 mol.) was added dropwise from a burette. The reaction proceeded with evolution of heat and copious fumes of HCl. When the reaction was complete, the flask was removed from the cold bath and evacuated with a water suction pump at room temperature for about 4 hr. until almost all the HCl formed had been removed. Water (500 ml.) was then added to the reaction mixture with stirring until the gummy mass formed was completely dissolved. The solution was made alkaline to pH 10.0 by addition of hot saturated baryta solution (approx. 400 ml.). The precipitated barium phosphate was centrifuged, washed twice with water (50 ml.), the washings were combined with the first supernatant and the volume was reduced to 50 ml. on a water bath. The solution was cooled and ethanol (80 ml.) was added in small quantities to precipitate the barium 2-aminoethanol 1-phosphate; it was then left at 0° for 2–3 hr. The white crystalline solid (8 g.) was centrifuged off and twice washed with ethanol (30 ml.) and ether and dried. The dry barium salt was recrystallized from water with ethanol and dried *in vacuo*. Yield, 7.5 g.

In order to obtain 2-aminoethanol 1-phosphate, the barium salt was dissolved in water (80 ml.) and H_2SO_4 (60 ml., 0.5N) was added to it until all the barium was completely precipitated as barium sulphate. The precipitate was centrifuged off, twice washed with water (25 ml.), the washings were combined with the original supernatant and the solution was concentrated on a water bath to 50 ml. The concentrate was filtered, made turbid by adding methanol (45 ml.) and left in the refrigerator overnight.