Phytochemistry, 1976. Vol. 15, pp. 887–892. Pergamon Press. Printed in England.

STARCH PHOSPHORYLASE ENZYMES IN DEVELOPING AND GERMINATING PEA SEEDS

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(*Received* 15 *November* 1975)

Key Word Index—*Pisum sativum*; Leguminosae; peas; starch phosphorylase; germination; seed development.

Abstract—Phosphorylase has been fractionated during development and germination of seeds of smooth and wrinkled-seeded peas. The total phosphorylase levels have been compared. In addition, a number of other pea tissues and other legumes have been examined. Some kinetic properties of the two enzymes present have been measured. Both enzymes have been further purified by affinity chromatography on Sepharose 4B-starch columns and by sequential gel filtration in the absence and presence of amylopectin. The MW and sub-unit structures of the two enzymes have been examined and their possible roles discussed.

INTRODUCTION

Multiple forms of starch phosphorylase (EC 2.4.1.1) have been detected in a range of plant tissues by a variety of separation techniques [1–13], including ion-exchange chromatography and gel electrophoresis. In an extensive examination of maize phosphorylase, chromatography on DEAE-cellulose showed the presence of two forms in the endosperm, one form in the embryo and another that was found in both parts of the seed. These various forms showed different primer requirements and all were inhibited by purine nucleotides [1–3]. Mistletoe leaves contained two phosphorylases which could be separated by DEAE-cellulose chromatography and the effects of nucleotides, metals, sugars, sugar phosphates and phenols on the activities of the two forms were examined [4]. In developing barley grains, two phosphorylases were separated chromatographically and the changes in the levels of primed and unprimed activities in endosperm extracts followed. The effects of nucleotides on activity were also studied [12]. Extracts of potato tubers gave a more complex pattern. Polyacrylamide gel electrophoresis gave nine bands and the MW of four of these were estimated as 1.8, 3.2, 5.2 and 6.0 × 10^5. However, gel chromatography on Sephadex G-200 produced a single retarded peak. Three fractions were eluted from DEAE-cellulose. Two fractions, which were separated by initial adsorption on starch granules and elution at pH 9, followed by DEAE-cellulose chromatography, showed different stimulation with primers [9–11]. In banana fruits, DEAE-cellulose chromatography gave multiple forms, two from the immature and three from the mature fruit. Inhibition studies in the presence of tyrosine and ATP showed a sigmoidal Glc-1-P saturation curve [7].

Total phosphorylase activity in pea seeds increases during development [14]. The levels also increase on germination and apparent differences have been found in different cultivars in the amount of increase during germination. Examination of the protein extract of germinating peas by polyacrylamide gel electrophoresis has indicated two bands of phosphorylase activity [8, 15].

In this paper, the changes in levels of phosphorylase in the cotyledons of two pea varieties (smooth-seeded, normal amylose and wrinkle-seeded, high amylose) as well as other pea tissues and some other legumes have been compared. The phosphorylase fractions have been purified and the MW determined. Some kinetic properties of the separated forms have been examined.

RESULTS AND DISCUSSION

After extraction, $(NH_4)_2SO_4$ precipitation and dialysis, extracts from the cotyledons of the two pea varieties (*Pisum sativum* cv Alaska, smooth-seeded and cv. Victory freezer, wrinkle-seeded) were chromatographed on DEAE-cellulose with a gradient to 60 mM citrate followed by a second gradient to 0.5 M NaCl in 60 mM citrate. The eluate was assayed for phosphatase activity with *p*-nitrophenyl phosphate and for phosphorylase by the release of inorganic phosphate and the formation of iodine staining glucan on incubation with Glc-1-P and primer. Two phosphorylase fractions (Fig. 1) that were free of phosphatase were separated from each cultivar and these re-chromatographed as single peaks at the same elution volume. Separation from a large amount of the total protein was also obtained. The two fractions were called phosphorylases I and II from their order of elution, after the convention of Tsai and Nelson [1]. For quantitative estimation of phosphorylase levels at various stages of development and times after imbibition, the fractions from the peaks were combined and assayed in the direction of synthesis by the determination of inorganic phosphate released. In Fig. 2 the changes in total phosphorylase (I plus II) are shown. During development, the phosphorylase level in both varieties increased and then decreased as described previously [14]. Total phosphorylase also increased during germination but the increase after imbibition was many times that during development. Previously, in separate studies [8, 15] quanti-

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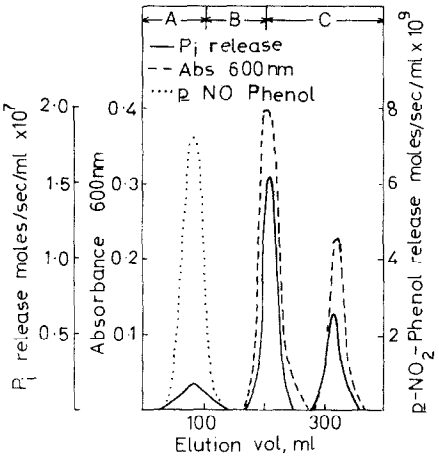


Fig. 1. Chromatography on DEAE cellulose of pea seed extracts. A—sample + wash; B—citrate gradient (20–60 mM); C—NaCl gradient (0–0.5 M).

tative differences in the amount of increase on germination in wrinkle-seeded (Fullpod) and smooth-seeded (Alaska) varieties had been found. This comparative study confirms these differences. Other pea tissues (Victory freezer) showed very much lower levels of total phosphorylase activities. The values for rapidly expanding leaves, fully expanded leaves, roots and etiolated shoots were 40, 20, 10 and 2 nkats per g wet wt respectively. These can be compared with typical values for maturing and germinating cotyledons of 270 and 3100. In developing lupin cotyledons, a non-starch storing

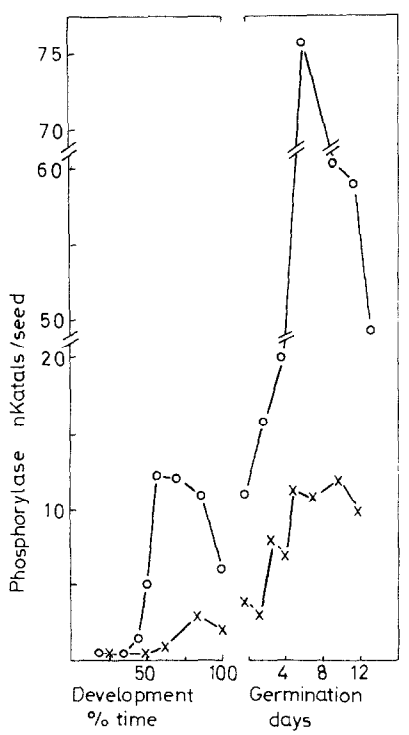


Fig. 2. Changes in total phosphorylase activities during development and germination of pea seeds. ×—Alaska (average wt of 1 seed, 187 mg); ○—Victory freezer (average wt of 1 seed, 250 mg).

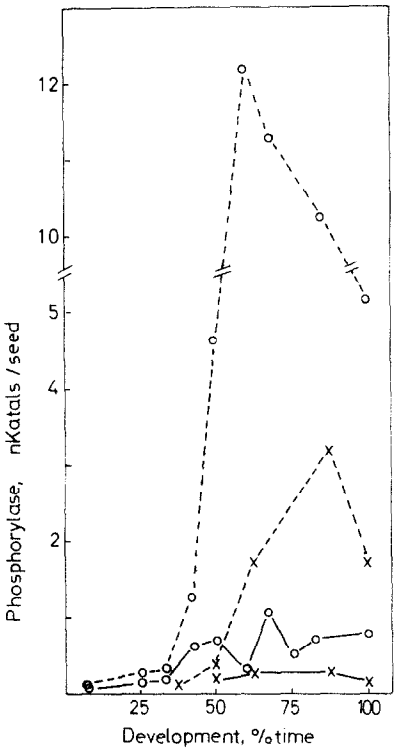


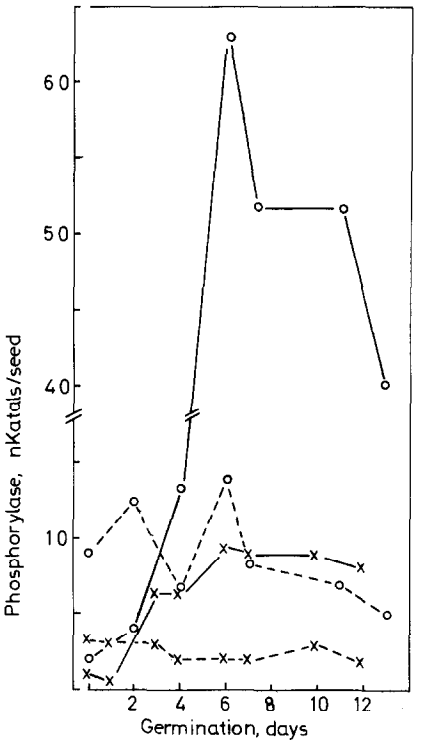
Fig. 3. Changes in the two phosphorylase levels during the development of pea seeds. × Alaska: ○ Victory freezer; —— phosphorylase I: – – – phosphorylase II.

legume seed, the levels were very much lower and constant, generally less than 5 nkats per g wet wt.

In Figs. 3 and 4 the quantitative changes in levels of phosphorylases I and II in both pea cultivars during development and germination are shown. The time to maturity of Alaska peas was only 8 weeks compared with 12 weeks for Victory freezer. The development stage is associated with low, relatively constant levels of phosphorylase I and higher and increasing amounts of II. The final level of enzyme II in Victory freezer was about twice that of Alaska. On germination, phosphorylase II remains relatively constant in both varieties but I increases rapidly and many fold. The increase in Victory freezer is about 7 times that in Alaska although the percentage dry wt content of the starch in Alaska is higher. This large increase in phosphorylase I on germination, when starch is being depleted suggests that at least this form is associated with starch degradation. Amylases could also degrade starch during germination but in peas these increase in quantity later than phosphorylase [8, 15].

Both of the forms found in cotyledons are present in rapidly expanding leaves, roots and etiolated shoots of peas in *ca* equal but low amounts. In fully expanded leaves the amounts are also low but there is about three times as much of enzyme II as of I. The presence of both enzymes in roots and etiolated shoots eliminates the possibility that either is associated exclusively with photosynthetically active tissue or the starch storage and depletion of seeds.

Two phosphorylase fractions have been reported in developing *Vicia faba* seeds [6]. To compare these with those in peas, *V. faba* was germinated and extracted. Two



g. 4. Changes in the two phosphorylase levels during the mination of pea seeds. ×—Alaska; ○—Victory freezer; —— phosphorylase I; --- phosphorylase II.

zymes were found in the ungerminated and germinat- g cotyledons and their chromatographic behaviour on EAE-cellulose was similar to the pea enzymes. Some antitative differences in levels of activity were found. ungerminated seeds the total phosphorylase was twice high as peas and like peas there was more phosphory- e II than I. However, on germination there was little ange in the amounts of either enzyme.

Some kinetic properties of the two phosphorylases om Victory freezer peas were examined following ther purification. After DEAE-cellulose chromatogra- y of an extract from germinating cotyledons, phos- orylase I had undergone a 14 fold purification and d a sp. act. of 14 nkats per mg protein. The recovery activity relative to the initial extract was 70%. Phos- orylase II was prepared by DEAE-cellulose chromato- aphy from mature seeds, giving a 24 fold purification, specific activity of 7.1 nkats per mg protein and a re- very of 61%. Both enzymes were further purified using o-gel A 1.5 (8% agarose) for phosphorylase I and pharose 6B for phosphorylase II. The sp. act. of phos- orylase I was 19 nkats per mg protein and the recov- y on the gel filtration step 53%. For phosphorylase the values were 16 nkats per mg and 70%.

Measuring in the direction of glucan synthesis, the K_m lues for phosphorylase I and II, for Glc-1-P in the esence of saturating amounts of glucan primer were and 3 mM Glc-1-P. The energy of activation of this nthetic reaction as catalysed by phosphorylase I was .3 kcal and by II 8.7 kcal. At saturating levels of lc-1-P, small inhibitions were found for both enzymes ith a variety of nucleotides, applied at concentrations om 1.7 to 6.7 mM. Similar behaviour was shown with the glycolytic intermediates Fru 1:6 di-P, phospho-enol pyruvate and 3-phosphoglyceric acid. Phenylalanine and tyrosone at 2.5 mM and 0.25 mM respectively were without effect. With phosphorylase I, AMP, ADP, ATP and ADPG all showed competitive inhibition. Assayed in the direction of glucan degradation by a coupled enzyme assay (phosphoglucomutase, Glc-6-P dehydrogenase—NADP) the K_m values with respect to inorganic phosphate were 14 mM for both enzymes and with respect to soluble starch 0.004%.

When the K_m for glucan substrates was measured in the direction of synthesis, using commercial Glc-1-P, both enzymes were self-priming after a lag period. Different rates of synthesis with various glucan primers in the presence of saturating amounts of Glc-1-P (50 mM) were found and these are shown in Table 1. Using maltotriose, the plot of phosphate released with time by phosphorylase I was identical with the no-substrate curve, showing a difference to enzyme II, which could use maltotriose as a substrate. If phosphorylase II functions as a synthetic enzyme during seed development, this difference could be significant.

The two enzymes showed different behaviour on freezing and thawing. Phosphorylase I retained 88% of activity, whereas phosphorylase II retained 11%. After refreezing and thawing three times, I still had 58% of the original activity but II had only 1%. Both enzymes slowly lost activity at a similar rate on storage at 4° (half life *ca* 11 days). The pH optima of the two enzymes are the same (pH 6). The values for half maximal activity were 5.2 and 7.6 for phosphorylase I and 5.2 and 8.0 for II. Both enzymes were completely and irreversibly inactivated by decreasing the pH to 5. Instability of potato phosphroylase at pH 5.5 has been noted [16].

Two additional techniques were used in further purification of the two phosphorylases to estimate MW. One procedure was to chromatograph on a gel which included the activity (8% agarose for I and 6% for II) and then re-chromatograph the activity on the same gel in the presence of amylopectin (10 parts to 1 part protein). The amylopectin and the activity were then excluded and separated from other protein which re-chromatographed with phosphorylase in the absence of amylopectin. A similar method has been used with yeast glycogen synthetase [17]. When applied to phosphorylase I after separation from II on DEAE-cellulose, a 16 fold purification to a sp. act. of 230 nkats per mg protein with a recovery of 44% was obtained. The values for phosphorylase II were 9 fold, 67 nkats and 46%. Phosphorylase activity was separated from amylopectin by chromatography

Table 1. Glucan synthesis by pea phosphorylases from glucan primers in the presence of adequate Glc-1-P

Substrate	K_m (% glucan)		Velocity relative to amylopectin = 100	
	Phospho-rylase I	Phospho-rylase II	Phospho-rylase I	Phospho-rylase II
Maltotriose	*	0.005	0*	95
Achroic dextrin	n.d.	n.d.	81	70
Soluble starch	0.0025	0.0035	97	103
Glycogen	0.001	0.013	100	65
Amylopectin	n.d.	n.d.	100	100

* See text.

again on DEAE cellulose when the amylopectin was not adsorbed. The method giving the maximum increase in sp. act., combined with a capacity to prepare large amounts of enzyme was bio-affinity chromatography [18] on a column of Sepharose 4B covalently linked to starch via a 6 carbon spacer. Several studies have shown that phosphorylase can undergo hydrophobic chromatography involving the hydrocarbon spacer [19, 20]. In the present study the following evidence indicates that a true bio-affinity chromatography occurred. At the ionic concentration at which chromatography was carried out there was no adsorption to Sepharose linked to the spacer arm alone. Earlier studies used much lower salt concentrations for elution. Also the enzyme could only be eluted with high concentrations of the substrate, soluble starch, indicating that bio-elution was taking place. In Fig. 5 the purification of phosphorylase I by this method is shown. The enzyme was then separated from starch by chromatography on DEAE-cellulose again. Applying this procedure to phosphorylase I that had been purified to the DEAE-cellulose stage gave a 12 fold purification with a 50% recovery for the affinity step. The sp. act. was 170 nkats per mg protein. The final method adopted for the preparation of phosphorylase I for MW determination, involved application of the original buffer extract of germinating cotyledons directly to a column of Sepharose-4B starch conjugate for affinity chromatography, followed by DEAE-cellulose chromatography, which removed starch and separated phosphorylase I from II. This was followed by chromatography on 8% agarose gel. The final product had a sp. act. of 220 nkats per mg protein. The advantage of this method compared to prior $(NH_4)_2SO_4$ precipitation, dialysis and DEAE-cellulose chromatography, was the high total recovery of active protein. Using this procedure the affinity column could be used only once. The best procedure for preparation of phosphorylase II, starting with mature seeds, was by DEAE-cellulose followed by gel chromatography on Bio-Gel P-150 on which the activity was excluded. This step gave a 4 fold purification with a 48% recovery. The next stage was affinity chromatography to a sp. act. of 140 nkats per mg of protein with a 5 fold purification for this step. This value could be increased to 200 by rejecting the later fractions of P-150 and affinity chromatography.

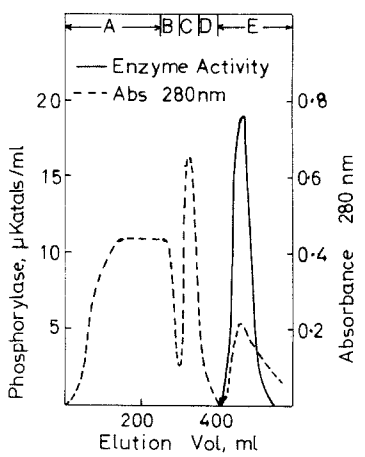


Fig. 5. Affinity chromatography of phosphorylase I on Sepharose 4B-hexyl-starch. A—sample application; B—buffer (20 mM citrate pH 6.5) wash; C—NaCl (1 M in 0.06 M citrate); D—buffer wash; E—NaCl (1 M in 0.06 M citrate), soluble starch (0–2%) gradient.

The two phosphorylase preparations were examined by polycrylamide gel electrophoresis and gels were stained for protein and also for phosphorylase activity with a mixture of Glc-1-P and glucan primer, followed by iodine. A range of 20 to 100 μg of protein was applied to cylindrical gels of 0.6 cm diameter. On staining for protein, phosphorylase I showed a main band with traces of two other bands, one faster and one slower than the main band. On staining for phosphorylase activity, one band corresponding to the main protein band was detected. Phosphorylase II, on protein staining, gave a major band and a more mobile minor band. On staining for activity one band corresponding to the major band was detected. The active bands of phosphorylase I and II had different mobilities. Electrophoresis on a slab with continuously increasing gel concentration (5–25%) clearly separated the two active bands and showed that the minor inactive band associated with phosphorylase II had a higher mobility than phosphorylase I indicating a significantly lower MW. SDS electrophoresis of phosphorylase I gave a single band with a MW of 90 000 and of phosphorylase II gave a major band with a MW of 110 000 and a minor band with a MW of 60 000. The linear graph of MW vs distance moved for phosphorylase I had the following characteristics: log MW = $-0.150 \times$ distance moved (cm) + 5.4 with a correlation coefficient of 0.998. The unknown protein moved 2.8 cm. The graph for phosphorylase II had a regression of: log MW = $-0.155 \times$ distance moved (cm) + 5.4 with a correlation coefficient of 0.996 and the protein travelled 2.4 cm. Treatment of a mixed solution of phosphorylases and I and II with mercaptoethanol and SDS followed by co-electrophoresis clearly differentiated the phosphorylase I band from the phosphorylase II bands.

Gel filtration of phosphorylase I on Sephadex G-200 with a series of standard proteins gave an apparent MW of 135 000. The linear regression equation was: log MW = $-0.0227 \times$ elution vol (ml) + 6.75 with a correlation coefficient of 0.995. The elution volume of the unknown was 71 ml. The elution volume of phosphorylase II was too close to the void volume to allow an assignment of MW, but the elution behaviour did indicate a much higher MW than that of enzyme I. Chromatography on Sepharose 6B-CL gave a value of 490 000. It eluted slightly ahead of urease. The regression line for MW vs elution volume was: log MW = $-0.0374 \times$ elution vol (ml) + 9.22. The correlation coefficient was 0.999 and the elution volume of the unknown was 94.5 ml. The gel filtration and gel electrophoresis data for phosphorylase I suggest a possible monomeric structure. Assuming that the lower MW minor band found on SDS gel electrophoresis derives from the non-active minor band found on gel electrophoresis without SDS, phosphorylase II is probably made up of 4 sub-units which are different in structure to phosphorylase I.

Since the discovery of the nucleotide diphospho-glucose starch synthesis pathway, a number of suggestions have been made about the function of starch phosphorylase in plants. It may function only in degradation, in both synthesis and degradation, to provide primer or in synthesis when cellular concentrations of Glc-1-P reach high levels, e.g. during seed development [2, 6, 21–23]. Although total phosphorylase increases during development, the increase on germination in peas

is much higher. All of this increase is associated with one form of the enzyme, which, as a monomer, has a relatively simple structure and suggests that at least phosphorylase I functions as a degradative enzyme. The increase in activity of a different phosphorylase (II) during the accumulation of starch in developing seeds indicates that it could function in synthesis. It differs from phosphorylase I in its ability to use maltotriose as a primer for glucan synthesis from Glc-1-P and the sub-unit structure is consistent with it having a more complex role, e.g. in synthesis, than phosphorylase I with a single sub-unit structure. However, a developing pea cotyledon shows similarities to a photosynthetic tissue as indicated by its chlorophyll content and, in the same way as a leaf, may utilize some of its starch during the night in glycolysis and synthesis. Degradation of starch under these conditions may need to be more controlled than during germination and the more complex phosphorylase II structure may reflect this.

The sub-unit MW values found for the phosphorylase activities are near to the rabbit muscle glycogen phosphorylase values, however there are significant differences from the animal glycogen phosphorylase system. The low MW enzyme is probably a monomer and does not require AMP for activity. The larger MW enzyme is possibly a tetramer of identical sub-units but these sub-units are not similar to the monomeric phosphorylase I. Potato phosphorylase has been the most actively studied plant starch phosphorylase. A sub-unit MW of 108 000 has been estimated by SDS polyacrylamide gel (24). Determinations on the active enzyme by ultra-centrifugation and gel filtration have generally indicated a MW of about 200 000 suggesting a dimeric structure [16, 25, 26], indicating a possible difference to pea phosphorylase II.

EXPERIMENTAL

Plant material. Legumes used were *Pisum sativum* (cv. Victory Freezer and cv. Alaska), *Vicia faba* (cv. Coles Dwarf Prolific) and *Lupinus luteus* (cv. Weiko III). In following changes during seed development, field-grown plants were labelled at flowering and for germination studies seeds were sterilized with 0.5% NaCl and, after washing, soaked in H_2O and planted in Perlite with 'Panasand' (BDH) at 30° in the d

Preparation of enzyme extracts. Cotyledons were homogenized in 0.5 M citrate (pH 6.5) with insoluble PVP at 4°. Citrate buffer was prepared from Na citrate and citric acid, and adjusted to pH with NaOH. After filtration through cheesecloth and centrifugation (30 000 ***g***, 10 min, 4°) $(NH_4)_2SO_4$ was added to the supernatant soln to 60% sat. The ppt. was dissolved in 20 mM citrate (pH 6.5) and dialysed for 18 hr. After centrifugation the extract (50 ml) was chromatographed on DEAE-cellulose (column vol 40 ml) using a wash of 20 mM citrate (pH 6.5) (50 ml) followed by a linear gradient to 60 mM citrate (50 ml 20 mM + 50 ml 60 mM) and then another linear gradient to 0.5 M NaCl–0.06 M citrate (100 ml 60 mM citrate + 100 ml 500 mM NaCl in 60 mM citrate).

Detection of phosphatase activity. An aliquot (0.1 ml) was incubated at 30° with 5 mM *p*-nitrophenyl phosphate in 0.1 M citrate (pH 6, 0.1 ml). Before 10% hydrolysis of the substrate had occurred, 5% Na_2CO_3 (3.8 ml) was added and the *A* at 410 nm determined.

Detection of phosphorylase activity by production of iodine staining glucan. An aliquot of extract (0.1 ml) and a soln of 0.1 M Glc-1-P and 0.2% soluble starch in 0.1 M citrate pH 6 (0.02 ml) were incubated for 10 min at 30°. Dilute I_2 (0.01% I, 0.1% KI in 0.2 M acetate, pH 4 (3.8 ml) was added and the *A* at 600 nm measured.

Assay of phosphorylase by determination of Pi released. Enzyme preparation (0.1 ml), 0.1 M Glc-1-P and 0.6% soluble starch in 0.1 M citrate (pH 6) (0.1 ml) were incubated at 30°. 5% TCA (3.8 ml) was added, the soln centrifuged if necessary, and Pi in the supernatant estimated [27]. The reaction was stopped before 2 μmol Pi was released.

Assay of phosphorylase by determination of Glc-1-P formation using Glc-6-P dehydrogenase. The assay procedure of ref 28 was adapted. An aliquot (0.1 ml) was mixed with Glc-6-P dehydrogenase (1 U), phosphoglucomutase (1.3 U), soluble starch (2 mg) in a final vol of 1 ml in which the conc of NADP was 0.63 mM, Mg Cl_2 10.5 mM, EDTA 1 mM and thioglycollic acid 1 mM, Glc-1-6 di-P 5×10^{-5} mM and bovine serum albumin 0.1%. The buffer was Tris–HCl (pH 6.5).

Determination of kinetic properties. K_m values in the direction of synthesis were measured in 50 mM citrate buffer, (pH 6) at 30°. The conc ranges were for Glc-1-P, 5-50 mM and for glucans, 0.006–0.3%. In the direction of degradation the buffer was 10 mM Tris HCl (pH 6.5) at 30°. The conc ranges were for Pi, 4–20 mM and for glucan, 0.0025–0.05%.

Preparation of Sepharose 4B n-hexyl-starch conjugate for affinity chromatography. Washed Sepharose 4B (5 g) and 2 M Na_2CO_3 (20 ml adjusted to pH 11.5) were mixed with cyanogen bromide (CNBr) (0.5 g) for 8 min at 15°. After filtering and washing (H_2O, 200 ml then 0.2 M $NaCHO_3$, 200 ml at 5°) the product was added to diaminohexane (2 g) in 0.2 M $NaHCO_3$ (20 ml, pH 9.5) at 5° and stirred for 18 hr, filtered and washed with 0.2 M $NaHCO_3$ and H_2O. Soluble starch, 0.2 g in 0.2 M $NaHCO_3$ (20 ml) was mixed with 80% CNBr in MeCN (0.05 ml) for 90 sec and added to the modified Sepharose-4B paste and stirred at 5° for 18 hr. After filtration, the solid was washed with 0.2 M $NaHCO_3$ H_2O. After packing into a column it was washed with 20 mM citrate (pH 6.5). The product stained with I_2.

Preparative gel filtration of phosphorylases I and II. Phosphorylase I and II were chromatographed on Biogel A 1.5 (8% agarose) and Sepharose 6B or Bio-Gel P-150 respectively in 0.02 M citrate (pH 6.5) and assayed by Pi release from Glc-1-P.

Preparative gel filtration in association with amylopectin. The product from gel filtration was isolated by pptn with $(NH_4)_2SO_4$ at 60% satn and re-dissolved in 0.02 M citrate (pH 6.5). The soln was mixed with an equal vol of potato amylopectin soln in the ratio of 10 mg of amylopectin for each mg of protein. Potato amylopectin was prepared by pptn of the amylose from NaCl soln of potato starch as the *n*-BuOH complex, after dispersion of the potato starch by DMSO. The residual amylopectin was ppted into 3 vol EtOH and washed with Me_2CO and Et_2O. The amylopectin–protein mixture was then re-chomatographed on the same column. Active fractions were combined and chromatographed on DEAE-cellulose to remove amylopectin which was not bound.

Affinity chromatography on Sepharose 4B-hexyl-starch conjugate. The phosphorylase preparation was applied and the column (40 ml), washed with citrate buffer (20 mM, pH 6.5) (25 ml) then 1 M NaCl–60 M citrate (25 ml). Activity was eluted with a linear gradient of 0–2% soluble starch in 1 M NaCl–0.06 M citrate (pH 7.0) (50 ml + 50 ml). The eluate was dialysed and soluble starch removed by chromatography on DEAE cellulose.

Polyacrylamide gel electrophoresis. This was carried out by the method of ref [29] using a 7% gel with 150V for 5 min followed by 210V and 25 mA for 12 gels or on a slab with a continuously increasing gel concentration (5–25%, Gradipore) at 50 V, 5 mA. Protein was stained with Amido Black or Coomassie Blue. Phosphorylase activity was detected after lightly rinsing the gels in buffer by incubating them in 20 mM Glc-1-P, 0.3% limit dextrin or 0.013% soluble starch for 1 hr, rinsing again and staining with dilute I_2 (0.01% I_2 in 0.1% KI).

SDS-polyacrylamide gel electrophoresis. The method of Weber [30] using mercaptoethanol was used with a 5% gel (8 mA/gel). The standards were muscle phosphorylase (sub-

unit MW 100 000), bovine serum albumin (68 000), catalase (60 000), fumarase (49 000), aldolase (40 000) and lactic dehydrogenase (36 000).

Determination of MW by gel filtration. In the chromatography of phosphorylase I, Sephadex G-200 with standards of urease (MW 480 000), rabbit muscle phosphorylase-a (380 000), bovine serum albumin (68 000) and myoglobin (17 000) was used. For phosphorylase II, urease, lactic dehydrogenase (150 000), bovine serum albumin and myoglobin on Sepharose 6B-CL were used. The solvent was 0.1 M Tris–HCl buffer (pH 6.5) in 0.1 M NaCl, 1 mM EDTA.

Acknowledgements—The authors wish to thank Professor J. F. Turner for helpful discussions. This work was supported by the University of Sydney Research Grant. One of us (R.H.R.) wishes to acknowledge the award of a Henry Bertie and Florence Mabel Gritton Post-graduate Research Scholarship.

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