THE EXTERNAL LOCATION OF PHOSPHATASE ACTIVITY IN PHOSPHORUS-DEFICIENT *SPIRODELA OLIGORRHIZA*

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Abstract

Phosphorus deficiency (P deficiency) caused a 10–20-fold increase in phosphatase activity of *S. oligorrhiza* homogenates. Specific staining showed that phosphatase in control plants was located primarily in and around the vascular strands, and in P-deficient plants it was located in the epidermis of the root and undersurface of the frond. Dissection experiments showed that roots of P-deficient plants contained a disproportionately high phosphatase activity. When plant and root homogenates were fractionated, phosphatase activity was recovered in the supernatant rather than in any cell particle fraction. However, intact plants released very little phosphatase to the external medium.

Intact plants were placed in solutions containing phosphatase substrates. Control plants hydrolysed the substrates at 4–8% of the rates of comparable homogenates. P-deficient plants hydrolysed substrate at 30–40% of the homogenate rate. When glucose 1-[^{32}P]phosphate was supplied to intact plants, more $^{32}P_i$ appeared in the medium than in the tissue. The pattern of appearance was consistent with hydrolysis of glucose 1-[^{32}P]phosphate in the external medium followed by accumulation of $^{32}P_i$ into the tissue; and was not consistent with hydrolysis of glucose 1-[^{32}P]phosphate in the tissue followed by loss of $^{32}P_i$ to the medium. We conclude that a large part of the phosphatase which arises in the plant *S. oligorrhiza* under the stress of P deficiency is located in either the cell wall or the external membrane, where its function is to utilize phosphate esters released to the medium by dying plants. Although a new isoenzyme appears during P deficiency it does not appear to function specifically as the externally active phosphatase.

I. Introduction

When the duckweed *Spirodela oligorrhiza* Kurz (Hegelm.) is grown in a minus-phosphorus medium, characteristic symptoms of phosphorus deficiency (P deficiency) develop (Bieleski 1968), notably a 10–50-fold increase in acid phosphatase activity (Reid and Bieleski 1970). Part of this increased activity is due to the appearance in P-deficient tissue of two new isoenzymes of lower molecular weight. When sections were stained to locate phosphatase, much of the activity in P-deficient tissue appeared to be localized in the epidermis (Reid 1968). Localization of phosphatase in *S.*

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oligorrhiza has now been studied in more detail. During P deficiency, much of the phosphatase becomes located in such a way that it is directly accessible to substrates in the external medium. It is suggested that this phosphatase may be in the outer cell membrane.

II. Materials and Methods

(*a*) *Plant Material*

Control *S. oligorrhiza* plants were grown for 6–7 days in a complete mineral culture medium, and P-deficient plants were grown for 10–24 days in a minus-phosphorus medium, under controlled and axenic conditions. Conditions of growth, the course and symptoms of P deficiency (Bieleski 1968), and the development of phosphatase activity (Reid and Bieleski 1970) were as previously reported.

(*b*) *Substrates for Phosphatase*

The course of hydrolysis of *p*-nitrophenyl phosphate (Sigma Biochemicals) was followed spectrophotometrically by measuring the production of *p*-nitrophenol.

Glucose 1-[^{32}P]phosphate (G1^{32}P) was synthesized by incubating a mixture of 1 ml crude potato phosphorylase, 1 ml 4% starch solution, 1 ml 0·04M, pH 6·7, phosphate buffer, 1 mCi (0·8 ml) $H_3{}^{32}PO_4$ (Amersham, England, carrier-free), and two drops toluene for 18 hr at 24°C. The mixture was then heated (100°C for 10 min) to denature protein, centrifuged, and the supernatant was shaken with 0·1 g IR120 (H^+) ion-exchange resin to remove divalent cations, then applied as a band to a single sheet, 45 by 50 cm, of Whatman 120 chromatography paper. The paper was chromatographed for 21 hr in n-propanol–ammonia–water (6 : 3 : 1, v/v), autoradiographed, and G1^{32}P was eluted and rechromatographed for 8 hr in n-propyl acetate–formic acid–water (11 : 5 : 3, v/v) (Bieleski 1965). G1^{32}P was eluted, and its purity checked: over 99% was hydrolysed in 7 min at 100°C in 1N HCl. For experiments, G1^{32}P (25 μCi/μmole) was diluted with carrier G1P (Nutritional Biochemicals, A grade). The course of hydrolysis was followed by appearance of $^{32}P_i$ in the reaction medium. Samples were chromatographed (on thin-layer plates, in n-propyl acetate–formic acid–water, 11 : 5 : 3, v/v), autoradiographed, and $^{32}P_i$ and G1^{32}P spots were cut out and their radioactivities measured (Bieleski 1965).

(*c*) *Assay of Phosphatase in Homogenates and Whole Plants*

Phosphatase activities of homogenates were measured as follows: 50 mg control or 20 mg P-deficient tissue were homogenized in 9 ml 0·05M imidazole buffer, pH 6·4; 1 ml 20 mM *p*-nitrophenyl phosphate was added and 1·0-ml aliquots were taken at 30-min (control) or 5-min intervals (P deficient) and added to 2·0 ml 1N KOH.

The apparent phosphatase activity of intact tissue was measured as follows: Rather than weigh tissue beforehand, and subject it to possible damage, the sample was weighed at the conclusion of the experiment. Flasks containing about 200 control fronds (about 130 mg) or about 40 P-deficient fronds (about 28 mg) were selected and growth medium was withdrawn by gentle suction. Distilled water was added and in turn withdrawn by suction. After two such washes, 10 ml reaction medium (0·01M imidazole buffer, pH 6·4, plus 0·4–2 mM *p*-nitrophenyl phosphate) was added, and samples were taken as before. Readings were corrected for the changing tissue : volume ratio. During incubation, the flasks were gently agitated on a Conway shaker.

When G1P was the substrate, similar procedures were used. Reaction medium contained 1 mM G1^{32}P in 0·01M imidazole buffer, pH 6·4, or else these plus 1 mM KH_2PO_4. The tissue : solution ratio was 50 mg to 1·0 ml (both tissues, homogenates and whole plants), and 10-μl samples were taken at $\frac{1}{4}$, $\frac{1}{2}$, or 1-hr intervals. Each sample was spotted directly onto a thin-layer plate, which was then put into an atmosphere of ammonia to inactivate any phosphatase prior to chromatography. Activities are expressed in enzyme units (E.U.; 1 E.U. hydrolyses 1 μmole substrate per minute at 25°C).

(*d*) *Gel Electrophoresis*

The phosphatase isoenzyme pattern was studied. Proteins in extracts were separated by flat-plate gel electrophoresis (Reid and Bieleski 1970). Afterwards, gel plates were washed in imidazole buffer, pH 6·4 (30 min, three changes), then one strip was cut from each plate and stained with α-naphthyl phosphate (0·005%) plus fast red tetrazolium salt (0·005%) (Burstone 1962) to reveal phosphatase. Areas in the unstained gel corresponding to regions containing phosphatase were assayed for phosphatase activity using *p*-nitrophenyl phosphate as substrate (Reid and Bieleski 1970).

(*e*) *Cell Fractionation*

Cell particles were prepared at 0°C by a modification of the procedure of Honda, Hongladarom, and Laties (1966): 0·25 g tissue was minced in 0·5 ml Honda medium (dextran 6%, Ficoll 2·5%, sucrose 8·5%, 4 mM mercaptoethanol, and 5 mM $MgCl_2$ in 0·05M Tris.HCl, pH 7·8) by chopping it on a polythene plate with five razor blades clamped together on a strip of spring steel mounted over the poles of an a.c. electromagnet. The homogenate with addition of 4·5 ml Honda medium was filtered through two layers of Miracloth, and the filtrate was centrifuged at 1500 *g* for 5 min (chloroplast fraction), 12,000 *g* for 15 min (mitochondrial fraction), 35,000 *g* for 30 min (light mitochondrial fraction), and 105,000 g for 60 min (microsomal fraction and supernatant). The supernatant was adjusted to pH 6·4 before assay. Each cell particle fraction was resuspended in Honda medium, recentrifuged, homogenized in 0·05M imidazole buffer, pH 6·4, and its phosphatase activity assayed.

(*f*) *Tissue Staining*

Intact plants were fixed in ethanol–0·1M acetate buffer (pH 5)–ethyl acetate–formalin (20 : 14 : 6 : 1, v/v) for 1½ hr. The fixative penetrated the tissue well and allowed recovery of about half the total phosphatase activity. The high molecular weight isoenzyme appeared to be slightly the more sensitive to inactivation. Air was evacuated with a water pump, and the plants were prodded to make them sink. Fixative was removed with four half-hour washes of acetate buffer. Plants were stained for phosphatase by the procedures of Gomori (1950) or Burstone (1962), embedded, sectioned, and stained in fast green.

(*g*) *Measurement of Phosphate Accumulation and Efflux*

Control or P-deficient plants were put into 1 mM or 10 mM $KH_2{}^{32}PO_4$ for 4–6 hr, their increase in radioactivity with time was measured, and accumulation rates were calculated. Plants which had accumulated $^{32}P_i$ in this way for 4–6 hr were then rinsed briefly in distilled water then put into non-radioactive 1 mM KH_2PO_4. The rate of appearance of $^{32}P_i$ in the medium was measured, the specific activity of the P_i in the tissue was measured, and the rate of efflux of P_i into the medium was calculated.

III. Results

(*a*) *Localization of Phosphatase in Roots and Leaves*

Plants were dissected into roots, and old, medium-aged, and young fronds. Each fraction was weighed, homogenized, and its phosphatase activity measured. In control plants, the activity of each fraction was proportional to its fresh weight (Table 1). In P-deficient plants the roots contained a disproportionately high phosphatase activity (Table 1). Thus the increase in phosphatase brought about by P deficiency occurred to a greater extent in the roots than in the fronds.

(*b*) *Phosphatase Isoenzymes in Root and Leaf*

During P deficiency, phosphatase activity increases and two low molecular weight isoenzymes of phosphatase appear (Reid and Bieleski 1970). Although the root shows the most marked increase in phosphatase activity, the ratio of low molecular weight to high molecular weight isoenzyme activity is much lower in the root than in the fronds (Table 2).

TABLE 1

RELATIVE PROPORTIONS OF FRESH WEIGHT AND OF PHOSPHATASE ACTIVITY IN HOMOGENATES OF VARIOUS ORGANS FROM CONTROL AND PHOSPHORUS-DEFICIENT *S. OLIGORRHIZA*

Total weight of tissue dissected: control, 498 mg; 11-day phosphorus-deficient plants, 591 mg; 14-day phosphorus-deficient plants, 563 mg. Total enzyme activity of tissue dissected: control, 0·25 E.U.; 11-day phosphorus-deficient plants, 1·58 E.U.; 14-day phosphorus-deficient plants, 2·13 E.U.

Plant organ	(A) Weight (as % of total)	(B) Enzyme activity (as % of total)	Ratio B/A
	Control plants		
Roots	6·6	6·1	0·92
Young leaves	7·6	6·5	0·85
Medium leaves	17·5	17·6	1·01
Old leaves	68·3	69·8	1·02
	11-day phosphorus-deficient plants		
Roots	15·3	40·6	2·65
Young leaves	4·2	2·9	0·69
Medium leaves	17·4	15·2	0·87
Old leaves	63·1	41·4	0·66
	14-day phosphorus-deficient plants		
Roots	10·8	34·7	3·21
Young leaves	4·8	1·8	0·38
Medium leaves	24·9	14·3	0·57
Old leaves	59·5	49·2	0·83

TABLE 2

ENZYME ACTIVITY OF HIGH AND LOW MOLECULAR WEIGHT ISOENZYMES IN EXTRACTS FROM ROOTS AND LEAVES OF PHOSPHORUS-DEFICIENT *S. OLIGORRHIZA*

Organ	Phosphorus status	Enzyme activity (E.U./g fresh wt.)		Ratio A/B
		Low molecular weight enzyme (A)	High molecular weight enzyme (B)	
Leaf	19-day deficient	0·66	1·35	0·49
Root	19-day deficient	0·35	3·06	0·11
Leaf	21-day deficient	0·31	0·73	0·43
Root	21-day deficient	0·30	4·47	0·07

(*c*) *Phosphatase Activities of Cell Fractions*

Over 90% of phosphatase activity released from chopped tissue was recovered in the supernatant fractions (Table 3). The remaining activity was distributed amongst all the particle fractions, and was probably due to adsorbed protein.

TABLE 3

PHOSPHATASE ACTIVITIES OF CELL PARTICLE FRACTIONS FROM CONTROL AND PHOSPHORUS-DEFICIENT *S. OLIGORRHIZA*

Phosphorus status of tissue	Cell fraction activities (as % of total activity)				
	Chloroplast	Mitochondrial	Light mitochondrial	Microsome	Supernatant
Control	0·8	0·8	1·0	0·0	97·4
12-day deficient	0·4	2·5	0·9	0·5	95·7
13-day deficient	2·0	4·1	1·8	1·1	91·0
19-day deficient	1·8	5·3	—	—	92·9
13-day deficient (roots only)	1·1	3·4	1·9	1·4	92·2

(*d*) *Phosphatase Activity in Growth Medium*

Activity per unit volume of phosphatase in the medium was less than 1/5000 that in the tissue. The total amount of phosphatase in the medium was 2–3% of that in the tissue which had been grown in the medium, and this proportion was more or less independent of the state of deficiency (Table 4).

TABLE 4

TOTAL PHOSPHATASE ACTIVITIES OF *S. OLIGORRHIZA* PLANTS AND THEIR GROWTH MEDIUM

A sample of medium was taken from each flask, then the tissue was harvested and homogenized. Each sample was made 0·01M with imidazole buffer (pH 6·4) and 2 mM with *p*-nitrophenyl phosphate, and its phosphatase activity was measured

Flask contents	Enzyme activity	Total enzyme activity per flask (mE.U.)*	Fraction of activity in medium
Control tissue (0·111 g)	0·286 (E.U./g)	31·8	0·03
+ control medium (20 ml)	0·00004 (E.U./ml)	0·8	
10-day deficient tissue (0·089 g)	3·96 (E.U./g)	353	0·02
+10-day deficient medium (20 ml)	0·00032 (E.U./ml)	6·5	
21-day deficient tissue (0·109 g)	4·75 (E.U./g)	519	0·03
+21-day deficient medium (20 ml)	0·00081 (E.U./ml)	16·2	

* 1 mE.U. = 0·001 E.U.

(*e*) *Comparison of Enzyme Activity of Whole Plants and Homogenates*

Generally, enzyme activities are assayed on homogenates. In the present experiment, apparent enzyme activities of intact plants were also measured. In various experiments with *p*-nitrophenol as substrate, intact control plants showed

activities 4–8% of those of comparable homogenates and P-deficient plants had 30–40% of the homogenate activity (Table 5).

TABLE 5

PHOSPHATASE ACTIVITY OF INTACT PLANTS COMPARED WITH THAT IN HOMOGENATES

Substrate	Status of tissue	(A) Whole plant activity (E.U./g fresh wt.)	(B) Homogenate activity (E.U./g fresh wt.)	Ratio A/B
2 mM *p*-nitrophenyl phosphate alone	Control	0·0159	0·263	0·06
	P-deficient	1·12	3·85	0·29
2mM *p*-nitrophenyl phosphate plus 1 mM KH_2PO_4	Control	0·0060	0·196	0·03
	P-deficient	0·68	1·91	0·36
1 mM glucose 1-phosphate plus 1 mM KH_2PO_4	Control	0·0010	0·0262	0·04
	P-deficient	0·049	0·153	0·32

Because control and P-deficient homogenates were respectively 200 and 2 μM with respect to P_i, the difference in behaviour could have arisen through differences in enzyme inhibition. Hence 1 mM KH_2PO_4 was added to reaction media: the same

TABLE 6

APPEARANCE OF INORGANIC PHOSPHATE IN SOLUTION AND TISSUE, FOLLOWING HYDROLYSIS OF GLUCOSE 1-PHOSPHATE BY INTACT PLANTS

Medium contained 1 mM glucose 1-[^{32}P]phosphate plus stated concentration of KH_2PO_4 in 0·01M imidazole buffer, pH 6·4. Tissue (of weight shown) was incubated in 1·0 ml medium for the periods indicated. Total radioactivity in solution at the start of the experiment I was 757,000 counts/min in glucose 1-[^{32}P]phosphate +23,000 counts/min in P_i (contaminant of glucose 1-phosphate), and in experiment II 908,000 counts/min in glucose 1-phosphate +26,500 counts/min in P_i

Phosphate status of tissue	Weight of tissue (mg)	KH_2PO_4 concn. in medium (mM)	Time of hydrolysis (hr)	$10^{-3}\times$ increase in $^{32}P_i$ in solution (counts/min)	$10^{-3}\times$ increase in $^{32}P_i$ in tissue (counts/min)	$^{32}P_i$ ratio, solution : tissue
			Experiment I			
Control	70	1	8	25·2	22·8	1·1
	65		8	19·4	18·4	1·1
P-deficient	50	1	4·5	366	122	3·0
	35		4·5	295	68	4·3
			Experiment II			
P-deficient	50	1	6	476	66	7·2
	50		6	494	111	4·5
P-deficient	50	10	6	372	27	13·8
	50		6	377	29	13·0

phenomenon was still observed (Table 5). *p*-Nitrophenyl phosphate and *p*-nitrophenol are potentially toxic, and could have affected the permeability of the living tissue. Hence the natural substrate G1P was used in the reaction mixture. The same phenomenon was still observed (Table 5).

When P-deficient plants were dissected into roots and fronds, and phosphatase activities of intact and homogenized organs compared, the roots showed a higher intact-tissue activity (64% of the homogenate activity) than either the old leaves (20%), the young leaves (12%), or the whole plants (36%).

(*f*) *Movement of Hydrolysis Products between Plants and Solutions*

Intact plants were floated on medium containing 1 mM G1^{32}P, 0·01M imidazole buffer, and either 1 or 10 mM KH_2PO_4. After part of the G1P had been hydrolysed, the plants were harvested and their radioactivity (mostly as $^{32}P_i$) was measured. The amount of $^{32}P_i$ in the external medium was also measured. There was more $^{32}P_i$ in the medium than in the tissue, more noticeably so with P-deficient tissue. Increasing the concentration of P_i in the medium enhanced the effect (Table 6).

Appearance of $^{32}P_i$ in both medium and tissue could have resulted from the hydrolysis of G1^{32}P in the medium, followed by accumulation of some of the resulting $^{32}P_i$ into the tissue. Equally, it could have resulted from hydrolysis of G1^{32}P in the

TABLE 7

APPARENT RATES OF MOVEMENT OF INORGANIC PHOSPHATE BETWEEN TISSUE AND SOLUTION DURING HYDROLYSIS OF GLUCOSE 1-PHOSPHATE BY WHOLE TISSUES

Medium contained 1 mM glucose 1-phosphate plus stated concentration of KH_2PO_4 in 0·01M imidazole buffer, pH 6·4. Data from experiments reported in Table 6. "Apparent uptake rate" is that rate of P_i uptake required to produce the observed radioactivity in the tissue if glucose 1-[^{32}P]phosphate was all hydrolysed outside the cell. "Apparent efflux rate" is the rate of P_i movement from tissue to medium required to produce the observed radioactivity of P_i in the solution if glucose 1-[^{32}P]phosphate was all hydrolysed inside the cell. See Section IV for further details

Phosphate status of tissue	KH_2PO_4 concn. in medium (mM)	Average hydrolysis rate (nmoles g^{-1} hr^{-1})	Apparent uptake rate (nmoles g^{-1} hr^{-1})	Apparent efflux rate (nmoles g^{-1} hr^{-1})
		Experiment I		
Control	1	112	1110	990
		95	1060	950
P-deficient	1	2870	2240	2150
		3040	2310	2470
		Experiment II		
P-deficient	1	2010	1100	1850
		2240	1540	1920
P-deficient	10	1460	4550	1450
		1480	4640	1470

tissue followed by efflux of some $^{32}P_i$ from the tissue into the medium. Therefore, P_i accumulation and efflux rates were measured. In different experiments, control plants accumulated P_i at 520 and 790 nmoles per gram fresh weight per hour from 1 mM P_i; P-deficient plants accumulated P_i at 1080, 1290, and 1320 nmoles per gram per hour from 1 mM P_i, and at 1840 and 2490 nmoles per gram per hour from 10 mM Pi. Efflux rates found were 44 nmoles P_i per gram per hour for control tissue

and 89 nmoles per gram per hour for P-deficient tissue. Note that these efflux measurements may have included some passive exchange of tissue-adsorbed $^{32}P_i$ with P_i in the medium, and that this would inflate the efflux values. In comparison, intact-tissue hydrolysis rates were: 60–100 nmoles G1P hydrolysed per gram fresh weight

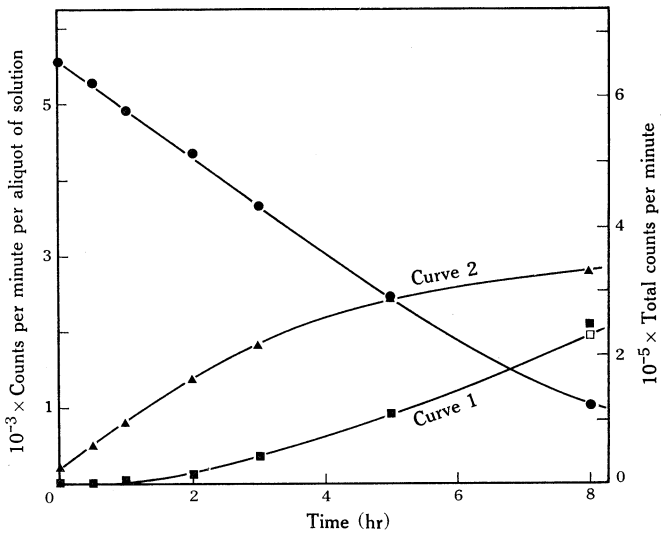
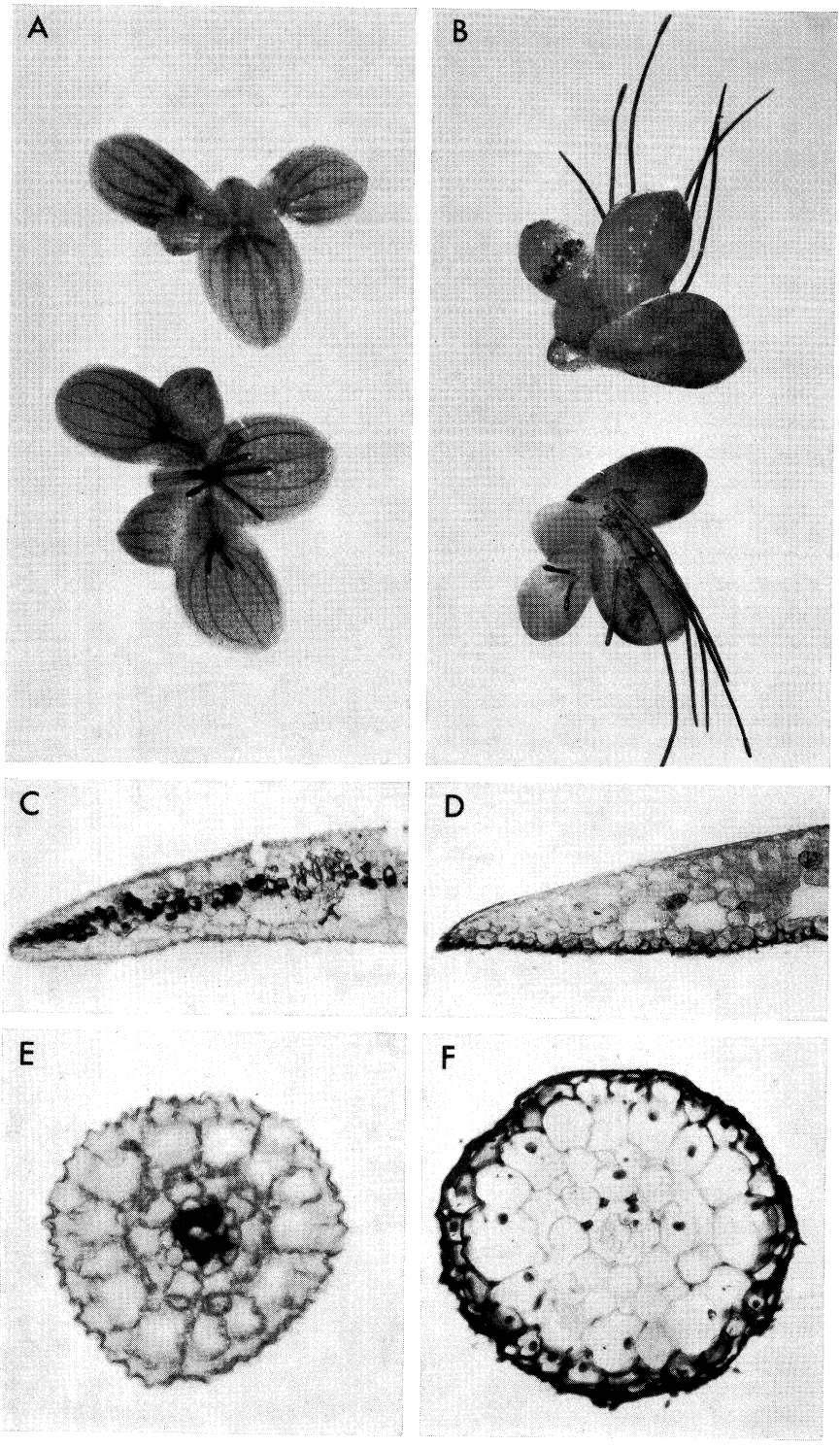


Fig. 1.—Time course of hydrolysis of glucose 1-phosphate (G1P) by intact phosphorus-deficient tissue; and of distribution of phosphate between solution and tissue. Intact phosphorus-deficient plants (60 mg) were incubated in 1 ml medium (1 mM G1^{32}P, 1 mM P_i, 10 mM imidazole buffer, pH 6·4). 10-μl samples were taken and chromatographed to separate G1P and P_i, thereby giving the total radioactivity of G1^{32}P and $^{32}P_i$ in the medium. The ^{32}P content of the tissue was measured by difference, as the loss of ^{32}P from the medium. At the conclusion of the experiment, the tissue was harvested and its ^{32}P content was measured directly. ● G1^{32}P in medium. ▲ $^{32}P_i$ in medium. ■ ^{32}P in tissue (by difference). □ ^{32}P in tissue (by direct measurement). Note that although the points connected by curve 1 are experimental points, the curve itself has been drawn from theoretical calculations (see Section IV). These calculations assumed that all G1^{32}P hydrolysis occurred in the external medium (rate, 2200 nmoles per gram fresh weight per hour) and that P_i was accumulated from the medium into the tissue at a steady rate of 2400 nmoles per gram fresh weight per hour.

per hour for control tissue, and 2800–3000 nmoles per gram per hour for P-deficient tissue (from Tables 5 and 7). Thus in P-deficient tissue, the rate of P_i production by hydrolysis was of the same order as the rate of P_i accumulation, but very much greater than the rate of P_i efflux.

Fig. 2.—Distribution of phosphatase in control (left) and phosphorus-deficient (right) *S. oligorrhiza* plants. Plants were fixed, stained by the procedure of Gomori, embedded, and sectioned. Note that control and phosphorus-deficient plants were stained for different lengths of time, to compensate for their different phosphatase activities. *A*, intact control plants (×5); *B*, intact phosphorus-deficient plants (×5); *C*, cross-section of control frond (×70); *D*, cross-section of phosphorus-deficient frond (×75); *E*, cross-section of control root (×300); *F*, cross-section of phosphorus-deficient root (×250). Note occurrence of phosphatase (black stain) in the region of vascular tissues in control plants (left), and in the epidermis of phosphorus-deficient plants (right).


A
B
C
D
E
F

(g) *Time Course of Hydrolysis*

The appearance of $^{32}P_i$ in P-deficient tissue and medium during hydrolysis of G1^{32}P was studied in more detail. Aliquots of the external medium were taken at various times and radioactivities of G1P and P_i were measured. Radioactivity in the tissue was found during the course of uptake by the disappearance of total ^{32}P from the medium, and at the end of the experiment by direct measurement (Fig. 1). Hydrolysis of G1P occurred at a constant rate until over half the substrate had been used, after about 5 hr. At first, very little ^{32}P appeared in the tissue, but progressively more went in as time went by, so that the curve relating tissue radioactivity to time was concave (curve 1, Fig. 1).

(h) *Cytochemical Localization of Phosphatase*

Control plants stained for phosphatase by the procedure of Gomori (1950) showed an overall light brown appearance. Leaf veins and root steles were densely stained (Fig. 2). Sometimes the stain formed a light covering on the frond surface, mostly on the underside. Transverse sections showed concentrations of stain in the epidermal and subepidermal cells, on the walls and chloroplasts of the lower layer of the palisade mesophyll cells, and particularly on the walls and within the cells of the vascular strands and their surrounding elongated sheath parenchyma cells.

P-deficient plants were much more heavily stained than control plants, with a heavy coat on the surface of roots and leaves. Leaf veins and root steles were relatively unstained. In transverse sections, cell walls of the epidermis and subepidermis, and sometimes the intercellular spaces within the subepidermis, particularly of the roots, were deeply stained. Contents of epidermal cells were erratically stained (Fig. 2).

Control and P-deficient plants showed a number of variable staining patterns, such as occasional staining of nuclei, and diverse patterns around the stomata. An attempt to stain fixed sections was unsuccessful. Similar results were obtained when the staining procedure of Burstone (1962) was used.

IV. Discussion

P deficiency causes a 4–12-fold increase in phosphatase activity in at least four lower organisms—in *Escherichia coli* (Torriani 1960), *Saccharomyces* (Suomalainan, Linko, and Oura 1960), *Euglena gracilis* (Blum 1965), and *Neurospora crassa* (Nyc 1967). In each case this increase involves the appearance of a new isoenzyme, the "inducible phosphatase", which appears to be located in the cell wall instead of in the body of the cell as in the case of the constitutive enzyme (above references and Malamy and Horecker 1961; McLellan and Lampen 1963; Weimberg and Orton 1964; Sommer and Blum 1965). Since P deficiency caused a 10–50-fold increase in phosphatase activity and the appearance of a new isoenzyme in the higher plant *Spirodela oligorrhiza* (Reid and Bieleski 1970), we asked whether a completely analogous situation might exist, and whether the new "induced" phosphatase might have an external location.

The most widely used procedure for studying the distribution of phosphatase has been the specific staining technique of Gomori (1950). When this was applied to *S. oligorrhiza*, the macroscopic pattern of phosphatase distribution was shown clearly.

In control plants, the vascular strands contained the highest phosphatase activity, and in P-deficient plants, the epidermis contained the highest activity. Unfortunately, variations in staining behaviour prevented the intracellular location from being established with any confidence. We believe this problem arose because staining solutions did not penetrate the cells evenly. *S. oligorrhiza*, adapted to life as a floating water plant, has a waxy coating that makes the air spaces almost impervious to water.

With dissection experiments, we confirmed that P-deficient growth caused phosphatase activity to increase to a far greater degree in the roots than in the remaining tissue. In one respect, this increase was unlike that recorded for the unicellular organisms. The low molecular weight "inducible" isoenzyme which appeared only in the P-deficient plants was much less prominent in the roots, where the greatest phosphatase increase took place. Even in the rest of the plant, only about 40% of the increase in phosphatase activity during P deficiency was due to the appearance of the induced enzyme: the rest was due to an increased activity of the constitutive enzyme itself (Reid and Bieleski 1970). It seems that in *S. oligorrhiza* a simple distinction between the two isoenzymes cannot be drawn, nor that separate and distinct functions can be ascribed.

In P-deficient tissues, the phosphatase must be segregated from the metabolically active part of the cell, since the amount present would hydrolyse the phosphate esters at about three times the observed rate of phosphate ester synthesis, if enzyme and substrate were in free contact (see Bieleski 1968). However, attempts to localize the enzyme in a lysosome-like body failed. The isolation procedure used was a gentle one, avoiding the shearing forces inherent in all homogenizers, and supplying materials found to help preserve fragile cell particle structure. Despite this, almost all the phosphatase was found in the supernatant fraction. We conclude that most of the phosphatase is held in some fragile structure that is completely disrupted during very mild homogenizing. Potential sites are within the vacuole, in a very fragile cell particle, or as part of a membrane system. Since much of the phosphatase in P-deficient plants is accessible to the external medium, a vacuolar site seems very unlikely and a site in the plasmalemma seems probable.

There are three facets to the evidence for the external location of phosphatase in P-deficient *S. oligorrhiza*. Firstly, whole plants exhibited a high phosphatase activity, and the products of hydrolysis were found primarily in the external solution. Secondly, these hydrolysis products were capable of entering the tissue fast enough but were not capable of leaving the tissue fast enough to produce the observed proportion of $^{32}P_i$ within and without the plant. Thirdly, the time course of hydrolysis was compatible with external hydrolysis followed by accumulation of products, but was not compatible with internal hydrolysis followed by subsequent loss of hydrolysis products to the medium. These are discussed in more detail below.

When whole plants were supplied with substrate, about 35% of the phosphatase in P-deficient plants behaved as if it had an external location, but only about 5% did in control plants (Table 5). The first value is probably an underestimate. The absolute rate of reaction was very high; in 1 hr, 20 mg tissue hydrolysed over 50% of the substrate in 5 ml medium, and although the plants and reaction medium were gently shaken the rate must have been limited to some degree by the rates at which substrate could diffuse through the liquid to the site of hydrolysis, and inhibitory P_i could diffuse away. A more vigorous shaking procedure markedly increased the hydro-

lysis rate. We believe that the enzyme activity may have been limited to approximately two-thirds its true rate in this way. The fraction of externally located phosphatase was much higher in the roots (64%) than in the whole plant (36%), possibly because of the much greater surface : volume ratio of the root lessening the diffusion barrier.

When G1^{32}P was hydrolysed by intact plants, $^{32}P_i$ appeared in both tissue and medium (Table 6). It is possible to calculate the rate at which P_i would need to be taken up by the tissue if all hydrolysis occurred in the external medium, and the rate at which P_i would need to leave the tissue if all hydrolysis occurred within the cell, in order to give the observed pattern. Assume first that G1^{32}P did not enter the control or P-deficient tissue, and that all hydrolysis occurred in the external solution. The radioactivity of P_i in solution would increase as G1^{32}P was hydrolysed. The P_i concentration, initially 1 mM, could also change, as P_i was both released into the solution by hydrolysis, and withdrawn by accumulation into the tissue; data in Table 6 show the concentration must have remained between 0·5 and 1·5 mM. From the average radioactivity and concentration of P_i in solution, we can calculate the amount of P_i that would have to have entered the tissue to give it its observed radioactivity. The hypothetical rate of P_i uptake calculated in this way (Table 7) was about twice the conventionally measured accumulation rate. Some difference of this order would be expected if hydrolysis of G1^{32}P were occurring at the cell surface. P_i at the surface of the cell would have a higher specific activity than that in the body of the solution: and thus P_i entering the tissue would have this higher specific activity, making the calculated uptake rate higher than it should be.

Assume next that hydrolysis of G1^{32}P occurred only within the cell. The total amount of $^{32}P_i$ produced within the tissue would be the same as the total amount of G1^{32}P hydrolysed. In P-deficient tissue, the amount of endogenous P_i (900 nmoles per gram) (Bieleski 1968) was much less than the amount of P_i released by hydrolysis (2500 nmoles per gram per hour); the specific activity of tissue P_i would therefore have been essentially the same as that of the G1^{32}P supplied, and the amount of P_i which passed from the tissue into the solution can be calculated directly (Table 7). In control tissue the situation is different because the endogenous P_i content was greater (30,000 nmoles per gram) and the amount of P_i released by hydrolysis was less (100 nmoles per gram per hour). We make the assumption that only about 12% of the 30,000 nmoles P_i per gram is present in the metabolic space, and thus in the same compartment as the G1P hydrolysis products (Bieleski 1968). From the initial and final specific activity of tissue P_i, calculated in this way, we can calulate the amount of P_i that would have to have moved out of the cell during the course of the experiment in order to yield the observed radioactivity of P_i in the external medium (Table 7). If all the endogenous P_i had equilibrated with the $^{32}P_i$ produced by hydrolysis, instead of 12%, the calculated efflux rate would need to be eight times greater; if none had, the rate would be one-quarter as great. In each case the measured rate of P_i efflux is much less than (1/5–1/150) this hypothetical rate of efflux. Increasing the concentration of P_i in the external medium increased the proportion of $^{32}P_i$ there as compared with the tissue (Table 6). This is to be expected if G1P hydrolysis occurred in the external medium; if it occurred in the tissue, increasing the external concentration of P_i should have decreased the efflux of $^{32}P_i$, and thus decreased the proportion of $^{32}P_i$ in the medium. Thus $^{32}P_i$ found in the

external medium was not produced within the cells. Teleologically speaking, it seems unlikely that a P-deficient plant would relinquish over two-thirds of the P_i produced within it from previously accumulated phosphate ester.

The same general conclusion is reached when the course of hydrolysis is followed in detail (Fig. 1). If G1^{32}P were hydrolysed externally, the specific activity of the 1 mM P_i carrier in the external medium would be low initially, but would increase more or less linearly with time. If P_i was accumulated into the tissue at a constant rate, the rate at which radioactive $^{32}P_i$ appeared in the tissue would be low at first, but would increase progressively with time as the specific activity of the external P_i rose, giving a concave curve. Curve 1 in Figure 1 is one such hypothetical curve of tissue radioactivity against time, obtained when an accumulation rate of 2400 nmoles P_i per gram fresh weight per hour is assumed, and the specific activity of P_i in the medium is as measured and shown in curve 2. There is a very close correspondence between the observed points and the calculated curve. Conversely, if G1^{32}P had been hydrolysed within the cell, the positions would be reversed: the internal $^{32}P_i$ radioactivity would rise linearly with time, and the specific activity of $^{32}P_i$ in the tissue would rise rapidly at first then approach, asymptotically, the specific activity of the G1^{32}P supplied. The relationship of tissue $^{32}P_i$ activity to time would then be a convex curve, not concave as observed (curve 1, Fig. 1). The radioactivity of $^{32}P_i$ reaching the external medium would at first be very low (because of the low initial specific activity of the tissue P_i) but would increase with time, so that the curve relating solution $^{32}P_i$ radioactivity to time would be concave, not convex as observed (curve 2, Fig. 1). If the rate of efflux were to increase as the internal P_i content of the tissue increased, the curve would be more noticeably concave.

Thus all the evidence strongly suggests that the G1^{32}P hydrolysis observed, when intact plants were supplied with substrate, was occurring in the external solution. It can be concluded that in the P-deficient plant, at least 35% and probably 60% of the phosphatase is accessible to external substrate. Following hydrolysis, there was a rapid accumulation of the P_i produced: the rate of accumulation in P-deficient tissue was of the same order as the rate of hydrolysis.

Can we envisage any advantage to the species in having so much phosphatase externally accessible? *S. oligorrhiza* has a very effective mechanism for accumulating P_i: it can withdraw P_i from solution down to about 3×10^{-7} M (Bieleski, unpublished data). On the other hand, there is sketchy evidence which suggests that plants do not normally take up phosphate esters very readily (e.g. Bieleski 1960). In *S. oligorrhiza* which is near death from P deficiency, the P_i and the phosphate ester contents are about the same, 600 nmole per gram fresh weight (Bieleski 1968). On death, these phosphorus compounds will be released to the medium. A nearby plant which possesses an active external phosphatase can lay claim to twice as much phosphate as one without. Competition for P_i by microorganisms might force the adoption of such a mechanism for scavenging phosphate esters. In keeping with this suggested role, the highest concentration of phosphatase in P-deficient plants is found in those part of the plant (roots and lower epidermis) which are in direct contact with the external medium, and this is a feature of P-deficient plants rather than control plants. At present, the relationship of the "inducible" enzyme to the overall phosphatase increase and the external localization during P-deficiency remains rather a mystery.

Logically, the inducible enzyme would be expected to be the enzyme primarily involved in these two phenomena; but the results of the dissection experiments make this seem unlikely. A final question concerns the generality of the phenomenon. *S. oligorrhiza* is a specialized higher plant from an unusual habitat. However, the occurrence of the same phenomenon in four lower plant groups suggests that, in turn, other less specialized plants will be found to show the same response to P deficiency (Woolhouse 1969). Whether this could have a diagnostic value for P deficiency remains to be seen.

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VI. References

Bieleski, R. L. (1960).—The physiology of sugar-cane. III. Characteristics of sugar uptake in slices of mature and immature storage tissue. *Aust. J. biol. Sci.* **13**, 203–20.

Bieleski, R. L. (1965).—Separation of phosphate esters by thin-layer chromatography and electrophoresis. *Analyt. Biochem.* **12**, 230–4.

Bieleski, R. L. (1968).—Effect of phosphorus deficiency on levels of phosphorus compounds in *Spirodela*. *Pl. Physiol., Lancaster* **43**, 1309–16.

Blum, J. J. (1965).—Observations on the acid phosphatases of *Euglena gracilis*. *J. Cell Biol.* **24**, 223–34.

Burstone, M. S. (1962).—"Enzyme Histochemistry and Its Application in the Study of Neoplasms." (Academic Press: New York.)

Gomori, G. (1950).—An improved histochemical technic for acid phosphatase. *Stain Technol.* **25**, 81–5.

Honda, S. I., Hongladarom, T., and Laties, G. G. (1966).—A new isolation medium for plant organelles. *J. exp. Bot.* **17**, 460–72.

McLellan, W. L., and Lampen, J. O. (1963).—The acid phosphatase of yeast. Localization and secretion by protoplasts. *Biochim. biophys. Acta* **67**, 324–6.

Malamy, M., and Horecker, B. (1961).—The localization of alkaline phosphatase in *E. coli* K_{12}. *Biochem. biophys. Res. Commun.* **5**, 104–8.

Nyc, J. F. (1967).—A repressible acid phosphatase in *Neurospora crassa*. *Biochem. biophys. Res. Commun.* **27**, 183–8.

Reid, M. S. (1968).—Response of *Spirodela* to phosphorus deficiency. Ph.D. Thesis, University of Auckland.

Reid, M. S., and Bieleski, R. L. (1970).—Changes in phosphatase activity in phosphorus-deficient *Spirodela*. *Planta* **94**, 273–81.

Sommer, J. R., and Blum, J. J. (1965).—Cytochemical localization of acid phosphatases in *Euglena gracilis*. *J. Cell Biol.* **24**, 235–51.

Soumalainen, H., Linko, M., and Oura, E. (1960).—Changes in the phosphatase activity of baker's yeast during the growth phase and location of the phosphatases in the yeast cell. *Biochim. biophys. Acta* **37**, 482–90.

Torriani, A. (1960).—Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. *Biochim. biophys. Acta* **38**, 460–79.

Weimberg, R., and Orton, W. L. (1964).—Evidence for an exocellular site for the acid phosphatase of *Saccharomyces mellis*. *J. Bact.* **88**, 1743–54.

Woolhouse, H. W. (1969).—Differences in the properties of the acid phosphatases of plant roots and their significance in the evolution of edaphic ecotypes. In "Ecological Aspects of the Mineral Nutrition of Plants". (Ed. I. H. Rorison.) pp. 357–80. (Blackwell Scientific Publications: Oxford.)