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# THE ACTIVITIES OF PHOSPHATASES, PYROPHOSPHATASES AND ADENOSINE TRIPHOSPHATASES FROM NORMAL AND BORON DEFICIENT BEAN ROOTS

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**Abstract**——The activities of the "soluble" fraction (100,000*g* supernatant) from *Vicia faba* var. *minor* roots for hydrolysis of phenylphosphate, pyrophosphate and adenosine triphosphate (ATP) have been determined at different pH values. At pH 5·1 all three substrates are hydrolysed by the same enzyme, an "acid" phosphatase which does not require $Mg^{2+}$. Hydrolysis of phenyl phosphate does not occur at pH 7·2 or 8·2, and the pyrophosphate and ATPase activities require $Mg^{2+}$ at these pH values. In the normal root the acid phosphatase and ATPase (at pH 7·2) activities increase up the root from a minimum in the tip, whereas pyrophosphatase (pH 7·2) shows a reverse distribution. Boron deficient roots show the same pattern of distribution of activities, with increases in acid phosphatase and ATPase and decreases in pyrophosphatase as compared with the normal root. Although the changes occur before the deficient root ceases to grow they are considered to be due to physiological or morphological changes in the root.

## INTRODUCTION

ENZYMIC activities for the hydrolysis of pyrophosphate and adenosine triphosphate (ATP) were observed in extracts of bean (*Vicia faba* var. *minor*) roots during the course of investigations on amino acid-dependent ATP–pyrophosphate exchange.[1] As the exchange activity in the soluble fraction of root homogenates was found to decrease in boron deficient tissue, it was considered desirable to investigate the effect of boron deficiency on other enzymic activities from the tissue and those for the hydrolysis of phosphates appeared suitable. Pyrophosphatase activity is of interest with regard to a number of synthetic processes in which pyrophosphate appears as a product, since the hydrolysis of the pyrophosphate would serve to displace the equilibrium towards the completion of the process. The investigation of ATPase activity was desirable in view of the observations of Maevskaya and Alekseeva[2] of increased ATPase activity in apical buds of boron deficient sunflower plants before signs of deficiency were visible.

## RESULTS AND DISCUSSION

Initial investigations of the soluble fraction from *Vicia faba* var. *minor* roots indicated the presence of activities for the hydrolysis of phenylphosphate, pyrophosphate and ATP. Phenylphosphatase activity was present at pH 5·1, very slight at pH 7·2 and absent at pH 8·2. The activity was unaffected by the concentration of $Mg^{2+}$ over the range 0–22 mM (Table 1). Pyrophosphatase activity was demonstrated at all three pH values, $Mg^{2+}$ being inhibitory

[1] R. W. HINDE, L. R. FINCH and S. CORY, *Phytochem.* **5**, 609 (1966).
[2] A. N. MAEVSKAYA and K. A. ALEKSEEVA, *Dokl. Akad. Nauk SSSR* **156**, 212 (1964).

at pH 5·1, but stimulating at higher pH. A similar result was observed for the hydrolysis of ATP.

It seemed possible that the activities observed at pH 5·1 might all be due to a non-specific acid phosphatase. If the substrates were competing for the one enzyme then, when two substrates were present at saturating concentrations, the rate might be expected to be intermediate between the rates with the substrates present individually at the same concentration as in the mixture. If the substrates were being acted upon by different enzymes then, if there

TABLE 1. EFFECT OF $Mg^{2+}$ ON PHENYLPHOSPHATASE, PYROPHOSPHATASE AND ATPASE ACTIVITIES FROM BEAN ROOTS

| Substrate (3 mM) | $MgCl_2$ concn. (mM) | pH of incubation | | |
|---|---|---|---|---|
| | | 5·1 | 7·2 | 8·2 |
| | | Rate of hydrolysis (nmoles/mg protein per min) | | |
| Phenylphosphate | 2·4* | 57 | — | — |
| Phenylphosphate | 12·9* | 57 | — | — |
| Phenylphosphate | 21·9* | 57 | — | — |
| Pyrophosphate | 2·4* | 189 | 55 | 46 |
| Pyrophosphate | 12·9 | 147 | 152 | 157 |
| ATP | 2·4* | 149 | 78 | — |
| ATP | 8·4* | 128 | 103 | — |
| ATP | 17·4 | 110 | 95 | — |
| Phenylphosphate | 0† | 102 | — | — |
| Phenylphosphate | 10† | 102 | — | — |
| Pyrophosphate | 0† | 177 | — | — |
| Pyrophosphate | 10† | 155 | — | — |
| ATP | 0† | 121 | | |
| ATP | 10† | 111 | | |

0·6 ml of the soluble fraction (100,000g supernatant) of a homogenate of bean root tips (8mm) in a total volume of 1 ml containing either 100 mM acetate buffer (pH 5·1), or 100 mM tris–HCl buffer (pH 7·2 and pH 8·2), with the stated substrates and concentrations of $MgCl_2$ was incubated for 10 min. at 27°. The reaction was stopped by the addition of 3 ml of cold 6% TCA and the concentration of $P_i$ estimated as described in the text. Zero time controls were carried out for all incubations.

* Extracts from 8 mm root-tips of bean seedlings germinated on vermiculite at 25° for three days.

† Extracts, dialysed for 16 hr against two changes of 2 l. of 1 mM tris buffer, pH 7·2, from 8 mm root-tips of bean seedlings germinated on vermiculite at 25° for 42 hr.

were no inhibitory effects, the rate with both substrates might be expected to be the sum of the values with the individual substrates. An experiment to test this possibility showed that the mixtures gave values which were intermediate between those with the individual substrates, giving a strong indication that only one enzyme was involved in the hydrolysis of the various substrates at pH 5·1, namely an acid phosphatase. Studies showing a parallel distribution of the activities in different root sections also supported the suggestion that only one enzyme was responsible for the hydrolysis of the three substrates in the incubations at pH 5·1.

Investigations of the distribution of the various activities in roots showed that the acid phosphatase increased away from the root tip, pyrophosphatase at pH 7·2 or 8·2 was greatest

in the tip and decreased up the root, whereas ATPase activity at these pH values exhibited a similar trend to the acid phosphatase. Comparison of normal and boron deficient roots established that the activities were affected by deficiency. Table 2 gives results with pyrophosphate as substrate. The activity at pH 5·1 (acid phosphatase) increased away from the tip and was greater in the deficient root than in the corresponding section of the normal root. The reverse result was found for the activity at pH 7·2 (alkaline pyrophosphatase). This was greatest in the tip and was less in the deficient root than in the corresponding section of the normal root. A factor which would have influenced the results shown in Table 2 is the inhibitory effect of orthophosphate ($P_i$) on all the activities investigated. The average $P_i$ values which are shown in Table 2 are the average of the initial and final $P_i$ concentrations in

TABLE 2. PYROPHOSPHATASE ACTIVITIES FROM VARIOUS SECTIONS OF NORMAL ($B_+$) AND BORON DEFICIENT ($B_-$) ROOTS

| | Normal tissue | | | Deficient tissue | | |
|---|---|---|---|---|---|---|
| pH of incubation | Tip (3 mm) | Section 2 (5 mm) | Section 3 (5 mm) | Tip (3 mm) | Section 2 (5 mm) | Section 3 (5 mm) |
| | Protein content of incubation mixture (mg/ml) | | | | | |
| | 0·53 | 0·32 | 0·26 | 0·49 | 0·32 | 0·37 |
| | Rate of hydrolysis (nmoles $P_i$ released/mg protein/min) | | | | | |
| 5·1 | 139 | 181 | 242 | 185 | 272 | 350 |
| | Average $P_i$ concentration of incubation mixture (mM) | | | | | |
| | 0·66 | 0·85 | 0·92 | 0·91 | 1·20 | 1·38 |
| | Rate of hydrolysis (nmoles $P_i$ released/mg protein/min) | | | | | |
| 7·2 | 200 | 151 | 139 | 185 | 141 | 108 |
| | Average $P_i$ concentration of incubation mixture (mM) | | | | | |
| | 0·82 | 0·88 | 0·89 | 0·95 | 1·09 | 1·12 |

Soluble fractions (100,000*g* supernatants) were prepared from sections of roots grown in liquid media with and without boron for 35 hr. 0·6 ml of the preparation in a total volume of 1·0 ml containing 3 mM $Na_4P_2O_7$ (adjusted to pH 7·2) and either 100 mM acetate buffer (pH 5·1), or 9·5 mM $MgCl_2$ and 100 mM tris–HCl buffer (pH 7·2) was incubated for 7 min at 27°. The extent of reaction was assayed as described in Table 1.

the incubation mixtures, that is, they are the sum of endogenous $P_i$ plus the average amount produced as a result of the incubation.

In the case of the acid phosphatase the inhibitory effect of $P_i$ in the incubation would not alter the qualitative comparison of the observed distribution of activities, since the highest $P_i$ values correspond to the highest activities. This is not so in the case of the alkaline pyrophosphatase however, since the highest activities observed corresponded to the lowest $P_i$ levels. Thus the high activities could have been an artifact resulting from the low $P_i$ concentration in the incubation. To exclude this possibility the enzyme preparations were dialysed free from $P_i$ and assayed under conditions allowing the estimation of minimal $P_i$ concentrations in the incubation. The results shown in Table 3 confirm those of Table 2.

Results of similar experiments on the distribution of activities for the hydrolysis of ATP in normal and deficient tissues are given in Table 4. The acid phosphatase activity measured at

TABLE 3. ALKALINE PYROPHOSPHATASE FROM VARIOUS SECTIONS OF NORMAL AND DEFICIENT BEAN ROOTS

| Time of growth in liquid medium (hr) | Normal roots ($B_+$) | | | Deficient roots ($B_-$) | | |
|---|---|---|---|---|---|---|
| | Tip (3 mm) | Section 2 (5 mm) | Section 3 (5 mm) | Tip (3 mm) | Section 2 (5 mm) | Section 3 (5 mm) |
| | Rate of hydrolysis (nmoles $P_i$ released/mg protein/min) | | | | | |
| 24 | 120 | 51 | 4 | 77 | 16 | Nil |
| 48 | 163 | 78 | 63 | 84 | 48 | 35 |

Soluble fractions (100,000*g* supernatants) were prepared from sections of 100 roots grown in liquid medium with and without boron for the stated times. The preparations were dialysed against two changes of 5 mM tris–HCl (pH 7·5), 0·5 mM EDTA for 20 hr. A 5 ml portion of a reaction mixture containing 100 mM tris–HCl (pH 7·2), 3 mM $Na_4P_2O_7$ (pH 7·2), 10 mM $MgCl_2$ and 0·5–1·5 mg of extract protein was incubated for 10 min at 27°. A 3 ml sample was taken and assayed for $P_i$ as described in the text. Non-enzyme controls were assayed concurrently and the enzyme preparations were demonstrated to be free of phosphate. The net growth of the roots given as the mean ± the standard deviation for 15 roots was, at 24 hr and 48 hr respectively,

$16{\cdot}93 \pm 1{\cdot}67$ and $39{\cdot}86 \pm 1{\cdot}91$ for $B_+$ roots
and $11{\cdot}31 \pm 0{\cdot}90$ and $14{\cdot}06 \pm 1{\cdot}25$ for $B_-$ roots

TABLE 4. ATPASE ACTIVITIES FROM VARIOUS SECTIONS OF NORMAL AND BORON DEFICIENT ROOTS

| pH of incubation | Normal tissue | | | Deficient tissue | | |
|---|---|---|---|---|---|---|
| | Tip (3 mm) | Section 2 (5 mm) | Section 3 (5 mm) | Tip (3 mm) | Section 2 (5 mm) | Section 3 (5 mm) |
| | Protein content of incubation mixture (mg/ml) | | | | | |
| | 1·13 | 0·84 | 0·53 | 1·07 | 0·74 | 0·58 |
| | Rate of hydrolysis (nmoles $P_i$ released/mg protein/min) | | | | | |
| 5·1 | 140 | 150 | 190 | 180 | 200 | 210 |
| 7·2 | 90 | 90 | 110 | 110 | 150 | 150 |
| 8·2 | 30 | 150 | 110 | 100 | 150 | 130 |
| | Average $P_i$ concentration of incubation mixture (mM) | | | | | |
| 5·1 | 1·07 | 2·26 | 1·99 | 1·76 | 2·21 | 2·35 |
| 7·2 | 1·01 | 2·03 | 1·94 | 1·61 | 2·12 | 2·30 |
| 8·2 | 0·77 | 1·88 | 1·69 | 1·41 | 1·96 | 2·01 |

Soluble fractions (100,000*g* supernatants) were prepared from sections of roots grown in liquid medium with and without boron for 26·5 hr. 0·6 ml of the preparation in a total volume of 1·0 ml containing 3 mM $Na_2H_2ATP$ and either 100 mM acetate buffer (pH 5·1), or 5·4 mM $MgCl_2$ and 100 mM tris–HCl buffer (pH 7·2 and pH 8·2) was incubated for 10 min at 27°. The extent of reaction was assayed as described for Table 1.

pH 5·1 with ATP as substrate showed the same pattern as with pyrophosphate. The ATPase activities at higher pH values also increased away from the tip and were greater in deficient than in normal tissue. The presence of $P_i$ during the incubation may have affected the results but would not have changed their qualitative relationship, because the highest $P_i$ levels were found in the extracts of highest activity.

The results presented relate to three enzymes, acid phosphatase, pyrophosphatase and ATPase. All three varied in activity between different sections of the root and between normal and deficient roots, as has been found with amino acid-dependent ATP-pyrophosphate exchange activity.[1] For all four activities the same type of change is found when the tissue is made boron deficient; the activities become more like that of a section in the normal root further away from the tip. As discussed in the previous paper,[1] it would seem likely that such an effect is due to general physiological and morphological changes which are far removed in the chain of causation from the original event that is susceptible to boron deficiency.

## MATERIALS AND METHODS

The growth, harvesting and preparation of the soluble fraction from sections of the radicle of "tick" or field beans (*Vicia faba* var. *minor*) were as described in the previous paper.[1] In some cases the preparations were freed of $Mg^{2+}$ and orthophosphate ($P_i$) by overnight dialysis at 0–4° against three changes of buffer (10 mM tris pH 7·2, 0·5 mM EDTA).

The activities were assayed by incubation at 27° with the appropriate substrate in 100 mM buffer (sodium acetate-acetic acid at pH 5·1 and tris–HCl at pH 7·2 and 8·2). The reactions were stopped by the addition of 3 ml of cold 6% TCA to the 1 ml of incubation mixture. Phosphate determinations were made on zero time samples carried through the phosphate estimation procedure with the test samples. This allowed for endogenous $P_i$ in the enzyme extract and for hydrolysis of substrate during the estimation. When it was desired to keep the phosphate level to a minimum during the incubation, the volume of the incubation mixture was increased to 5 ml with a corresponding dilution of the enzyme concentration, and the sample size was increased to 3 ml. The incubation was stopped by the addition of 1·5 ml of a mixture of 15% w/v TCA-19% w/v $HClO_4$, the protein precipitate was spun down and the supernatant used directly for $P_i$ estimation by the addition of the amidol and molybdate reagents. Non-enzyme and non-substrate controls were assayed concurrently in these experiments

The method of Allen[3] was used for the estimation of $P_i$.

Protein was determined by the method of Cleland and Slater.[4]

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[3] R. J. L. Allen, *Biochem. J.* **34**, 858 (1940).
[4] K. W. Cleland and E. C. Slater, *Biochem. J.* **53**, 547 (1953).