CHAPTER I

SECTION A

LEVELS OF DIFFERENT PHOSPHATE METABOLIZING ENZYMES IN

THE SEEDS OF V. sinensis DURING GERMINATION
INTRODUCTION

During the early stage of germination, many enzyme systems begin to operate in the germinating seeds. Storage materials are broken down and part of the breakdown products are transported from the cotyledons or endosperms to the developing axis of the embryo. These series of events are well documented and some recent reviews of these sequences have appeared (3, 98, 106-107). Phosphate plays an extremely important role in a variety of reactions in seeds. Thus, the phosphates are required for the formation of nucleic acids, which in turn, intimately connected with protein synthesis and the hereditary constituent of the plant cell. The function of phospholipid, such as, lecithin, in controlling surface properties and permeability of cells and of its organelles, is well-established today. The various sugar phosphates and nucleotides are closely linked to the energy producing processes in the cell during germination. Phosphorus appears in seeds primarily in the organic form and very little seem to be present as inorganic orthophosphate. Among the phosphorus-containing compounds are the nucleic acids, phospholipids, phosphate esters of sugars and nucleotides and the hexaphosphoric acid esters of inositol in the form of mixed calcium and magnesium salts known as phytin which is frequently present in many seeds and may constitute up to 80% of the total phosphorus content of the seeds. Usually there is a good correlation between the rapidity of phytin breakdown and the phytase activity of the seed and the seedling (108). It has been found that in the dry wheatseed about 80% of
the phytase is present in the endosperm and only 1% in the embryo. In addition to phytase, seeds contain many phosphatases, the activity of which are increased during germination. This phosphatase can account for the turnover of all the phosphate esters present in the seed.

The phosphatase enzymes from plant sources which differ from animals have been examined and some of the reported results do not agree with each other. The possible identity of the phosphomonoesterase and pyrophosphatase in higher plant has been suggested by some workers (53), whereas the independent nature of those enzymes in bacteria and yeast has been claimed by others (55). On the other hand, the relation of phosphatase to phytase, which has been believed to be distinct enzyme (56) has also been criticized (57). However, the presence of the isozymes for the plant phosphatase is well established (109, 110).

The germination of seeds offers a field of study in the biochemistry of development which is of particular interest in other living systems. Although we know a lot about the progress, we have only now begun to answer important questions concerning the metabolic control of germination. The determination of the activity of an enzyme has a wide range of application. Enzyme assays are of importance in the field of food, agriculture, forensic and clinical chemistry, especially in the detection of various diseases of the body. Section I of this Chapter is mainly concerned with the studies on the changes in various enzymes involved in phosphate metabolism e.g., acid phosphatase, acid and alkaline pyrophosphatases, phytase etc. in the germinating seeds of V. sinensis.
MATERIALS

The substrates viz. p-nitrophenyl phosphate, Na-pyrophosphate and Na-phytate were obtained from Sigma Chemical Co. Mo., U.S.A. p-Nitrophenol was also purchased from Sigma Chemical Co., U.S.A. Triton X-100 was obtained from Rohme Haas Company, Philadelphia, Pa, U.S.A. Seeds of *Vigna sinensis* (Linn) Savi were purchased from local market. All other common chemicals used, were of analytical grade and purchased from BDH and E. Merck.

METHODS

Seeds of *V. sinensis* (Linn) Savi were germinated according to the method of Das *et al.* (111). Required amount of wholesome seeds were taken. These seeds were washed in tap water several times and for surface sterilization these were immersed in 0.02% *HgCl₂* for 10 min followed by several wash with distilled water. The seeds were then immersed in distilled water and kept in dark place for at least 3-4 h at room temperature (25° - 30°C). After that the seeds were taken out, peeled and washed with distilled water and spread over moist blotting paper (containing 0.01% chloramphenicol) in a plastic tray and allowed to germinate in dark place at room temperature (25° - 30°C). For half-seed experiments two cotyledons of the ungerminated seeds were separated from each other as 'cotyledons with embryo' and 'cotyledon without embryo' just after peeling. After every 24 h, germinating seeds were taken out, washed with distilled water and again spread over moist blotting paper to continue germination for next
Whenever required, inhibitors or other chemicals at certain doses were given at the initial stage of germination.

**Preparation of Cell-free Extract**

After completion of different period of germination, seeds were taken out, washed 3-4 times with distilled water and then cotyledons were separated from embryo and kept in ice-cold condition. In the half-seed experiment the adhering embryo was also separated from the cotyledon before extraction.

After washing, a 10% homogenate of the seeds were made in ice-cold buffer (10 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100) by grinding with seensand in a mortar-pestle. The homogenate was kept overnight at 4°C and was strained through double layer of cheese cloth to remove coarser cell debris. The homogenate was then centrifuged at 600 x g for 20 min at 4°C in a refrigerated centrifuge (Model B-20) to remove remaining cell debris. The supernatant thus obtained was further centrifuged at 10,000 x g for 20 min at 4°C. The pellet was discarded and the supernatant was taken as the enzyme source for enzyme assay.

**Enzyme Assay**

**Acid phosphatase** - The activity of acid phosphatase was estimated according to the following method as described by Michel et al. (112). The assay system was a total volume of 2 ml containing 0.5 ml of 0.1 M acetate buffer (pH 5.0), 0.05 ml substrate (0.025 M p-nitrophenyl phosphate), 0.005 ml of enzyme source and rest distilled water. Incubation was carried out for 10 min.
at $37^\circ$C. The reaction was stopped by the addition of 4 ml of 0.1 M NaOH. The liberated p-nitrophenol was measured at 410 nm in a colorimeter (Bausch & Lomb, Spectronic-20). The amount of p-nitrophenol liberated was estimated by comparing with standard values. The specific activity of the enzyme was expressed as μmole of p-nitrophenol liberated/h/mg of protein.

Acid pyrophosphatase & Alkaline pyrophosphatase: The activities of acid and alkaline pyrophosphatases were estimated according to the following method as described by Naganna et al. (50). In the case of acid pyrophosphatase, the incubation mixture was a total volume of 0.5 ml containing 0.2 ml of 0.03 M veronal-acetate buffer (pH 4.0), 0.05 ml of neutralized pyrophosphate (0.025 M), 0.05 ml of enzyme source and 0.2 ml of neutral distilled water. The incubation period was 15 min at $37^\circ$C. The enzyme reaction was stopped with 0.5 ml of 10% (w/v) trichloroacetic acid (TCA). After centrifugation, the orthophosphate in the supernatant was estimated by the method of Lowry and Lopez (113), using freshly prepared ascorbic acid solution.

In the case of alkaline pyrophosphatase, the assay method was similar to that applied in the case of acid pyrophosphatase except 0.05 M Tris-glycine buffer (pH 8.0), 0.1 M MgCl$_2$ were used. For the assay of the enzyme, the incubation mixture, in a total volume of 0.5 ml, contained 0.2 ml of 0.05 M Tris-glycine buffer (pH 8.0), 0.05 ml of 0.1 M MgCl$_2$, 0.05 ml of neutralised pyrophosphate (0.025 M), 0.05 ml of enzyme source and 0.15 ml of neutral distilled water. Other details were the same as those given under acid pyrophosphatase assay. The specific activities of the two enzymes.
were expressed as μmole of orthophosphate liberated/h/mg of protein.

Phytase: The phytase activity was assayed according to the method as described by Gibbins et al. (114). The assay system was a total volume of 1.0 ml, containing 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.01 ml of 10 mM phytate, 0.1 ml enzyme source, and 0.64 ml of neutral distilled water. Incubation was carried out for 3 h at 37°C. The reaction was stopped by adding 1 ml of 10% ice-cold trichloroacetic acid and the denatured protein was eliminated by centrifugation. The orthophosphate in the supernatant was measured according to the method of Lowry and Lopez (113). The specific activity of the enzyme was expressed as μmole of orthophosphate liberated/h/mg of protein.

Estimation of Protein:

Samples withdrawn from cell-free extract were added to double volume of 10% (w/v) ice-cold trichloroacetic acid in 10 ml of tapered centrifuge tubes, well mixed and allowed to stand for 30 minutes in ice cold condition. The protein-free supernatant was removed by centrifugation. After dissolving the precipitated protein in 0.1 N NaOH, the protein was estimated by the Folin-phenol method of Lowry et al. (115). Bovine serum albumin of known concentration was taken as a standard.
It appears from the data given in the Table 1 that the level of free inorganic phosphate in the cotyledons increases with the time of germination. In contrast, the embryonic inorganic phosphate has been found to increase sharply up to 24 h of germination and then decreased slowly as the germination proceeded. The inorganic phosphate content in the 'cotyledon with embryo' (half-seeds) appeared to be greater than that in 'cotyledon without embryo' (half-seeds). From the Table 2 it is evident that the phytase activity increased in the 'cotyledon with embryo' and also in the embryo during the course of germination. But in the 'cotyledon without embryo' the phytase activity remained constant. It appears from the data given in Table 3 that the acid phosphatase activity increased in the cotyledon with increase in time of germination. But in the embryo acid phosphatase activity increased sharply from 15 h of germination and reached a maximum in between 24-48 h and then started to decline in the latter stages of germination. In the cotyledon the change in acid phosphatase activity was different. The acid phosphatase activity in 'cotyledon with embryo' and 'cotyledon without embryo' started to rise after 4 h and 24 h of imbibition respectively and gradually increased up to 96 h of germination. The acid phosphatase activity in 'cotyledon with embryo' was greater than that in 'cotyledon without embryo'. The maximum activity of acid phosphatase was found in the embryo. The effect of temperature on the development of acid phosphatase activity during germination was also studied. The results (Fig. 1) show that with increase in temperature of germination, there was
increased acid phosphatase activity. In this experiment, chloramphenicol (0.01%) was used as an antibacterial agent. It was observed that this concentration of chloramphenicol had no effect on germination, as well as on the acid phosphatase activity in the germinating seeds of *V. sinensis*.

The data given in Fig. 2 indicate that the acid phosphatase activity in the cotyledon increased with increase in time of germination, however, the activity was found to be decreased when the half-seeds were kept in cycloheximide (100 μg/ml) at 28°C after 2 h of imbibition.

The results depicted in Fig. 3 also indicate that acid phosphatase activity increased in both embryo and cotyledon (half-seeds with and without embryo) with increase in time of germination. In both the cotyledons (half-seeds) the acid phosphatase activity increased gradually after 15-24 h of germination. However, the overall activity was much higher in 'cotyledon with embryo'. The effects of different inhibitors have been studied on the developmental process of acid phosphatase during germination (Fig. 3). Rifampicin did not have any effect on the development of acid phosphatase activity in the germinating seeds of *V. sinensis*. However, the increase in acid phosphatase activity was inhibited by cycloheximide. It should be noted that the increase in acid phosphatase activity was not inhibited by chloramphenicol. Another interesting observation is that the extent of inhibition of the acid phosphatase activity during germination was more pronounced with cycloheximide than that observed either with actinomycin D or with ethidium bromide.
The effects of different plant growth substances e.g.,
gibberellic acid (GA3) and indole acetic acid (IAA), have been
studied (Fig. 4). GA3 was found to have an inhibitory effect on
the development of acid phosphatase activity in the germinating
half-seeds (cotyledon without embryo) of *V. sinensis*. Similar in­hibitory effect was also found with another growth promoter namely
indole acetic acid (IAA).

The effects of cell-free extract of dry cotyledons and that
of embryo from 96 h germinating seeds on the acid phosphatase ac­tivity present in the cell-free extract of embryo from 24 h germina­ted seeds of *V. sinensis* have been studied (Table 4). But no in­hibition was found in either of the cases.

Like acid phosphatase, both acid and alkaline pyrophospha­tase activities were found to increase in embryo and in cotyledon of *V. sinensis* during germination (Tables 5 & 6). However, the
activities of both the enzymes were much higher in embryo. The rise in activities of both the enzymes in cotyledon as well as in
embryo appeared to be inhibited by cycloheximide (Figs. 5 & 6).

Fig. 8 shows that the development of acid phosphatase
activity was inhibited by cycloheximide treatment after 15 and 24 h
of germination (as indicated in the Figure by arrow mark). But no
inhibitory effect of cycloheximide on the acid phosphatase activity
was found after 72 h of germination. Interestingly, the increase
in acid phosphatase activity in germinating seeds of *V. sinensis*
was completely inhibited if the seeds were imbibed in a cyclohexi­mide solution for 2 h at room temperature followed by normal ger­
It was found (Figs. 9A & 9B) that the increase in acid phosphatase activity was inhibited by the addition of phosphate and pyrophosphate. But suppressive effect of pyrophosphate was five times greater than that of phosphate. The extent of suppression increased in both the cases with increase in concentration. The acid phosphatase activity remained constant at 100 mM of phosphate and 20 mM pyrophosphate concentration. But at 50 mM pyrophosphate concentration the acid phosphatase activity decreased with increase in time of germination. Another interesting finding (Fig. 9C) shows that the specific activity of acid phosphatase was not further reduced (compared to the extent of inhibition by cycloheximide) by the addition of phosphate when the enzyme synthesis was blocked by cycloheximide. The results (Table 7) show that there was no effect of phosphate or pyrophosphate treated extract on the acid phosphatase activity in the control system.
### Table 1

*Changes in free phosphate in the cotyledon end embryo of *V. sinensis* during germination*

<table>
<thead>
<tr>
<th>Period of germination (Hours)</th>
<th>Cotyledon without embryo</th>
<th>Cotyledon with embryo</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.98</td>
<td>9.98</td>
<td>27.69</td>
</tr>
<tr>
<td>24</td>
<td>20.81</td>
<td>22.05</td>
<td>109.43</td>
</tr>
<tr>
<td>48</td>
<td>29.58</td>
<td>31.88</td>
<td>68.35</td>
</tr>
<tr>
<td>72</td>
<td>30.67</td>
<td>38.96</td>
<td>65.29</td>
</tr>
<tr>
<td>96</td>
<td>27.07</td>
<td>33.73</td>
<td>45.33</td>
</tr>
</tbody>
</table>

### Table 2

*Developmental changes in phytase activity in the cotyledon and embryo of *V. sinensis* during germination*

<table>
<thead>
<tr>
<th>Period of germination (Hours)</th>
<th>Phytase activity (µmole of phosphate liberated/h/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cotyledon without embryo</td>
</tr>
<tr>
<td>0</td>
<td>0.10</td>
</tr>
<tr>
<td>24</td>
<td>0.10</td>
</tr>
<tr>
<td>48</td>
<td>0.10</td>
</tr>
<tr>
<td>72</td>
<td>0.10</td>
</tr>
<tr>
<td>96</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Table 3

Developmental changes in acid phosphatase activity in the cotyledon and embryo of V. sinensis during germination

<table>
<thead>
<tr>
<th>Period of germination (Hours)</th>
<th>Acid phosphatase activity (µmole of p-nitrophenol liberated/h/mg of protein)</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.34</td>
<td>4.34</td>
</tr>
<tr>
<td>15</td>
<td>3.97</td>
<td>7.03</td>
</tr>
<tr>
<td>24</td>
<td>5.48</td>
<td>11.22</td>
</tr>
<tr>
<td>48</td>
<td>10.48</td>
<td>19.55</td>
</tr>
<tr>
<td>72</td>
<td>11.49</td>
<td>19.83</td>
</tr>
<tr>
<td>96</td>
<td>11.10</td>
<td>21.01</td>
</tr>
</tbody>
</table>
Table 4

Effects of cell-free extracts of dry cotyledon and 96-h germinating embryo on the acid phosphatase activity in the cell-free extract of 24-h germinating embryo of seeds of 7. sinensis

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amount of protein added (μg)</th>
<th>Activity (AOD at 410nm/10 min)</th>
<th>Percentage of activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 24-h germinating embryo</td>
<td>53.5</td>
<td>0.75</td>
<td>100.0</td>
</tr>
<tr>
<td>2a. Dry cotyledon</td>
<td>107.5</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>b. Dry cotyledon + 24-h germinating embryo</td>
<td>107.5</td>
<td>0.86</td>
<td>97.3</td>
</tr>
<tr>
<td>3a. Dry cotyledon</td>
<td>215.0</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>b. Dry cotyledon + 24-h germinating embryo</td>
<td>215.0</td>
<td>0.94</td>
<td>93.3</td>
</tr>
<tr>
<td>4a. Dry cotyledon</td>
<td>430.0</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>b. Dry cotyledon + 24-h germinating embryo</td>
<td>430.0</td>
<td>113.0</td>
<td>96.0</td>
</tr>
<tr>
<td>5a. 96-h germinating embryo</td>
<td>36.0</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>b. 96-h germinating embryo + 24-h germinating embryo</td>
<td>36.0</td>
<td>0.88</td>
<td>100.0</td>
</tr>
<tr>
<td>6a. 96-h germinating embryo</td>
<td>72.0</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>b. 96-h germinating embryo + 24-h germinating embryo</td>
<td>72.0</td>
<td>0.985</td>
<td>100.0</td>
</tr>
</tbody>
</table>

(Continued)
Table 4 (Contd.)

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amount of protein added (μg)</th>
<th>Activity (AOD at 410 nm/10 min)</th>
<th>Percentage of activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a. 96-h germinating embryo</td>
<td>144.0</td>
<td>0.42</td>
<td></td>
</tr>
</tbody>
</table>
| b. 96-h germinating embryo + 24-h germinating embryo | 144.0 | 53.5 | 1.18 | 100%

Cotyledon and embryo were homogenized and centrifuged as described in 'Methods' in this Section. Activity in 53.5 μg protein of 24-h germinating embryo was taken as 100 per cent and the activity was measured in the presence of increasing protein of the cell-free extracts of dry cotyledon and 96-h germinating embryo.

Table 5
Developmental changes in acid pyrophosphatase activity in the cotyledon and embryo of V. sinensis during germination

<table>
<thead>
<tr>
<th>Period of germination (Hours)</th>
<th>Acid pyrophosphatase activity (μmole of phosphate liberated/h/mg of protein)</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'Cotyledon without embryo'</td>
<td>'Cotyledon with embryo'</td>
</tr>
<tr>
<td>0</td>
<td>1.97</td>
<td>1.97</td>
</tr>
<tr>
<td>15</td>
<td>1.43</td>
<td>1.78</td>
</tr>
<tr>
<td>24</td>
<td>1.99</td>
<td>3.33</td>
</tr>
<tr>
<td>48</td>
<td>3.08</td>
<td>5.0</td>
</tr>
<tr>
<td>72</td>
<td>3.54</td>
<td>5.26</td>
</tr>
<tr>
<td>96</td>
<td>5.35</td>
<td>7.11</td>
</tr>
</tbody>
</table>
Table 6

Developmental changes in alkaline pyrophosphatase activity in the cotyledons and embryo of *V. sinensis* during germination

<table>
<thead>
<tr>
<th>Period of germination (Hours)</th>
<th>Alkaline pyrophosphatase activity (µmole of phosphate liberated/1mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'Cotyledon without embryo'</td>
</tr>
<tr>
<td>0</td>
<td>1.45</td>
</tr>
<tr>
<td>15</td>
<td>1.55</td>
</tr>
<tr>
<td>24</td>
<td>1.75</td>
</tr>
<tr>
<td>48</td>
<td>1.84</td>
</tr>
<tr>
<td>72</td>
<td>3.06</td>
</tr>
<tr>
<td>96</td>
<td>4.12</td>
</tr>
</tbody>
</table>
### Table 7

Effects of dialyzed cell-free extracts of 72-h phosphate and pyrophosphate treated cotyledon on the acid phosphatase activity present in the cell-free extract of cotyledon from 72-h germinating seeds of *V. sinensis*.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amount of protein added (µg)</th>
<th>Activity (A_405 nm/10 min)</th>
<th>Percentage of activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 72-h germinating cotyledon</td>
<td>40.0</td>
<td>0.84</td>
<td>100.0</td>
</tr>
<tr>
<td>2a. 72-h phosphate treated cotyledon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. 72-h germinating cotyledon + 72-h phosphate treated cotyledon</td>
<td>33.3</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>3a. 72-h phosphate treated cotyledon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. 72-h germinating cotyledon + 72-h phosphate treated cotyledon</td>
<td>66.6</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>4a. 72-h phosphate treated cotyledon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. 72-h germinating cotyledon + 72 h phosphate treated cotyledon</td>
<td>133.2</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amount of protein added (μg)</th>
<th>Activity (A.O.D. at 410 mp/10 min)</th>
<th>Percentage of activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. 72-h germinating cotyledon</td>
<td>400</td>
<td>0.84</td>
<td>100.0</td>
</tr>
<tr>
<td>5a. 72-h pyrophosphate treated cotyledon</td>
<td>33.3</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>b. 72-h germinating cotyledon + 72-h pyrophosphate treated cotyledon</td>
<td>400</td>
<td>0.84</td>
<td>94.5</td>
</tr>
<tr>
<td>7a. 72-h pyrophosphate treated cotyledon</td>
<td>66.6</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>b. 72-h germinating cotyledon + 72-h pyrophosphate treated cotyledon</td>
<td>400</td>
<td>0.82</td>
<td>99.0</td>
</tr>
<tr>
<td>8a. 72-h pyrophosphate treated cotyledon</td>
<td>133.2</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>b. 72-h germinating cotyledon + 72-h pyrophosphate treated cotyledon</td>
<td>400</td>
<td>0.96</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Cotyledons from different systems were homogenized and centrifuged as described in 'Methods' of this Section. The cell-free extracts were dialysed against 2 litres of 10 mM Tris-HCl buffer (pH 7.4) for 12 h with several changes. Activity in 40 μg protein of 72-h germinating cotyledon (nontreated) was taken as 100 per cent. The activity was measured in the presence of increasing protein of the dialysed cell-free extract of 72-h germinating cotyledon (phosphate and pyrophosphate treated).
FIG. 1

Specific Activity (μmole of PNP liberated/hr/mg of protein)

期内 of Germination (Hours)

37°C
30°C
20°C
10°C
FIG. 2

Period of Germination (hours)

Specific Activity

Mole of PNP liberated/hr/mg of protein

Control
Cycloheximide
FIG. 3

Period of Germination (Hours)

Specific Activity

(a mole of pNP liberated/1 mg of protein)
**FIGURE LEGENDS**

**Fig. 1:** Effect of temperature on the development of acid phosphatase in the seeds of *V. sinensis* during germination.

**Fig. 2:** The developmental changes of acid phosphatase activity in the cotyledon of germinating seeds of *V. sinensis;* effect of cycloheximide on the process.

**Fig. 3:** Effects of various inhibitors for transcription and translation on the development of acid phosphatase activity in the cotyledon and embryo of the seeds of *V. sinensis* during germination. Inhibitors were added after 4-h imbibition in sterile distilled water.

- **Control:**
- **0 — 0** Cycloheximide (100 µg/ml);
- **Δ-Δ** Chloramphenicol (100 µg/ml);
- **▲▲** Rifampicin (100 µg/ml);
- **▽▽** Ethidium bromide (100 µg/ml);
- **▼▼** Actinomycin D (100 µg/ml).

**Fig. 4:** Effects of various growth promoters on the developmental process of acid phosphatase activity in the half seeds (cotyledon without embryo) of *V. sinensis* during germination.
FIG. 7

Specific Activity
(mole of PMP liberated/hr/mg of protein)

Period of Germination (Hours)
The developmental changes of acid pyrophosphatase in the cotyledon and in the embryo of seeds of *V. sinensis* during germination. Effects of some inhibitors of protein synthesis on the development of acid pyrophosphatase in the growing embryo.

\[ \Delta - \Delta : \text{Chloramphenicol} ; \quad \Delta - \Delta : \text{Rifampicin} ; \]

\[ \bullet - \bullet : \text{Control} ; \quad \bigcirc - \bigcirc : \text{Cycloheximide} . \]

The developmental changes of alkaline pyrophosphatase in the cotyledon and in the embryo of the seeds of *V. sinensis* during germination. Effects of some inhibitors of protein synthesis on the development of alkaline pyrophosphatase in the growing embryo.

\[ \Delta - \Delta : \text{Chloramphenicol} ; \quad \Delta - \Delta : \text{Rifampicin} . \]

Effect of cycloheximide on the development of acid phosphatase in the cotyledon of seeds of *V. sinensis* during germination.

\[ \bullet - \bullet : \text{Control} ; \quad \bigcirc - \bigcirc : \text{Cycloheximide} \]

\[ \Delta - \Delta : \text{Seeds were imbibed in cycloheximide solution (100 \( \mu \)g/ml) for 2-h at room temperature. Seeds were then etiolated and washed thoroughly with sterile distilled water and allowed to germinate in sterile distilled water at 28^\circ C.} \]
FIG. 9

A

Specific Activity (nmole of ATP per mg of protein)

Period of Germination (Hours)

Control
20 mM
50 mM
100 mM

B

Specific Activity (nmole of ATP per mg of protein)

Period of Germination (Hours)

Control
5 mM
20 mM
60 mM

C

Specific Activity (nmole of ATP per mg of protein)

Period of Germination (Hours)

Control
5 mM
20 mM
60 mM
Effect of cycloheximide on the development of acid phosphatase activity in the cotyledon of seeds of *V. sinensis* during germination. Cycloheximide was added in the growing medium after 15, 24, 72-h of germination (indicated by arrow).

Fig. 9A: Effect of phosphate on the developmental process of acid phosphatase in the cotyledon of seeds of *V. sinensis* during germination. Phosphate was added in the growing medium after 4-h of imbibition.

Fig. 9B: Effect of pyrophosphate on the development of acid phosphatase in the cotyledon of seeds of *V. sinensis* during germination. Pyrophosphate was added in the growing medium after 4-h of imbibition.

Fig. 9C: Combine effects of phosphate, pyrophosphate and cycloheximide on the developmental process of acid phosphatase in the cotyledon of seeds of *V. sinensis* during germination. Phosphate, pyrophosphate and cycloheximide were added in the growing medium after 4-h of imbibition.

- O, Cycloheximide; • Delta, Phosphate; • Delta, Pyrophosphate; • Delta, Cycloheximide & Pyrophosphate.
Phosphate is an important constituent of the living system. Specific aspects of phosphate metabolism during germination have been examined by many workers (20, 116). The free phosphate was increased in the cotyledon with germination while the embryonic phosphate increased sharply up to 24 h of germination and then decreased slowly at the later stage of germination (Table 1). This type of phosphate accumulation in the seeds during germination was reported by Mayer and Maber (117) who showed a good correlation between the rapidity of phosphate accumulation and phosphatase activity of the seeds or seedlings during germination. The increase in free phosphate in the embryo and also in cotyledon may be due to the increase in phosphatase activity during germination. It was presumed by some workers (118) that the free phosphate in the reserve tissue was transported to the developing axis and utilized for the synthetic reaction, but these results are not sufficient to give the clear idea about inorganic phosphate migration. The increase in phosphate in the cotyledon suggests the breakdown of phosphorus-containing compounds mainly phytic acid during germination. A sharp increase in phosphate in the embryo during early stage of germination is probably due to the accumulation of free phosphate formed by the breakdown of phytic present in the embryo or by the transportation of inorganic phosphate from the storage tissue. The remarkable decrease of the free phosphate in the embryo in the later stage of germination may be due to the utilization of inorganic phosphate by the developing axis for its various synthetic reaction of essential compounds like phospho-
lipids, phosphoproteins, nucleic acids and phosphate sugars etc.

The phytase activity was also found in the germinating seeds of *V. sinensis*. Higher phytase activity was found in the 'cotyledon with embryo' than that of 'cotyledon without embryo' which indicates that the higher phytase activity is required to supply the inorganic phosphate from the cotyledon to the embryo (growing axis). The increase in phytase activity in the germinating pea seeds was also reported by others (59, 19).

The acid phosphatase activity increases with germination in cotyledon. But in embryo it reaches a maximum in between 24-48 h and then starts to decline in the later part of germination (Table 3). This finding indicates that embryo needs the higher acid phosphatase activity in the early stage of germination for its physiological requirement. The increase in temperature of germination within the physiological range enhances the growth and reduces the duration of growth period (120). The effect of temperature on the acid phosphatase activity during germination was also studied to correlate the development of acid phosphatase with the rate of germination (Fig. I). The results show that the increase in acid phosphatase activity depends on the germinating temperature (on growth). It also indicates that the development of acid phosphatase activity during germination may be due to the continuous synthesis of enzyme protein but not due to hydration. Another observation, where acid phosphatase activity is inhibited by cycloheximid during germination indicates that the increase in acid phosphatase activity is mainly due to the de novo synthesis of enzyme protein, rather than the activation of the already synthesized.
enzyme protein present in the dry seeds. It is important to recognize the time scale of the appearance of different enzymes during germination. Some enzyme systems become active instantaneously as soon as water is imbibed. These enzyme systems are already existing in the active state, are produced during seed development and a second group of enzymes becomes active only after some delay, usually requiring several hours. These latter enzymes require something in addition to imbibition in water for attaining the activity. A third group of enzymes becomes active even later; the appearance requires protein synthesis but not formation of mRNA. Lastly some activity appears even later, and these require the entire apparatus of protein synthesis, mRNA synthesis and gene activation. The appearance or increase in enzyme activity in absence of protein synthesis may be defined by activation of enzyme system. Hence the results presented in Table 3 and Fig. 1 are in well agreement with the conclusion that the rise in acid phosphatase activity in the cotyledon of germinating seeds of V. sinensis is not due to the activation of the pre-existing enzyme proteins.

Fig. 3 also shows that acid phosphatase activity increases in both embryo and in cotyledons (half-seeds with embryo and without embryo) with increase in time of germination. In order to characterize the nature of increase in acid phosphatase activity in germinating seeds, the effects of different inhibitors have been studied on the developmental process. Rifampicin, an inhibitor mainly for prokaryotic RNA synthesis (121), does not have any effect on the development of acid phosphatase activity in the germinating seeds. However, the increase in acid phosphatase
activity is inhibited by cycloheximide. Similar type of inhibition was also observed with actinomycin D and ethidium bromide, the specific inhibitors for transcription (122-123). These results strongly suggest that the increase in acid phosphatase activity during germination is due to the de novo synthesis of enzyme protein where the synthesis of mRNA is also required at least partially. It should be noted that the increase in acid phosphatase activity is not inhibited by chloramphenicol, specific inhibitor for prokaryotic and mitochondrial protein synthesis (124, 125). As the changes of acid phosphatase activity in the germinating seeds of V. sinensis is unaffected by chloramphenicol, it appears that the enzyme protein(s) is not the product of mitochondrial protein synthesizing machinery. The results given in Fig. 3 also suggest that the observed increase in acid phosphatase activity in germinating seeds is not due to the contamination of bacteria. Another interesting observation is that the extent of inhibition of the acid phosphatase activity during germination is more pronounced with cycloheximide than that observed either with actinomycin D or with ethidium bromide. From such observation it would not be unjustified to conclude that the increase in the acid phosphatase activity (particularly in cotyledon) could be due to the translation of enzyme protein(s) from two types of mRNA viz., preformed mRNA and newly synthesized mRNA. It is worth to mention at this point the existence of long-lived pre-formed mRNA has already been documented in germinating seeds (126, 127).

During germination the acid phosphatase activity is always higher in 'cotyledon with embryo' than that in 'cotyledon without
embryo' (Table 3 and Fig. 3). These results give rise to the idea that the embryo may play some important role in the development of acid phosphatase activity in cotyledon. Similar conclusion was also drawn by Yomo (128) while studying other enzymes in germinating seeds. The developments of protease (129), α-amylase (130) and citrate lyase (131) activities in the germinating seeds are controlled by a substance which originates in axial tissue and is transported to the cotyledon. Some of the workers conclude that this control is hormonal in nature.

Different plant growth substances GA₃, cytokinin and abscisic acid are known to affect dormancy and therefore their effects on metabolism during germination have been studied to a greater extent. It is well established that GA₀ and GA₃ appear to be the most efficient inducers of acid phosphatase formation (132). For these reasons the metabolic effects induced by GA₃ are of special interest. After the initial discovery of the effect of gibberellic acid, intensive work was carried out on its effect on the metabolism of cereal seeds during germination. This work was facilitated by early observation that the effect of GA₃ on the endosperm could be studied in half-seeds of the cereal, from which the endosperm had been removed. Such half-seeds respond to GA₃ applied exogeneously in the same way that the entire seed responds to the hormone from the embryo. The half-seeds respond to GA₃ by the rapid hydrolysis of starch, hydrolysis of proteins in the aleurone layer and also by the appearance of inorganic phosphate (133). Some of the metabolic changes have also been found to be induced in the barley endosperm by GA₃ (134). Varner et al. in 1965 demonstrated that the α-amylase formed in the
algarone arose by de novo synthesis of protein and also show that this protein synthesis was dependent on the synthesis of new mRNA and could be prevented by inhibitors of DNA dependent RNA synthesis (135). Pollard in 1969 studied the sequential effect of GA₃ on a number of enzymes in barley and wheat half-seeds (29). The first enzyme to be affected was a β-1, 3-glucanase and this was followed by increase in activity of phosphomonoesterase, ATPase, phytase and other enzymes. Keeping these views in mind the effect of GA₃ on the acid phosphatase activity in half-seeds (cotyledon without embryo) of V. sinensis during germination has been studied. Moreover, another reason for carrying out this experiment is to explore the probable role of embryo on the development of acid phosphatase during germination of seeds of V. sinensis. Unlike its effect in barley endosperm, GA₃ was found to have an inhibitory effect on the development of acid phosphatase activity in the half-seeds of V. sinensis during germination. Similar inhibitory effect was also found with another growth promoter namely indole acetic acid (IAA) (Fig. 4), whose role in germination has long been in dispute.

In the early stage of germination the lower activity of acid phosphatase in cotyledon could be due to the presence of some natural inhibitor as has been found in the cases of acid DNase (136), acid RNase (137) and nitrate reductase (138). Similar possibility may also exist in embryo where acid phosphatase activity in the early period of germination is increased sharply and then goes down with the same rate. This sharp decrease could be due to the production of some natural inhibitor in the embryo.
which may in turn control the acid phosphatase activity in the germinating seeds. In order to explore the presence of such inhibitor, the effects of cell-free extract of dry cotyledon and that of embryo from 96 h of germinating seeds on the acid phosphatase activity present in the cell-free extract prepared from the embryo of 24 h germinating seeds (where the enzyme activity is maximum) have been studied. The cell-free extract of cotyledons of dry seeds does not inhibit the acid phosphatase activity present in the cell-free extract of the embryo from 24 h germinating seeds (Table 4). Hence, it would be justified to rule out the possibility of existence of such natural inhibitor of acid phosphatase in the cotyledons of dry seeds. The data given in the Table 4 also rule out the development of any natural inhibitor of acid phosphatase in the embryo during later stage of germination.

Recently an inhibitor of phosphatase has been identified and characterized from the rabbit skeletal muscle (139).

Results given in Tables 5 and 6 show that both acid and alkaline pyrophosphatase activities were increased in embryo and in cotyledon during germination of seeds of *V. sinensis*. The activities of both the enzymes in cotyledon as well as in embryo were inhibited by cycloheximide. These results suggest that the increase in acid and alkaline pyrophosphatase activities are also due to the *de novo* synthesis of enzyme proteins. Increase in activities of acid and alkaline pyrophosphatase at least in embryo are not inhibited by chloramphenicol and rifampicin (Figs. 5 & 6).

The results presented in Fig. 2 suggest that the increase in enzyme activity is due to the continuous synthesis of enzyme.
protein. Increase in acid phosphatase activity in germinating seeds of *V. sinensis* is completely inhibited if the seeds are imbibed in a solution of cycloheximide for 2 h followed by normal germination (Fig. 1). These results strongly advocate the fact that for the steady increase in acid phosphatase activity in the cotyledon of germinating seeds of *V. sinensis*, the synthesis of new protein and its mRNA are required.

In some higher plants such as *Lemna minor* (140), *Spirodea oligorrhiza* (141), as well as *Escherichia coli* (142), *Neurospora crassa* (143) and *Euglena gracilis* (144), the synthesis of phosphatase is known to be controlled by phosphate. It was previously observed that in cultured tobacco cells XD-6 the release of phosphatase into the culture medium is increased by phosphate deficiency and suppressed by the addition of phosphate and cycloheximide (145). It was also found in contrast to acid phosphatase activity that the activities of β-amylase, β-galactosidase, succinic dehydrogenase and catalase did not change markedly by phosphate.

Results given in Fig. 9A and 9B show the inhibitory effects of phosphate and pyrophosphate on the development of acid phosphatase in the germinating seeds of *V. sinensis*. Liedtke and Ohmann (140) reported that in *lemna* the alkaline phosphatase which was induced by phosphate deficiency, was inactivated after the addition of phosphate. In the present study the specific activity of acid phosphatase was not reduced by the addition of phosphate when the enzyme synthesis had been blocked by cycloheximide. This indicates that after the addition of phosphate, the acid phosphatase synthesis is repressed but the inactivation of the pre-existing...
enzyme protein does not occur. There might be another possibility that the apparently low activity of acid phosphatase may be due to the presence of phosphate or pyrophosphate in the extract which are the potent inhibitor of acid phosphatase. Therefore, to eliminate this possibility the extracts were dialysed against 10 mM Tris-HCl buffer, pH 7.4 for 24 h with four changes. Another experiment (Table 7) shows that there is no effect of the phosphate and pyrophosphate treated extract on the acid phosphatase activity present in the control system. At the same concentration, pyrophosphate is more potent inhibitor than that of phosphate, probably because of the formation of two molecules of phosphate by the intracellular breakdown of one molecule of pyrophosphate. Similar type of observation was found by Ratajczak (146) on the decotyledonized pea embryo which were grown on the Heller's medium with different phosphate concentration.

It is now well established that seed germination may be regarded as the resumption of active growth by the embryo. It has been demonstrated that the protein synthesis is one of the essential steps in seed germination (147). The requirement of RNA synthesis for seed germination, until recently, was less certain. It has been shown that dry seeds contain pre-formed mRNA (148) and that polyribosomes and polypeptides synthesis occur during early hours of imbibition (149). In recent years the involvement of long-lived mRNA in germination process has been discussed extensively (150). It has been reported that mRNA synthesis did not occur in the first 24 h and protein synthesis appeared to be directed by the pre-formed mRNA (151). Studies
with germinating cotton seeds led to the similar conclusion (152). These studies are consistent with the observation that $^3$H-uridine incorporation into the RNA occurred only after 12 h of germination of wheat (26). The fact that lettuce seed germination was not inhibited by actinomycin D (153), but by nucleotide base analogue (154) suggested the involvement of RNA synthesis in the seed germination. Rejman and Buchowicz (155) found RNA synthesis within the first 3 h of germination followed by 8 h imbibition at 20°C.

Recent studies support the involvement of RNA synthesis in seed germination. Synthesis of RNA including that of mRNA occurs in the first few hours of germination of wheat (156), rye (105), barley (157), corn (158), red bean (159) and pea (160) seeds. From literature it appears that both protein and RNA synthesis are essential for germination of many seeds.
CHAPTER I

SECTION B

MULTIPLE FORMS OF ACID PHOSPHATASE IN THE GERMINATING SEEDS OF V. sinensis.
The term isozyme was first coined by Pferket and Miller to refer to multiple forms of an enzyme within the same organism (74). Isozymes are physically distinct forms of an enzyme, catalysing the same reaction. The occurrence of multimolecular forms of an enzyme is now known to be a common characteristic in most organisms. Cellular differentiation involves, at least in part, differential gene activity and control of synthesis and degradation of given proteins (enzymes) at specific times and places in the various cells and tissues of the developing organism. Investigation of the phenotypic expression of biochemical traits is of great importance for the knowledge of the molecular genetic mechanisms of gene function and gene interaction during ontogenesis (161). Isozymes, the stage specific proteins of organisms, have been proved to be especially convenient markers of the gene activity and they are now frequently employed in genetic and developmental analysis using experimental organisms such as mice (162) and Drosophila (163).

The developmental patterns of isozymes have provided a rich source of information about many aspects of enzyme biology. Not only are these progressions of value related to the developmental biologists in assessing the time-scale of differential gene function, but also in relation to such diverse and significant aspects as the structure and compositional inter-relationships of the proteins, the control of enzyme synthesis, the correlation between organ-specific patterns, and the distinctive physiological
roles of isozymes, the etiology of diseases, and many other significant aspects of cell and tissue function (164).

We have already reported that the acid phosphatase activity increases in the cotyledons of germinating seeds of *V. sinensis* (165). From these results it is evident that acid phosphatase activity is related to the growth i.e. the activity of acid phosphatase in the cotyledons is increased with the increasing time of germination. The change in the total activity of acid phosphatase in the course of development is found to be a composite of several isozymes each of which undergoes a change. These isozymes permit one to ask questions not merely about quantitative changes in total enzyme activity during development, but also about qualitative changes. How many isozymes do exist? How do the isozymes change in space and in time during development, and what are the factors that control these changes? As a consequence, many characteristic ontogenic changes in the isozyme patterns have been found to reflect the stages of differentiation. Quite conceivably, the isozymes of acid phosphatase may serve as good gene markers for analyzing cellular differentiation of seeds of *V. sinensis*.

In *V. sinensis* (L) Savi, the regulation of the isozymes of acid phosphatase has been the subject of intense investigations in our laboratory for the past few years. Although at present our understanding of gene regulation in eukaryotes is meagre, it is apparent that gene expression is controlled at several levels involving not only transcriptional controls but also translation and post-translation mechanism (166). The isozyme patterns of
acid phosphatase in germinating seeds of *V. sinensis* have become an excellent probe for studying the control of gene expression in an eukaryotic system, since a number of regulatory mechanisms appear to contribute to the overall pattern of acid phosphatase expression during bean development. The analysis of isozymes, therefore, is an important aspect of the studies of the genome-phenotypic product relationship which is the central problem in current developmental biology.

In this section of our study we have concentrated on the acid phosphatase activity as related to the developmental pattern in embryo and cotyledons (with and without embryo) and also the mode of expression of particular isozymes of acid phosphatase which changes during development in the seeds of *V. sinensis*.

**MATERIALS**

*p*-naphthyl phosphate, Fast blue RR were purchased from Sigma Chemical Company, Mo, U.S.A.; Triton-X-100 was obtained from Rohme Hass Company, Philadelphia, Pa, U.S.A.; Acrylamide and NN'-methylene bisacrylamide were obtained from BDH Chemical, England. *N*,*N*,*N',*N'-tetramethylethylene diamine was obtained from Fluka AG, Buchs SG Company, Switzerland. All other common chemicals used are of analytical grade and purchased from BDH and E. Merck.
Procedures of seed germination and inhibitor treatment were described in the Section A of this Chapter.

Preparation of Soluble Supernatant:

After completion of desired period of germination seeds were taken out, washed 3-4 times with distilled water and then cotyledons were separated from embryo and chilled. In the half-seed experiment the attached embryo was also separated from the cotyledon before extraction. The cotyledons/embryos were homogenized with ice-cold buffer (10 mM Tris-HCl, pH 7.4 containing 0.1% Triton X-100). The homogenate was kept overnight at 4°C and centrifuged at 10,000 x g for 20 min in cold centrifuge (Model B-20). The pellet was discarded and the supernatant was again centrifuged at 105,000 x g for 60 min in an ultracentrifuge (Spinco, Model L). The supernatant was saved for using as the enzyme source for gel electrophoretic studies.

Determination of Isozyme pattern of acid phosphatase by polyacrylamide gel electrophoresis:

7.0% polyacrylamide gels were prepared with 0.004 M histidine buffer (pH 8.5) as gel buffer according to the method described by Davis (167). Sample, prepared by mixing 200 µg - 250 µg of protein present in the soluble supernatant with glycerol was charged on gel after 30 min pre-electrophoresis. 10 µl of bromophenol blue (0.05%) was used as an indicator dye. Electrophoresis was carried out at 4°C for 2.5 h to 3 h with a constant
current at 3 mA/gel tube. In the case of 12.0% gel, electrophoresis was also carried out at 4°C for 4 h with a constant current of 5 mA/gel tube. After electrophoresis, the activity of acid phosphatase (isoenzyme pattern) was visualized by the diazo dye method described by Brewer (168). The staining mixture contained 0.01 M Na-acetate buffer (pH 5.0), 0.3 M NaCl, 5 mg of α-naphthyl phosphate and 5 mg of "Fast blue HR" in a volume of 10 ml. Gel dipped in staining solution was incubated at 37°C for 30-60 min. Staining reaction was stopped by replacing gels in 30% ethanol and stored at 4°C in the same solution.
It has been reported in the earlier Section that the total acid phosphatase activity increased through the first 48 h of germination, followed by the attainment of a constant activity in the later stage. From the zymogram pattern of acid phosphatase in polyacrylamide gel electrophoresis, it appeared that in the cotyledons of germinating seeds of *V. sinensis*, there were at least six different isozymes of acid phosphatase which were designated as AP-I, AP-II, AP-III, AP-IV, AP-V and AP-VI. Figure 10 reveals that the resolution of isozymes of acid phosphatase was better obtained in the alkaline pH (pH 8.5). So this pH was generally used in the experiments. Zymogram of acid phosphatase from the cotyledons and embryo at various stages of seed germination showed that the number and position of the isozymes under study were quite variable. The developmental changes of acid phosphatase activity in the germinating seeds of *V. sinensis* appeared to be correlated to the growth of the seeds. The distinct change in isozyme pattern of acid phosphatase appearing at different stages of germination is shown in Fig. 11. There were at least five different isozymes in the cotyledons of dry seeds (Tube No. A; Fig. 11). After 12 h of germination few of these isozymes appeared (AP-VI in Tube No. B; Fig. 11) and some of these disappeared (AP-IV and AP-V in Tube No. B; Fig. 11). In between 12-15 h of germination AP-IV reappeared (Tube No. C; Fig. 11). The reappearance time of AP-V was in between 24 to 48 h of germination. Similar changes occurred in the cotyledons without embryo, where identical isozyme pattern of acid phosphatase was observed (Tube No. F, G...
and Fig. 11), The developmental patterns of different isozymes of acid phosphatase have been found to follow the similar course in embryo too (Tube No. I, J and K; Fig. 11). Figure 12 shows the isozyme pattern of acid phosphatase in the growing embryo. These results also correlate with the earlier observation depicted in Fig. 11. The appearance time of the two most rapidly moving isozymes (AP-IV and AP-V) might further be confirmed by separating these two isozymes in 12% polyacrylamide gel and subsequent staining. Isozyme patterns of acid phosphatase shown in Fig. 13 are in well agreement with the fact that the appearance time of AP-IV and AP-V in cotyledons with embryo (half-seeds) are in 24 h and 48 h of germination respectively (Tube No. A and B; Fig. 13). But these two isozymes (AP-IV and AP-V) were not detected if the seeds were germinated in the presence of cycloheximide (Fig. 14). Another important observation shown in Fig. 15 is that these two isozymes exist also in cotyledons without embryo (half-seeds). The only difference between the two systems viz., cotyledons with and without embryo lies in the quantity of the synthesised enzyme during germination. The appearance and disappearance of the two fast moving isozymes of acid phosphatase (AP-IV and AP-V) have also been examined using 10% polyacrylamide gel as shown in Fig. 16. The result agrees well with the previous finding presented in Fig. 13. Figure 17 shows the influence of germination temperature on the appearance of the acid phosphatase isozymes. At 20°C, one of the fast moving isozymes (AP-IV) appeared after 48 h of germination but at 30°C AP-IV appeared at 24 h and AP-V at 48 h of germination. Similar appearance of these two isozymes was also found at 37°C where most of the isozymes appeared (Tube No. L; Fig. 17) after
24 h of germination. The appearance of the two most rapidly moving isozymes (AP-IV and AP-V) and also a few others (AP-III) were inhibited by ethidium bromide (Tube No. F and G; Fig. 18), by actinomycin D (Tube No. H and I; Fig. 18) and by cycloheximide (Tube No. J and K; Fig. 18). One of the two fast moving isozymes (AP-V) was inhibited completely at the initial stage of germination, but the other one (AP-IV) was inhibited partially during early period of germination by the transcriptional inhibitors. At 48 h of germination, complete inhibition of that particular isozyme by the inhibitors of RNA synthesis has been observed (Tube No. S and T; Fig. 18). The picture might be better visualised in 8% polyacrylamide gel (Fig. 19).
Fig 16

Fig 17
Fig. 14: Gel electrophoretic pattern (in 12% polyacrylamide gel) of acid phosphatase activity in cycloheximide treated half-seeds of *V. sinensis* (cotyledon with embryo). A, B, C and D represent respectively the enzyme activity from 24, 48, 72 and 96-h cycloheximide treated seeds.

Fig. 15: Gel electrophoretic pattern (in 12% polyacrylamide gel) of acid phosphatase from germinating half-seeds of *V. sinensis* (cotyledon without embryo). A, B and C represent respectively the enzyme activity from 24, 48 and 72-h germinating half-seeds.

Fig. 16: Gel electrophoretic pattern (in 10% polyacrylamide gel) of acid phosphatase activity from germinating half-seeds of *V. sinensis*. A, B, C and D represent respectively the enzyme activity from dry, 15, 24 and 48-h germinating half-seeds (cotyledon with embryo); E, F and G represent the enzyme activity from 15, 24 and 48-h germinating half-seeds (cotyledon without embryo).

Fig. 17: Gel electrophoretic pattern (in 7% polyacrylamide gel) of acid phosphatase from seeds of *V. sinensis* germinated at different temperature. A represents the enzyme activity from 2-h imbibed seeds; B, C, D, E and F represent respectively the enzyme activity from 15, 24, 48, 72 and 96-h germinated seeds at 20°C; G, H, I and J represent respectively the enzyme activity...
from 15, 24, 48 and 72-h germinating seeds at 30°C and K, L, M, N and O represent respectively the enzyme activity from 15, 24, 48, 72 and 96-h germinating seeds at 37°C.

Figs. 18 & '19: Gel electrophoretic pattern (in 7% & 8% polyacrylamide gel) of acid phosphatase activity from germinating half-seeds of V. sinensis (cotyledon with embryo). A, B, C, D and E represent respectively the enzyme activity from dry, 12, 15, 24 and 48-h germinating seeds; F and G represent the enzyme activity from 24 and 48-h ethidium bromide treated seeds; H and I represent the enzyme activity from 24 and 48-h actinomycin D treated seeds; J and K represent the enzyme activity from 24 and 48-h cycloheximide treated seeds.
DISCUSSION

The increase in acid phosphatase activity during germination has been reported in the previous section. It is found that the total increase in acid phosphatase activity during germination is due to the cumulative changes of several isozymes of acid phosphatase. Six isozymes of acid phosphatase have been recognised by employing polyacrylamide gel electrophoresis. Similar type of isozyme pattern of acid phosphatase has been reported by many workers in plants (14, 17), in animal systems (169-171), in microorganisms (172-174) and in insects (175, 176). Three isozymes (AP-I, AP-II and AP-III) are found at all stages of development of seeds of V. sinensis while the other three isozymes (AP-IV, AP-V and AP-VI) appear only in the later stages of germination (Fig. 11). As the development of acid phosphatase isozymes is unaffected by the chloramphenicol (data not given), an inhibitor of prokaryotic protein synthesis (124), the formation of the newly formed isozyme in the later stage of germination is not due to the bacterial contamination. The differential appearance of acid phosphatase isozyme suggests that the last three isozymes may be necessary for the terminal stages of development of embryo. These high levels of two fast moving isozymes (AP-IV and AP-V) observed in the case of cotyledons of 24-48 h germinating seeds of V. sinensis may indicate that these two isozymes are necessary for the maximal supply of nutrient and energy from the store house (cotyledons) of the germinating seeds to the delicate embryo which is essential for its proper development.
Another interesting observation is that these two fast moving acid phosphatases (AP-IV and AP-V) exist in the half-seeds viz., 'cotyledons with embryo' and 'cotyledons without embryo'. The only difference between the two systems is relative rate of the enzyme protein synthesis during the initial period of germination. Higher activities of two isozymes of acid phosphatase (AP-IV and AP-V) in the 'cotyledons with embryo' than that in 'cotyledons without embryo' rectify the previous speculation that these two isozymes are responsible for the maximum supply of essential nutrients to the developing embryo. These results also give rise to the idea that embryo may have some definite role in the development of acid phosphatase activity in the cotyledons during germination of seeds of *V. sinensis*. The role of embryo on the acid phosphatase activity in the germinating seeds of *V. sinensis* has been mentioned in the earlier section. The role of embryo may be either of the following: (a) some activators or stimulators which after being transported into the cotyledons enhances the rate of synthesis of different isozymes of acid phosphatase there; (b) some of the acid phosphatase activity after synthesis is transported from the embryo to the cotyledons.

The most likely candidates for such activation or stimulation as mentioned in the first possibility may be the plant growth hormones. To explore this possibility we have examined the effects of different plant growth hormones viz., GA₃, and indole acetic acid on the developmental pattern of acid phosphatase activity in cotyledons kept in germinating condition. No remarkable difference has been observed with any of the above mentioned plant growth substances (data not given). Further, the results obtained
after mixing and assaying the tissue extracts from the seeds of *V. sinensis* germinated for different time periods do not reveal the presence of activators or inhibitors, indicating that the changes were due to the differences in the amount of enzyme proteins. Regarding the second possibility, nothing conclusive could be suggested from the data through the developmental pattern of acid phosphatase in embryo correlates with the cotyledons with embryo. Further studies are already in progress in our laboratory to conclude something definite in this direction. The role of embryo on the development of acid phosphatase isozymes (AP-IV & AP-V) is clearly visualized in 12% polyacrylamide gel electrophoresis (Fig. 13).

Shaw (177), classified isozymes into two major categories (a) those which are distinctly different molecules and are presumably produced from different genetic sites, and (b) those which result from secondary alteration in the structure of a single polypeptide species. The formation of the fast moving isozymes may be due to any one of these two processes. Therefore, to understand the mechanism of the formation of these two phosphatases the effect of cycloheximide (a specific inhibitor of protein synthesis in eukaryotic system) on the development has been studied. Previous observation of inhibition of acid phosphatase activity by cycloheximide and the zymogram pattern of acid phosphatase indicates that the fast moving acid phosphatase arises from the de novo synthesis of enzyme protein and not due to the alteration or modification of an already existing form of the acid phosphatase. Other experimental data (the effect of germination
temperature on the development of acid phosphatase) also corroborate with this observation. Such change in isozyme pattern of acid phosphatase is very much related to growth, rather than to the activation or alteration of the enzyme protein in the imbibed condition.

Density labelling and immunological techniques and also cross breeding experiments made in several inbred lines appear necessary to explore whether the formation of these newly appeared isozymes of acid phosphatase may be due to the control of different genes in the same loci or to the products of allelic genes or to the modification of the major phosphatase. But cross-breeding experiment is very difficult to carry out because of the unavailability of eukaryotic mutants in this system.

Results of inhibition study mediated by the use of translational and transcriptional inhibitors explore an interesting events of appearance and disappearance of the isozymes of acid phosphatase during germination. The appearance of the two most rapidly moving isozymes (AP-IV & AP-V) and also of few others (AP-II & AP-III) are inhibited by ethidium bromide (Tube No. F & G; Fig. 11), by actinomycin D (Tube No. H & I; Fig. 11) and by cycloheximide (Tube No. J & K; Fig. 11). However, the AP-IV isozyme of acid phosphatase is inhibited partially during early period by the transcriptional inhibitors. But the complete inhibition of that particular isozyme with the inhibitors has been observed (Tube No. G & I; Fig. 11) at the latter stage of germination. The other fast moving isozyme of acid phosphatase (AP-V) is completely inhibited by these inhibitors. The extent...
of inhibition of these isozymes during germination is more pronounced with cycloheximide than those observed either with actinomycin D or with ethidium bromide. From these results it may be logical to speculate that some mRNA for that particular protein is already present in the dry seeds and this class of mRNA is translated first followed by the appearance of newly transcribed one. This type of long-lived mRNA present in the dry seeds have already been documented (126).

In addition to controlling the rate of synthesis of the enzyme protein at either transcriptional or translation level, control of the quantity of cellular enzymes can be achieved by turnover. From the above mentioned results (Figs. 18 & 19) a gross idea about the tentative turnover of the particular isozymes of acid phosphatase in the germinating seeds of V. sinensis can also be imagined. From the results of inhibition by cycloheximide, it appears that all the phosphatase activities decrease with the increase in time of germination. All phosphatases are subjected to dynamic turnover so that their immediate level represent the steady-state balance between their rate of synthesis and decay. When new phosphatase synthesis is prevented by the inhibitor of protein synthesis like cycloheximide, the acid phosphatase which is originally present, now gradually disappears with increasing time of germination. One of the fast moving isozymes whose appearance time in imbibed seeds is 12 h disappear on or before 48 h of germination. The persistence time of these phosphatases may lie in between 12-36 h after its appearance. This persistence time of these two isozymes of acid phosphatase in the germinating
seeds is again assured by other experimental facts. AP-IV & AP-V which are present in the dry seeds disappear after 12 h of imbibition.

Although the biological significance of isozymes in cellular metabolism is poorly understood, nevertheless, its role can hardly be over emphasized. Their periodic existence under a variety of environmental conditions clearly reflects how a cell is nicely equipped to cope with the diverse biochemical reactions that are needed during the course of its sustenance and propagation. Even more interesting is the tissue specificity of the isozymes wherein the specialised metabolic functions of a particular organ of tissue is carried out efficiently by the individual isozyme according to the need of that particular tissue or organ. In this way the isozymes beautifully shares the metabolic complexity of an organism in its totality.

The disappearance and reappearance of these two acid phosphatase (AP-IV & AP-V) during germination indicate their vital role on the growth of the seedling of *V. sinensis.*