Plant Physiol. (1975) 56, 292-299

Histochemical and Biochemical Observations on Storage Protein Metabolism and Protein Body Autolysis in Cotyledons of Germinating Mung Beans[1]

Received for publication August 26, 1974 and in revised form February 21, 1975

N. HARRIS AND MAARTEN J. CHRISPEELS
Department of Botany, University of Durham, Durham, England and Department of Biology, University of California, San Diego, La Jolla, California 92037

ABSTRACT

Storage protein hydrolysis in the cotyledons of germinating mung beans (*Phaseolus aureus* Roxb.) was examined by histochemical techniques, and the autolytic capacity of isolated protein bodies was studied with biochemical methods. The localization of endopeptidase activity within the cotyledons was studied using an India ink-gelatin film technique. After 24 hours of imbibition, a low level of endopeptidase activity was found throughout the storage tissues of the cotyledons. A marked increase in activity was noted in cells farthest from the vascular bundles 48 to 60 hours after the start of imbibition. The decrease in storage protein followed the same spatial distribution starting in the cells farthest from the bundles. The cotyledons contain a population of cells in various stages of endopeptidase activity enhancement and storage protein degradation. A wave of endopeptidase activity moves progressively through the cotyledons towards the vascular bundles leaving behind areas devoid of stored reserves and low in endopeptidase activity. Observations on the morphology of protein bodies during germination indicate that the membrane surrounding them remains intact, while the reserves disappear. This result suggests that the protein bodies may be undergoing autolysis. To determine whether this may indeed be the case, protein bodies were isolated from the meal of mung bean seeds using an aqueous medium containing 80% glycerol. The protein body preparations and the cytoplasm were assayed for the presence of a number of enzymes which may be involved in the breakdown of the storage proteins. The protein bodies contained all, or nearly all, of the carboxypeptidase, α-mannosidase, N-acetyl-β-glucosaminidase, and caseolytic activity. The cytoplasm contained all, or most, of the leucine aminopeptidase and the trypsin-like activity (benzoyl arginine-*p*-nitroanalide as substrate). Incubation of the isolated protein bodies resulted in the release of amino acids. An analysis of the products of hydrolysis indicated that very little, if any, storage protein was being hydrolyzed during the incubation. Hydrolysis of the storage proteins present in the protein bodies was greatly accelerated by the addition of extracts from the cotyledons of 4-day-old seedlings. The results suggest that new enzymic activities not present in the protein bodies isolated from dry seeds must either be activated or synthesized and possibly added to the protein bodies before storage protein breakdown can begin.

The cotyledons of leguminous seeds contain large amounts of reserve proteins localized in specialized organelles called aleurone grains or protein bodies (9, 22). Germination of these seeds is often accompanied by the swelling of the protein bodies (1, 4, 15). This swelling is probably the result of protein hydrolysis leading to an increase in osmotic pressure which in turn causes the protein bodies to take up more water. In an earlier paper (5), we presented evidence for the appearance of endopeptidase activity in the cotyledons of germinating mung beans along with the role of this enzyme in storage protein metabolism. We found a temporal relationship between the enhancement of endopeptidase activity and the breakdown of storage proteins. Using histochemical techniques, we have now investigated the spatial relationship between the enhancement of endopeptidase activity and protein body degradation within the cotyledons. The membranes surrounding the protein bodies appear to remain intact while the storage proteins are being metabolized, suggesting that the protein bodies may be undergoing autolysis. Using biochemical techniques, we investigated the enzyme complement of protein bodies isolated from seed meal and determined their capacity for autolysis.

The pattern of storage protein breakdown has been reported in several other legumes. In *Phaseolus vulgaris,* breakdown starts in the cells farthest removed from the vascular bundles (15), but throughout the cotyledons; in *Pisum arvense* breakdown starts at the periphery of the cotyledons (20), while in *Vicia faba* initiation of digestion near the vascular bundles has been reported (4). Using a histochemical method to localize protease activity, Yomo and Taylor (26) showed that in cotyledons of germinating *Phaseolus vulgaris* protease activity is highest in the cells farthest from the vascular bundles and matches the pattern of protein degradation found by others (15). The present study was undertaken to confirm these observations for another species and extend them to a time study of the spatial correlation between protease activity and storage protein breakdown.

The second part of this investigation concerns the potential of protein bodies to function as lysosomes and digest the

[1] The major portion of this work was completed while M. J. C. was on sabbatical leave from the University of California, San Diego. This sabbatical leave was supported by a fellowship from the John S. Guggenheim Foundation, and the research was supported by National Science Foundation Grants GB 30235 and GB 37224X.

storage proteins contained within them. In animal cells digestion of macromolecules is accomplished by the acid hydrolases present in the lysosomes (6, 7). The initial observation by Matile (12) that plant vacuoles contain a large number of acid hydrolases led to the suggestion that they function as plant lysosomes (14). This suggestion was confirmed by electron microscopical evidence showing that vacuoles often contain cell organelles in various states of disintegration and apparent digestion (23). Lysosomes, containing acid hydrolases, have been isolated from plant cells (18), but it is not clear whether they represent a distinct organelle or are actually identical with vacuoles. Electron microscopical observations on the formation of protein bodies during seed development and their final fate during germination suggest a close similarity between vacuoles and protein bodies. During the development of the seed, the reserve proteins are synthesized on the rough endoplasmic reticulum (2) and then deposited in pre-existing vacuoles (2, 16). When, in the course of germination, the protein reserves have been exhausted, the membranes surrounding the empty protein bodies fuse and form one or more large vacuoles (4, 15).

These structural similarities between vacuoles and protein bodies are paralleled by biochemical ones. Protein bodies have also been shown to contain various acid hydrolases (13, 17, 24), including proteases (13, 19, 21, 24), and it is this observation which led to the hypothesis that they may be autolytic organelles. To test this hypothesis, we determined the enzymic content of isolated protein bodies from mung beans and their capacity for self-digestion. This work is part of a study to determine how the metabolism of storage proteins is regulated in the cotyledons of leguminous seeds.

MATERIALS AND METHODS

Histochemical Observations. Dry seeds of mung beans (*Phaseolus aureus* Roxb., also called *Vigna radiata* [L.] Wilczek) obtained from a local dealer were sterilized with 10% commercial bleach for 15 min, rinsed with H_2O, and germinated as described (5). The India ink-gelatin film method of Yomo and Taylor (26) was followed to localize the endopeptidase activity within the cotyledons. Sections from the cotyledons were cut by freehand, fixed in 3 to 4% formaldehyde buffered with 0.02 M phosphate citrate at pH 5.5 for 30 sec, washed six times with the same buffer, blotted, and placed on the gelatin films. Incubation was usually for 40 to 60 min at room temperature, in a closed Petri dish. The sections were then carefully removed from the gelatin films, and the resulting cell prints were photographed at low magnification. Control sections were soaked in 1 mM *p*-chloromercuribenzoic acid for 30 min prior to incubation on gelatin films.

Protein distribution was determined by staining freehand sections of cotyledons with toluidine blue. All the sections used in these experiments were obtained by cutting single cotyledons transversely. Only the sections from the middle third of the cotyledon were used. The sections shown in Figure 1 have been positioned in such a way that the abaxial side (round side) of the cotyledons is at the top and the adaxial side (flat side) at the bottom. The rounded end of the section was adjacent to the seed coat hilum.

Material for electron microscopy was fixed at 4 C in 2.5% glutaraldehyde and 1% osmic acid in 50 mM cacodylate buffer, pH 7. After alcohol dehydration and embedding in Spurr's resin, the tissue was examined in an AEI EM 6B electron microscope. Sections (1 μm thick) were cut from the blocks and stained with 1% toluidine blue in borax for light microscope observations.

Isolation of Protein Bodies. Protein bodies were always isolated from seeds which had first been sterilized with bleach, rinsed, and dried. The dry seeds were ground in an electrically driven mill (three bursts of 10 sec each), and the meal was sieved through a 36-mesh sieve. The meal (10 g) was homogenized in a mortar and pestle in 60 ml of 80% glycerol containing 25 mM citrate-phosphate buffer, pH 5, and the slurry was centrifuged at room temperature at 2000*g* for 10 min. The supernatant was decanted, layered over 90% glycerol, and centrifuged at 35,000*g* for 45 min at a temperature of 15 C. The sediment was resuspended in 25 mM citrate-phosphate buffer, pH 5, containing 2 mM 2-mercaptoethanol. The supernatant, diluted with an equal volume of the same buffer, was used as a source of cytoplasmic enzymes.

Electron microscopical observation of several protein body preparation revealed that they were not as free of contaminants as protein body preparations made from imbibed seeds using sucrose gradients (8). The isolation of protein bodies from seed meal resulted in preparations which were contaminated with cell wall fragments and contained aggregates of protein bodies. The preparations contained a larger proportion of intact protein bodies, however, and breakage was usually as low as 10%. To make sure that our findings were not due to the presence of the contaminants, we compared protein bodies isolated from imbibed seeds using sucrose gradients with those isolated from seed meal using the glycerol method. One such comparison, in which we tested for the presence of typical protein body enzymes (see below), is shown in Table I. The specific activity of the enzymes is somewhat lower in the protein bodies isolated on sucrose gradients. This result is probably due to the greater breakage of the protein bodies isolated from imbibed seeds on sucrose gradients (8), coupled with a selective loss of protein-body enzymes as compared to storage proteins.

Incubation of Protein Bodies. Release of amino acids from protein bodies as a result of autolysis was studied on protein body preparations which had been dialyzed against 25 mM citrate-phosphate, pH 5, containing 2 mM 2-mercaptoethanol. This dialyzed preparation was made 10 mM with respect to 2-mercaptoethanol and then incubated in closed vials at 26 C with gentle shaking. Aliquots were removed at appropriate times, and the proteins were precipitated by the addition of an equal volume of 15% trichloroacetic acid. The coagulated proteins were removed by centrifugation, and the amino acid content of the supernatant was determined by the method of Yemm and Cocking (25) using ninhydrin as a reagent. The results are expressed as μmoles of amino acid released per mg of protein in the preparation.

Table I. *Comparison of Protein Bodies Isolated from Imbibed Seeds Using Sucrose Gradients and from Bean Meal Using Aqueous Glycerol*

The enzyme activities are expressed as specific activities: α-mannosidase and[1] N-acetyl-β-glucosaminidase, units/mg of protein; carboxypeptidase, μmoles of amino acid released/hr·mg of protein.

Enzyme	Method of Isolation	
	Glycerol	Sucrose
α-Mannosidase	0.66	0.49
N-Acetyl-β-glucosaminidase	2.90	2.20
Carboxypeptidase	0.45	0.42

The effect on *in vitro* storage protein hydrolysis of enzymes present in the extracts of cotyledons of seeds germinated for 1 or 4 days was tested in the following way. A (1-ml) aliquot of a preparation of protein bodies (about 10 mg/ml of protein) was combined with 3 ml of buffer (25 mM citrate-phosphate pH 5) or 3 ml of cotyledon extract and incubated for various lengths of time. At the beginning and at the end of the incubation, samples were removed and prepared for SDS acrylamide gel electrophoresis to check the integrity of the various polypeptides which make up the two principal storage proteins vicilin and legumin (8). Extracts of the cotyledons were prepared by homogenizing 40 cotyledons in 15 ml of 0.1 M sodium borate buffer, pH 8, containing 2 mM 2-mercaptoethanol; the homogenate was centrifuged at 15,000*g* for 20 min and dialyzed against 25 mM citrate-phosphate buffer, pH 5, containing 2 mM 2-mercaptoethanol. The storage proteins precipitated during dialysis and they were removed by centrifugation; the clear supernatant was used as a source of proteolytic enzymes. In some experiments, this extract was treated with 3 mM N-ethylmaleimide for 12 hr at 0 C to inactivate the endopeptidase activity (5).

Total Protein and Reserve Proteins. Total protein and reserve proteins were estimated according to the method of Lowry *et al.* (11). Reserve proteins were precipitated by adjusting the pH to 4.7 (25 mM citrate-phosphate) and allowing the suspension to remain at 0 C for 24 hr. This precipitated most of the globulins which were recovered after centrifugation at 15,000*g* for 20 min.

Enzyme Assays. Enzymes were assayed as described elsewhere (5). Trypsin-like activity (BAPNAase[2]) using benzoyl arginine-*p*-nitroanilide as a substrate was assayed in the following way. The substrate (1 ml of 1 mM in 10 mM sodium phosphate, pH 7.5), an aliquot of the extract, and H_2O to make a total volume of 2 ml were incubated for 60 min at 37 C. The reaction was stopped by the addition of 1 ml of 1 N acetic acid. The coagulated proteins were removed by centrifugation, and the absorbance of the supernatant solution was determined at 410 nm. One unit of activity corresponds to a change in absorbance of 1.0 A/hr.

Acrylamide Gel Electrophoresis. Polypeptides were separated by electrophoresis on acrylamide gels (10% acrylamide) containing 0.1% SDS as described elsewhere (5).

Amino Acid Analysis. The amino acid composition of the amino acid mixture released as a result of the incubation of isolated protein bodies was determined with an automated amino acid analyzer.

RESULTS AND DISCUSSION

The disappearance of protein from the cotyledons and the appearance of proteolytic activity in the cotyledons of germinating mung beans was recorded by a protein stain (toluidine blue) and by digestion of gelatin-India ink films on glass microscope slides. Circumstantial evidence indicates that this proteolytic activity was primarily due to the presence of the endopeptidase discussed in a previous paper (5). The proteolytic activity was greatly enhanced in the course of germination (see below) and was inhibited by *p*-chloromercuribenzoic acid. Similarly, when endopeptidase activity was measured quantitatively with a viscosimetric assay using gelatin as a substrate, it was found that the activity was inhibited by reagents which block sulfhydryl groups and was enhanced more than 10-fold in the course of germination.

[2] Abbreviation: BAPNA: benzoylarginine-*p*-nitroanilide.

Loss of Protein from Cotyledons. The sequence of protein degradation in the cotyledons is shown in Figure 1 (A through D). The distribution of the large starch grains and the smaller protein bodies in the cotyledon cells of 1-day germinated seeds is shown in Figure 1A. Germination is accompanied by the gradual disappearance of protein from the cells, first to one side of each cotyledon, adjacent to the seed coat hilum, and later on in the cells farthest from the vascular bundles. Although the vascular bundles cannot be seen in Figure 1 B, C, or D, one or two vascular bundles occur at the center of each dark staining area. A typical distribution of vascular bundles in a cross-section through a cotyledon is shown in Figure 2A, while the relationship between the vascular bundle, the protein-rich cells, and the cells which have lost their reserves can be seen in Figure 2B. Figure 1 B, C, and D, show that the zones of protein-rich cells surrounding the vascular bundles became smaller and smaller as germination progressed. Nine days after the start of germination, the cotyledons had lost nearly all their storage proteins (5). At that time, the zone of protein-rich cells surrounding each vascular bundle was only a few cells thick (data not shown).

Localization of Endopeptidase. The sequence of endopeptidase activity enhancement in the cotyledons of germinating mung beans is shown in Figure 1, E through H, which records the patterns of digestion of the gelatin film. Thus Figure 1, E through H, and Figure 2B are photographs of cell and tissue imprints on the gelatin films; the lighter areas represent the endopeptidase activity. An initial low level of enzyme activity was found throughout the storage tissues after 24 hr of imbibition at 20 C (Fig. 1E) as evidenced by the digestion of the gelatin film underneath each cell. The level of activity was such that incubation needed to be carried out at 37 C (for 40 min). This histochemical result confirms the biochemical observation (4) that low levels of endopeptidase activity are present at the start of germination. Yomo and Taylor (26) did not find any protease activity in *Phaseolus vulgaris* seeds germinated for 1 or 2 days, although work in our laboratory has shown endopeptidase to be present in this species and at this time.

At 2 to 2.5 days from the start of imbibition, an area of high endopeptidase activity developed to one side of each cotyledon (Fig. 1F) adjacent to the seed coat hilum. The other storage areas showed no increase in activity from the low levels found after 1 day, but by the 4th day a clear pattern had emerged of differential endopeptidase activity between tissues of the cotyledons (Fig. 1G). Activity was greater in the cells farthest from the vascular bundles with an area of low activity surrounding each vascular bundle. The zones of low activity became smaller as germination progressed, although it was apparent by the 5th day that the areas of highest activity were in cells adjacent to the residual low activity areas (Fig. 2B). The groups of cells which had previously shown high endopeptidase activity now showed a lower level of activity. With increased incubation times, this result was usually masked by complete dissolution of the gelatin between the vascular bundles (as in Fig. 1, F, G, and H).

The cotyledons of germinating mung beans contain a population of cells in various stages of endopeptidase enhancement and related storage protein mobilization. There is apparently a close spatial relationship between the activity of this enzyme and reserve protein breakdown, confirming our earlier conclusion (5) that this enzyme plays an important role in the metabolism of the reserve proteins.

The general pattern of endopeptidase activity in mung beans is similar to that observed by Yomo and Taylor (26) for *Phaseolus vulgaris*, although they did not record any loss of

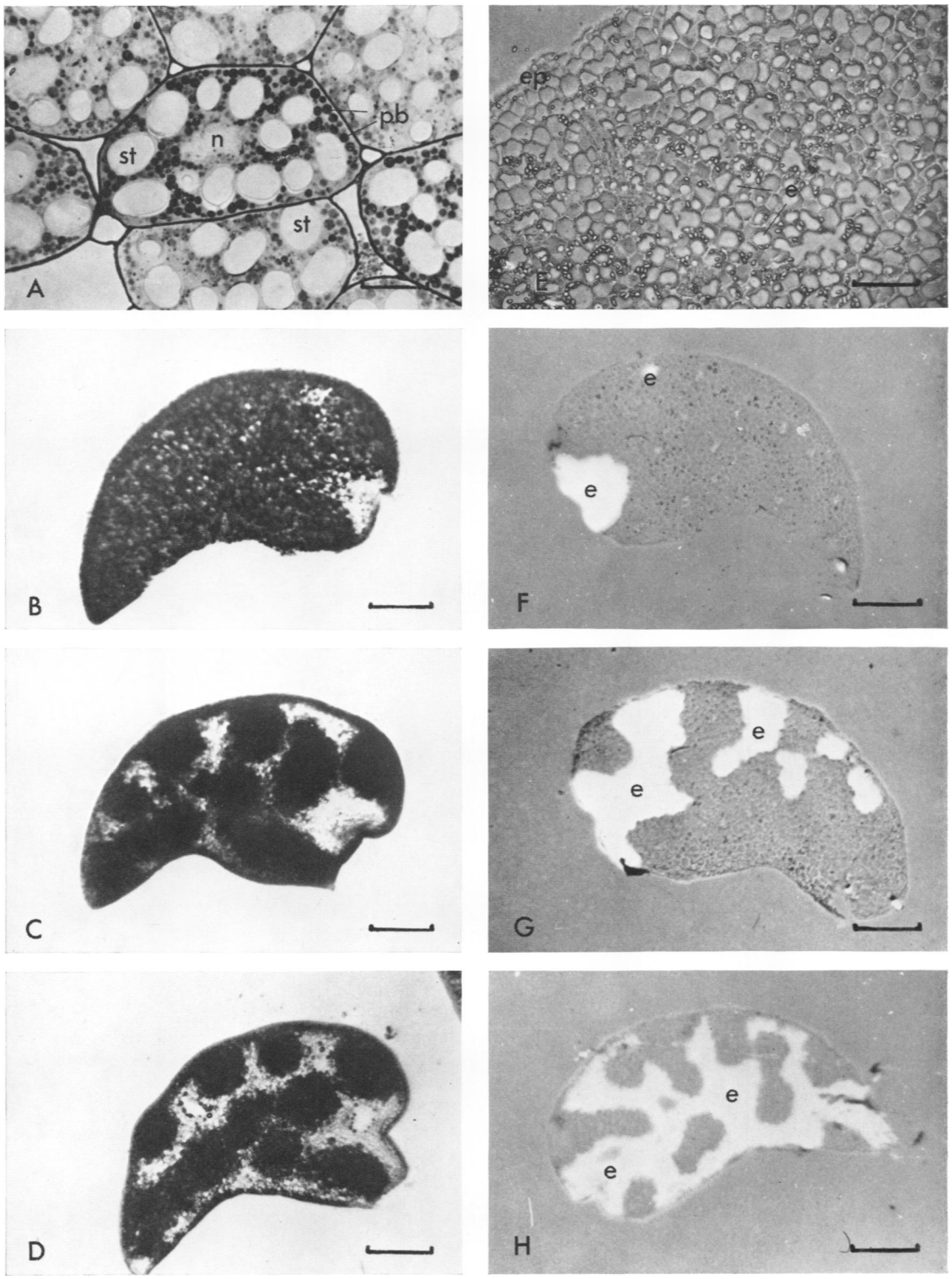

FIG. 1. A: Thin (1 μm) section of embedded 24-hr imbibed mung bean cotyledon. The section has been stained with toluidine blue and shows starch grains (st), protein bodies (pb), and a nucleus (n). Marker: 10 μm; × 1000. B-D: Freehand sections from cotyledons of germinating mung beans stained with toluidine blue to show protein distribution. Germination for 2 days (B), 4 days (C), or 6 days (D). Marker: 1 mm; × 10. E-H: Photomicrographs of gelatin film tissue prints. The pale areas in the tissue prints represent the areas of highest endopeptidase activity (e). E: 24-hr imbibed seed; a low level of endopeptidase activity (e) is apparent throughout the storage tissue as shown by the digestion of the film within each cell of the cell print; epidermis (ep). Marker: 250 μm; × 40. F, G, H: Germinated for 2, 4, and 6 days, respectively. Marker: 1 mm; × 10.

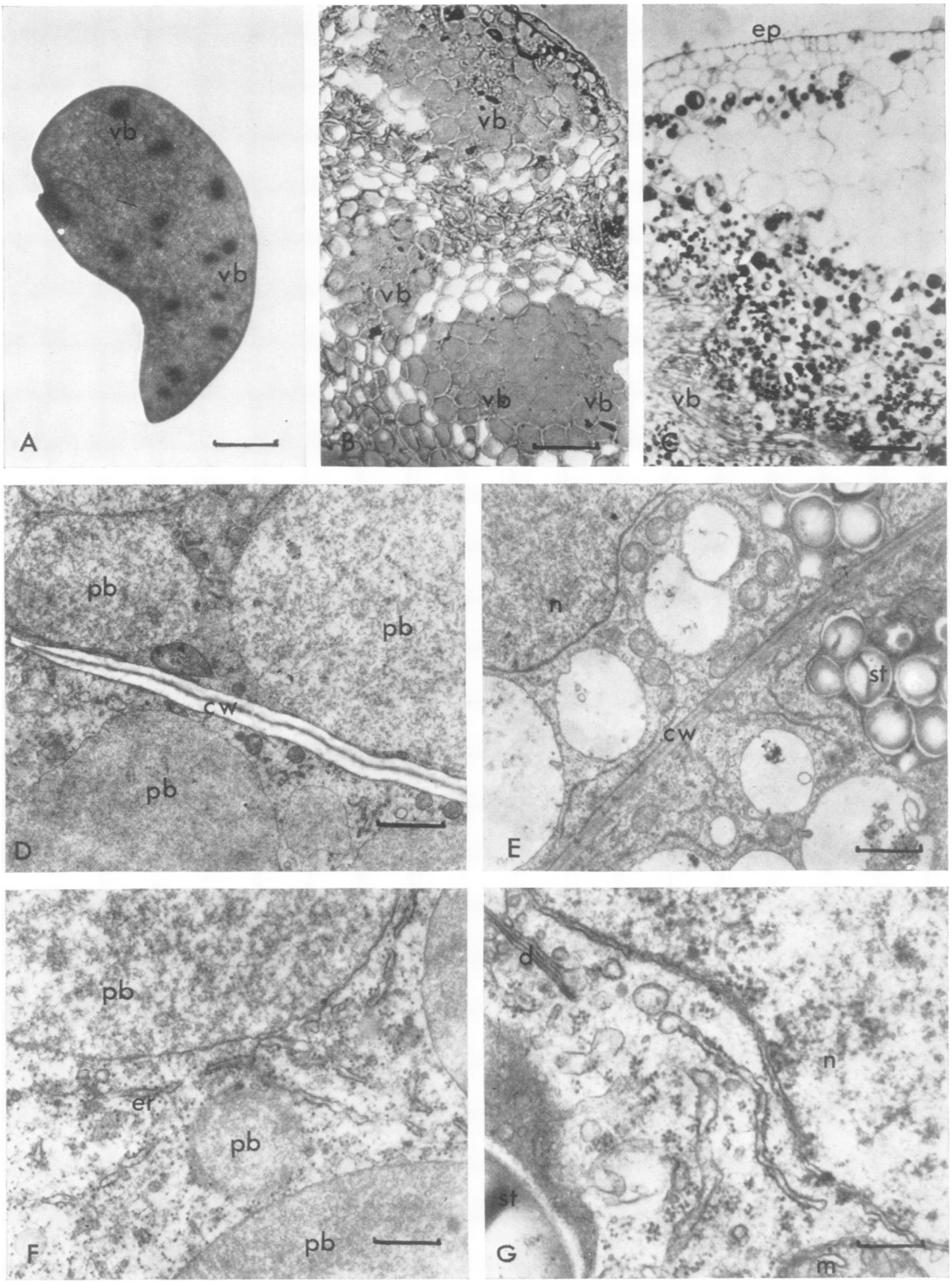

FIG. 2. A: Distribution of vascular bundles (vb) in a section through a mung bean cotyledon similar to those used for protease localization. Marker: 1 mm; × 10. B: Gelatin film cell print of a section from a 4-day germinated cotyledon showing the relationship between the vascular bundles (vb) consisting of numerous small cells, cells with low endopeptidase activity (gray areas), and with high endopeptidase activity (white areas). Marker: 250 μm; × 40. C: Thin (1 μm) section of embedded 6-day germinated cotyledon showing depletion of reserves from cells furthest from vascular bundles and from epidermis (ep). Marker: 70 μm; × 150. D, F: Electron micrographs of 1-day imbibed cotyledons. Protein bodies (pb) are still intact and full of protein. The cytoplasm contains numerous ribosomes and cisternae of the endoplasmic reticulum (er); cell wall (cw). Markers: 1 μm and 0.4 μm, respectively; × 10,000 and 25,000, respectively. E, G: Electron micrographs of 4-day germinated cotyledons. These cells are in the process of losing their reserves. The protein bodies appear empty while the starch grains (st) have not yet been degraded; nucleus (n); dictyosome (d); mitochondria (m). The ribosomes now appear in small aggregates. Markers: 1.4 and 0.3 μm, respectively; × 7,500 and 35,000, respectively.

activity in cells depleted of reserves. The pattern of reserve protein digestion differs from that found in *Pisum arvense* (20), where mobilization starts at the periphery, but is similar to that in *Phaseolus vulgaris* (15) and *Arachis hypogaea* (1).

Ultrastructure. The organization of the cytoplasmic organelles was examined with the electron microscope. The cotyledons of seeds germinated for 1 day contain numerous protein bodies (Fig. 2, D and F) with amorphous proteins and a limiting membrane. The cytoplasm has many ribosomes as well as short cisternae of the endoplasmic reticulum. The ribosomes appear to be randomly distributed throughout the cytoplasm. After 3 days of germination, many protein bodies appear to have lost their storage proteins (Fig. 2E), the cytoplasm contains much longer cisternae of endoplasmic reticulum (Fig. 2, E and G), and the ribosomes occur in small aggregates (Fig. 2G). These observations suggest that profound changes take place not only in the protein bodies but also in the morphology of other cytoplasmic organelles. How these changes relate to storage protein breakdown remains to be determined.

Enzymic Activities in Protein Bodies. The disappearance of the storage protein from the protein bodies without the apparent breakdown of the limiting membrane suggests that these organelles may be capable of autolysis. To test whether this might indeed be the case, we assayed for the presence or absence of a number of enzymes which could be involved in the metabolism of the reserve proteins. Homogenates of seed meal were fractionated into protein bodies and cytoplasm using the aqueous glycerol method, and the distribution of the enzymes between the protein bodies on the one hand and the cytoplasm on the other was determined. The results are presented in Table II. Each fraction was also assayed for globulins; the presence of globulins in the cytoplasmic fraction indicated that a certain proportion of the protein bodies had been ruptured during the isolation procedure. More than 90% of the globulins were found in the protein body fraction. The calculated enzyme distribution (see Table II) includes a correction based on the observation that 8% of the protein bodies ruptured and spilled their contents. It is quite obvious from these results that a number of enzymic activites are located primarily in the protein bodies, while others are confined to the cytoplasm. Carboxypeptidase, α-mannosidase, N-acetyl-β-glucosaminidase, release of amino acids through self-digestion, and caseolytic activity are associated primarily with the protein bodies. All these enzymes have a pH optimum around 5 or slightly higher (4). These results confirm the observation that protein bodies, like lysosomes, contain a variety of acid hydrolases (13, 17, 24). Two other enzymes involved in protein metabolism, leucine aminopeptidase and a trypsin-like enzyme using BAPNA as substrate, were found nearly exclusively in the cytoplasm. These two enzymes have a pH optimum near neutrality (3, 5) or in the alkaline pH range (3). It is of interest to note that these two cytoplasmic enzymes declined in the course of germination (3, 5, 10), while the protein body enzymes remained constant or increased (5). The distribution of these enzymes with their different pH optima is in agreement with the often expressed notion that the cytoplasmic pH is near neutrality while that of the vacuoles is acidic. The protein bodies also contained measurable amounts of endopeptidase (gelatin as substrate) confirming a similar observation on protein bodies isolated from hempseed (20) and barley (16).

Autodigestion of Protein Bodies. These results encouraged us to think that protein bodies may be capable of autolysis and this was tested directly by incubating isolated protein bodies at 26 C in 25 mM citrate phosphate at pH 5 containing 10 mM 2-mercaptoethanol. The progress of proteolysis was followed by measuring the increase in amino acids and other α-amino groups with ninhydrin. The optimum pH was determined in a preliminary experiment. Proteolysis was quite low below pH 3 and above pH 8 and was at a maximum around pH 4.8 to 5. A time course of amino acid release in shown in Figure 3. The decreasing rate of amino acid release could have been due to the inactivation of the proteolytic enzymes or to the shortage of a suitable substrate. Amino acid release was enhanced by the addition of casein or an equivalent amount of protein from protein bodies heated to 100 C (Fig. 3). Casein and denatured reserve proteins are probably more suitable substrates for the proteolytic enzymes present in the protein bodies.

Table II. *Distribution of Enzymic Activities between Protein Bodies and Cytoplasm*

The enzyme activities are expressed as total units in this particular preparation: α-mannosidase, N-acetyl-β-glucosaminidase, BAPNAase, and leucine aminopeptidase. Self-digestion, caseolysis, and carboxypeptidase: μmoles of amino acid released/hr; endopeptidase: change in relative viscosity/hr; Globulins were recovered after precipitation in the cold for 24 hr at pH 4.7 in 25 mM citrate-phosphate buffer. The calculated distribution takes account of the fact that 8% of the protein bodies were ruptured, as shown by the distribution of globulins.

Enzyme	Enzyme Activity		Calculated Distribution in Protein Bodies
	Protein bodies	Cytoplasm	
			%
Self-digestion	0.70	0.04	
Caseolysis	2.90	0.80	86
Carboxypeptidase	3.90	0.60	95
α-Mannosidase	21.60	3.80	94
N-Acetyl-β-glucosaminidase	28.80	7.60	87
BAPNAase	0.01	0.37	2.5
Leucineaminopeptidase	0.03	9.20	0.3
Endopeptidase	0.13	ND[1]	
Globulins	8.06 mg	0.73 mg	100

[1] ND: not determined

The effect of synthetic protease inhibitors was tested to determine which enzymes may be involved in the proteolytic digestion of the protein bodies. Phenylmethylsulfonylfluoride inhibits proteases with a serine residue in their active site and was found to inhibit mung bean carboxypeptidase (4), whereas N-ethylmaleimide inhibits proteases with a sulfhydryl group in their active site. Both inhibitors were only partially effective in preventing amino acid release from the protein bodies (Fig. 4), even though they completely inhibited their target enzymes. Even a combination of the two inhibitors did not completely stop amino acid release, suggesting the presence of at least three types of proteolytic enzymes.

Reserve proteins of leguminous seeds have a distinctive amino acid composition and are usually rich in aspartyl and glutamyl residues. The composition of the amino acids released after an 18-hr incubation of isolated protein bodies was determined to find out whether reserve proteins had actually been broken down. The four most abundant amino acids were alanine (12.6%), valine (11.4%), threonine (9.6%), and serine (9.4%). Vicilin, the principal storage protein of mung beans, has quite a different composition. The four most abundant amino acids are glutamic acid (19.9%), aspartic acid (13.4%),

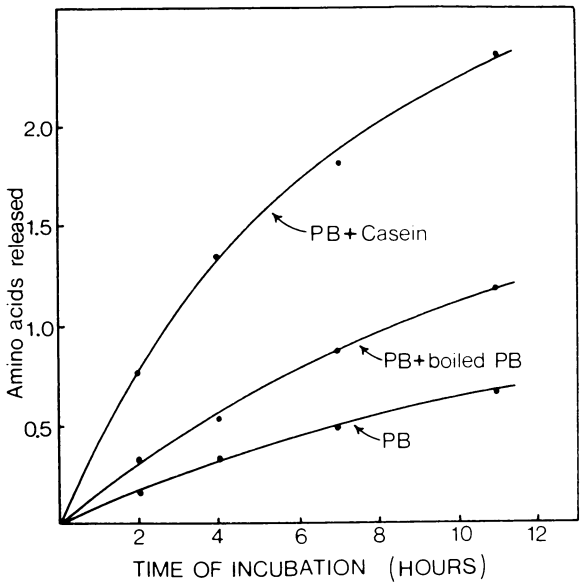

FIG. 3. Release of amino acids during incubation of isolated protein bodies. The protein bodies were isolated as described, resuspended in 25 mM citrate-phosphate containing 2 mM 2-mercaptoethanol, dialyzed against the same buffer, and incubated at 26 C with 10 mM 2-mercaptoethanol. The protein body preparation contained 8 mg/ml of protein. PB (protein bodies): 2 ml of the protein body preparation with 2 ml of 25 mM citrate-phosphate buffer; PB + boiled PB: 2 ml of the protein body preparation + 2 ml of the same preparation heated to 100 C for 8 min. The boiled PB preparation released no amino acids upon incubation. PB + casein: 2 ml of the protein body preparation + 2 ml of 1% casein (4).

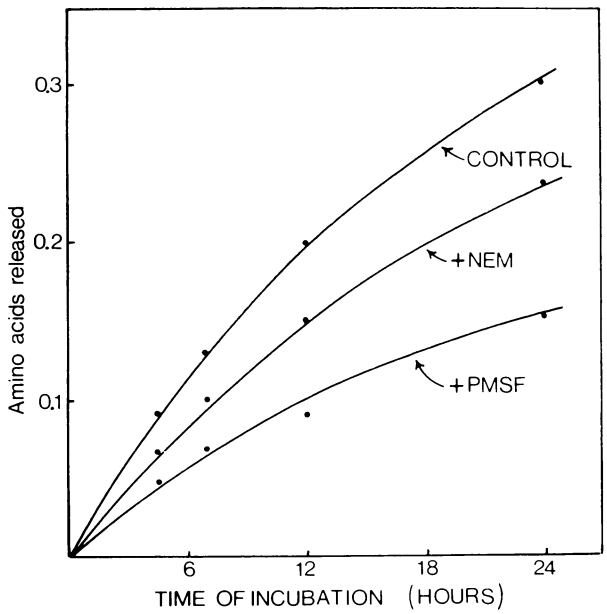

FIG. 4. Effect of synthetic protease inhibitors on the release of amino acids during incubation of isolated protein bodies. The dialyzed protein body preparation was treated for 12 hr at 0 C with either 1 mM phenylmethylsulfonylfluoride (PMSF) or 3 mM N-ethylmaleimide (NEM). The addition of 10 mM 2-mercaptoethanol before incubation was omitted from the NEM-treated samples.

leucine (9.4%), and serine (7.3%) (8). These results suggest that there was little or no complete digestion of the reserve proteins, but they do not exclude the possibility that extensive partial digestion took place.

Effect of Endopeptidase on Protein Body Digestion. Digestion of the reserve proteins was determined in an entirely different way by comparing the molecular sizes of the major polypeptides before and after protein body incubation. This comparison was done by separating the polypeptides by electrophoresis on SDS-acrylamide gels. The gels were then stained for protein with amino black, and the results are presented as gel scans (Fig. 5). Similar aliquots were used in each case. Before incubation the protein bodies contained five major bands (Fig. 5A): two of high mol wt (around 50,000 daltons) and three of low mol wt (around 25,000 daltons). These bands represent the polypeptides present in the two major reserve proteins legumin and vicilin. It is assumed that more than one polypeptide may be present in each band. After the protein bodies had been incubated for 18 hr at 26 C, the band pattern looked exactly the same (Fig. 5B) both qualitatively and quantitatively. This result suggests that incubation of the protein bodies resulted in very little or no storage protein hydrolysis. This result may have been due to the fact that the conditions of incubation did not favor proteolysis. That conditions were indeed suitable for proteolysis to occur was shown by measuring the effect on reserve protein hydrolysis of extracts obtained from cotyledons of beans which had been allowed to germinate for different lengths of time. Mung beans were germinated for either 1 or 4 days, and their cotyledons were extracted to obtain the proteolytic enzymes which increased in activity in the course of germination (4). The extracts, from which most reserve proteins had been removed, were then combined with isolated protein bodies and

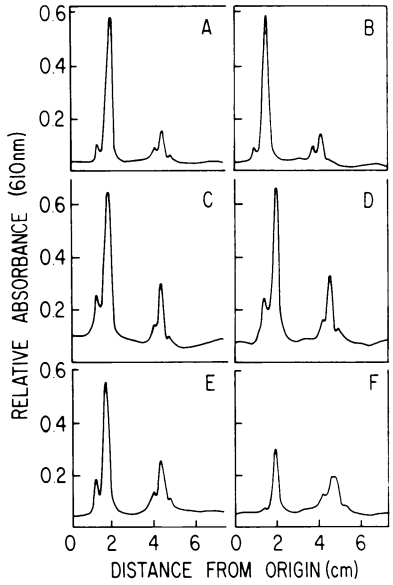

FIG. 5. Digestion of reserve proteins after incubation of isolated protein bodies as revealed by SDS-acrylamide gel electrophoresis. A, B: Gel scans of the polypeptides present in isolated protein bodies before (A) and after (B) incubation for 18 hr at 26 C. C, D: The same protein bodies treated with an extract of cotyledons obtained from seeds germinated for 1 day before (C) and after (D) incubation. E, F: As C and D, but the seeds had been allowed to germinate for 4 days before (E) and after (F) incubation. For details see "Materials and Methods." Decreasing molecular weights from left to right.

the mixture incubated for 18 hr at 26 C. The polypeptides present at the beginning and at the end of the incubation were separated and visualized as described. The results clearly show that extracts of cotyledons obtained from seeds germinated for 4 days contained enzymes capable of hydrolyzing the two larger polypeptides (Fig. 5, E and F). These enzymes were absent from seeds germinated for 1 day (Fig. 5, C and D). The proteolysis due to the addition of extract from cotyledons if seeds germinated for 4 days was abolished when the extract was preincubated with 3 mM N-ethylmaleimide. This result suggests that the endopeptidase, whose activity has been shown to increase during germination (5), is capable of hydrolyzing the reserve proteins of isolated protein bodies.

CONCLUSION

The metabolism of storage proteins in the cotyledons of germinating mung beans depends on the enhancement of endopeptidase-type protease(s) (5). There is a close temporal and spatial relationship between the appearance of this enzyme activity and the metabolism of storage proteins. Ultrastructural observations suggest that protein bodies may be autolytic organelles. Our results point to the conclusion that protein bodies isolated from dry seeds contain several hydrolytic enzymes, but are not capable of autolysis, and that the reserve proteins cannot be metabolized until the protein bodies have acquired new proteolytic activities. Whether the proteolytic activites result from the activation of enzymes already present in the protein bodies of dry seeds or from the addition of newly synthesized enzymes to the protein bodies remains to be determined.

Acknowledgment—We are indebted to Professor Donald Boulter for his continuing interest in our research and for many stimulating discussions about the biosynthesis, characterization, and utilization of storage proteins in legume seeds.

LITERATURE CITED

1. Bagley, B. W., J. H. Cherry, M. L. Rollins, and A. M. Altschul. 1963. A study of protein bodies during germination of peanut (*Arachis hypogaea*) seed. Am. J. Bot. 50: 523–532.
2. Bailey, C. J., A. Cobb, and D. Boulter. 1970. A cotyledon slice system for the electron autoradiographic study of the synthesis and intracellular transport of the seed storage protein of *Vicia faba*. Planta 95: 103–118.
3. Beevers, L. 1968. Protein degradation and proteolytic activity in the cotyledons of germinating pea seeds (*Pisum sativum*) Phytochemistry 7: 1837–1844.
4. Briarty, L. G., D. A. Coult, and D. Boulter. 1970. Protein bodies of germinating seeds of *Vicia faba*. Changes in fine structure and biochemistry. J. Exp. Bot. 21: 513–524.
5. Chrispeels, M. J. and D. Boulter. 1975. Control of storage protein metabolism in the cotyledons of germinatng mung beans: role of endopeptidase. Plant Physiol. 55: 1031–1037.
6. Coffey, J. W. and C. de Duve. 1968. Digestive activity of lysosomes. I. The digestion of proteins by extracts of rat liver lysosomes. J. Biol. Chem. 243: 3255–3263.
7. de Duve, C. and R. Wattiaux. 1966. Functions of lysosomes. Annu. Rev. Physiol. 28: 435–492.
8. Ericson, M. C. and M. J. Chrispeels. 1973. Isolation and characterization of glucosamine-containing storage glycoproteins from the cotyledons of *Phaseolus aureus*. Plant Physiol. 52: 98–104.
9. Graham, T. A. and B. E. S. Gunning. 1970. Localization of legumin and vicilin in bean cotyledon cells using fluorescent antibodies. Nature 228: 81–82.
10. Hobday, S. M., D. A. Thurman, and D. J. Barber. 1973. Proteolytic and trypsin inhibitory activities in extracts of germinating *Pisum sativum* seeds. Phytochemistry 12: 1041–1046.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 236: 1372–1379.
12. Matile, Ph. 1966. Enzyme der Vakuolen aus Wurzelzellen von Maiskeimlingen: Ein Beitrag zur funktionellen Bedeutung der Vakuole bei der intrazellulären Verdauung. Z. Naturforschg. 21b. 871–878.
13. Matile, Ph. 1968. Aleurone vacuoles as lysosomes. Z. Pflanzenphysiol. 58: 365–368.
14. Matile, Ph. 1968. Vacuoles as lysosomes of plant cells. Biochemistry J. 111: 26–27.
15. Öpik, H. 1966. Changes in cell fine structure in the cotyledons of *Phaseolus vulgaris* L. during germination. J. Exp. Bot. 17: 427–439.
16. Öpik, H. 1968. Development of cotyledon cell structure in ripening *Phaseolus vulgaris* seeds. J. Exp. Bot. 19: 64–76.
17. Ory, R. L. and K. W. Henningsen. 1969. Enzymes associated with protein bodies isolated from ungerminated barley seeds. Plant Physiol. 44: 1488–1498.
18. Pitt, D. and M. Galpin. 1973. Isolation and properties of lysosomes from dark-grown potato shoots. Planta 109: 233–258.
19. Schnarrenberger, C., A. Oeser, and N. E. Tolbert. 1972. Isolation of protein bodies on sucrose gradients. Planta 104: 185–194.
20. Smith, O. L. and A. M. Flinn. 1967. Histology and histochemistry of the cotyledons of *Pisum arvense L.* during germination. Planta 74: 72–85.
21. St. Angelo, A. J., R. L. Ory, and H. J. Hansen. 1969. Localization of an acid proteinase in hempseed. Phytochemistry 8: 1135–1138.
22. Varner, J. E. and G. Schidlovsky. 1963. Intracellular distribution of proteins in pea cotyledons. Plant Physiol. 38: 139–144.
23. Villiers, T. A. 1971. Lysosomal activities of the vacuole in damaged and recovering plant cells. Nat. New Biol. 233: 57–58.
24. Yatsu, L. Y. and T. J. Jacks. 1968. Association of lysosomal activity with aleurone grains in plant seeds. Arch. Biochem. Biophys. 124: 466–471.
25. Yemm, E. W. and E. C. Cocking. 1955. The determination of amino acids with ninhydrin. Analyst 80: 209–213.
26. Yomo, H. and M. P. Taylor. 1973. Histochemical studies on protease formation in the cotyledons of germinating bean seeds. Planta 112: 35–43.