Intracellular Distribution of Proteins in Pea Cotyledons[1, 2]

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Introduction

As a part of a study of organ and cellular senescence in plants, we are delineating the structural and functional changes which occur in the cotyledons of germinating peas. As might have been expected from earlier reports that cotyledon cells are capable of performing oxidative phosphorylation, incorporation of labeled amino acids into protein (4) and de novo synthesis of certain enzymes (5), examination by electron microscopy showed the presence of all structures normally present in plant cells. In addition, a major fraction of the cell volume was found to be occupied with relatively large roughly spherical bodies, with no visible internal structure. The data presented here indicate that the reserve globulins are localized in these structures. These structures are easily isolated as a pellet following centrifugation of a pea cotyledon homogenate. The presence of similar protein bodies in peanut cotyledons has already been reported (1, 3).

Material & Methods

The peas (*Pisum sativum*, var. Early Alaska) were soaked in 1 % sodium hypochlorite for 30 minutes, rinsed in sterile distilled water, and transferred to moist sand in sterile Petri dishes. After one to three days' incubation at 25 C, the cotyledons were excised and examined microscopically and chemically. For electron microscopy 1 mm^3 blocks were cut from that part of the cotyledon distal to the axis attachment and fixed for 2 hours at room temperature in 4 % potassium permanganate in 0.1 % uranyl nitrate. The samples were then rinsed in distilled water, dehydrated over a period of 2 hours in 50, 70, and 100 % ethyl alcohol and embedded in either methacrylate or epoxy resin. Thin sections (500–1000 A) were cut on a Porter-Blum microtome with glass knives and examined with a Siemens Elmiskop Ia.

For chemical and enzymic analysis, the excised cotyledons were ground with sand in 10 ml. of 0.10 M Tris (pH 7.1) with a mortar and pestle at 0 to 5 C. The homogenate was centrifuged at 1500 × *g* for 10 minutes. The light green precipitate overlying the firm starch pellet was removed with a spatula. This green precipitate constitutes the pellet fraction of figure 7.

The supernatant fraction was further centrifuged at 144,000 × *g* for 60 minutes. The 144,000 × *g* supernatant fraction was dialyzed against 0.01 M Tris (pH 7.8). A separate pellet fraction prepared as above was extracted at room temperature for 4 hours with 0.2 M NaCl and 0.05 M Tris (pH 7.8), and centrifuged at 1500 × *g*. The 1500 × *g* supernatant fraction was dialyzed against 0.01 M Tris (pH 7.8) to the point of incipient precipitation and then added to a DEAE column. The whole extract was prepared by grinding pea cotyledons in a mortar and pestle with 0.2 M NaCl, 0.05 M Tris (pH 7.8) and continuing the extraction for 4 hours. This extract was then dialyzed in preparation for separation on a DEAE column. The globulin fraction was prepared from the whole extract according to Danielsson (2). Pyrophosphatase activity was determined by incubating a 1.0 ml aliquot of each fraction for 60 minutes at 30 C with 300 μmoles acetate-Tris buffer (pH 5.5) and 20 μmoles of pyrophosphate in a final volume of 4.0 ml. Adenosine triphosphatase activity was determined at pH 7.1 in the presence of Mg^{++} ions (4).

Results & Discussion

Although no attempt will be made at present to describe exhaustively the morphology of pea cotyledon cells, nuclei, mitochondria, golgi bodies, chloroplasts, and starch grains are readily visible in suitable sections. Cells in an early stage of germination (approximately 12 to 24 hrs' imbibition) exhibit local concentrations of endoplasmic reticulum in areas that appear randomly dispersed throughout the cytoplasm.

For the purpose of this report it is significant to observe the large number of roughly spherical bodies, averaging 2 microns in diameter, that are scattered at random throughout the cytoplasm of such cells as in figure 1. The data presented here allow this particular component of germinating pea cotyledon cells to be designated as *protein bodies*—a term already suggested by Dieckert (3). At the stage il-

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lustrated in figure 1 no morphological continuity between the protein bodies and other cytoplasmic constituents can be demonstrated by the fixation procedure used here.

Preliminary observations of peas at various stages of development and maturation suggest that the appearance of these bodies follows the time course of globulin synthesis. Upon germination the bodies disappear gradually over a period of several days. It is concluded that they are not artifacts arising upon the dessication of peas following maturation.

Although the micrographs shown suggest the presence of a limiting membrane around the protein bodies, we believe that further observations of this tissue preserved with different fixatives are necessary to establish this feature unequivocally.

Figures 3 and 4 illustrate the composition of the globulin pellet. The main bulk of the pellet consists of relatively intact protein bodies obviously identical to the cytoplasmic bodies observed in whole tissue cells (fig 1). Some contamination with cellular debris and artifacts resulting from the preparative procedure is apparent and expected since no attempt has been made to wash the pellet. This fraction consists of a distinct cytoplasmic component : the protein bodies which preserve their identity upon homogenization and fractionation of whole cells. It may be regarded as a morphological expression of a stage in the protein economy of such cells.

Analysis of the pellet and supernatant fractions by the extraction and precipitation procedure of Danielsson (2) indicate (table I) that the pellet protein consists almost entirely of globulins. The small proportion of albumin in the pellet is probably accounted for by the fact that the pellet was not washed. It also appears that there is little globulin outside the protein bodies in solution in the cytoplasm because the globulins are soluble in the homogenizing medium. The necessary conditions for preserving the protein bodies in aqueous suspensions have not been investigated extensively. These bodies become soluble when maintained in 0.1 M Tris (pH 7.1). Time is adequate, however, for the kind of separations and analyses reported here.

Figure 5, 6, and 7 show the results of the chromatography of the various fractions on DEAE columns and further support the conclusion that the main component of the pellet and the protein bodies is the globulin fraction and that the globulin fraction is the major protein fraction of the pea cotyledon cells. It is interesting to note that the chromatographic separation suggests the presence of four components in the globulin fraction while ultracentrifugal analyses indicate two major components (2).

The supernatant fraction chromatographed in figure 8 was from cotyledons of 3-day old germinating peas. The first pyrophosphatase peak may be an artifact. The three major peaks always appear during chromatography and the last pyrophosphatase peak increases greatly during the third to sixth days of germination. The small adenosine triphosphatase peak results from the low activity of the corresponding pyrophosphatase peak toward ATP at pH 7.1. The major adenosine triphosphatase peak increases greatly during germination and is the enzyme studied earlier by Young and Varner (5). The proteins which become labeled in vivo from C^{14}-labeled amino acids (4) are in the albumin fraction and separate into five or six discrete peaks under the chromatographic conditions used here. By a combination of in vivo introduction of labeled amino acids into proteins and the separation of these proteins by column chromatography, we hope to be able to identify those enzymes which increase in activity as a result of de novo synthesis and those which increase as a result of some kind of activation.

Table I

Distribution of Globulins and Albumins in Homogenate of Pea Cotyledons

	Albumins*	Globulins*
	mg	mg
Supernatant fraction	120	22
Pellet	20	150
Debris	56	180
Total	196	352
0.2 M NaCl extract of ground cotyledons	165	410

* The albumins and globulins of each fraction were determined by the biuret procedure after separation according to Danielsson (2).

Summary

Electronmicrographs of pea cotyledon cells show that roughly spherical bodies of about two microns in diameter are a prominent feature of the cells. Isolation and physical characterization of this fraction indicate that these bodies contain the reserve globulins of the pea seed.

Acknowledgments

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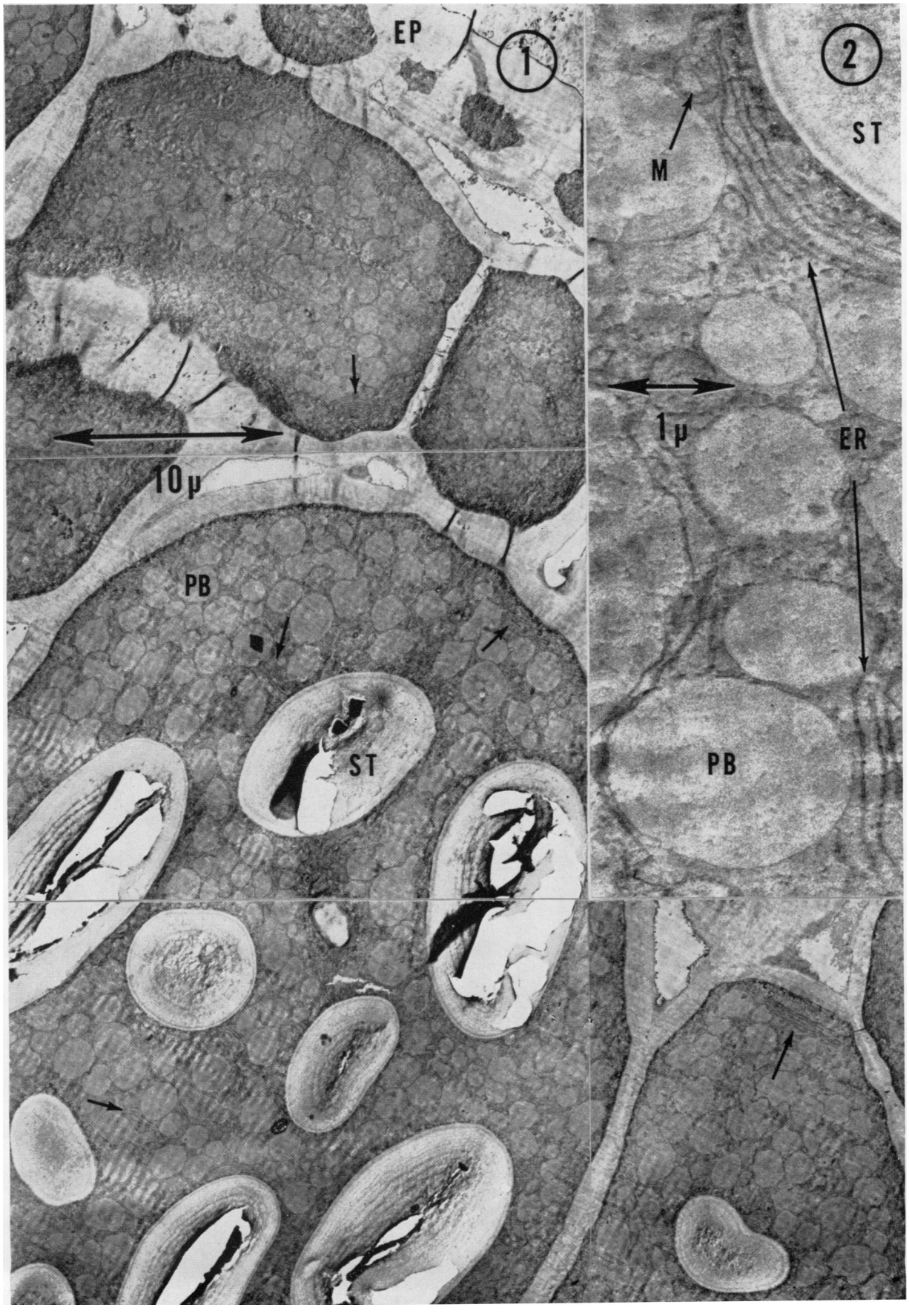
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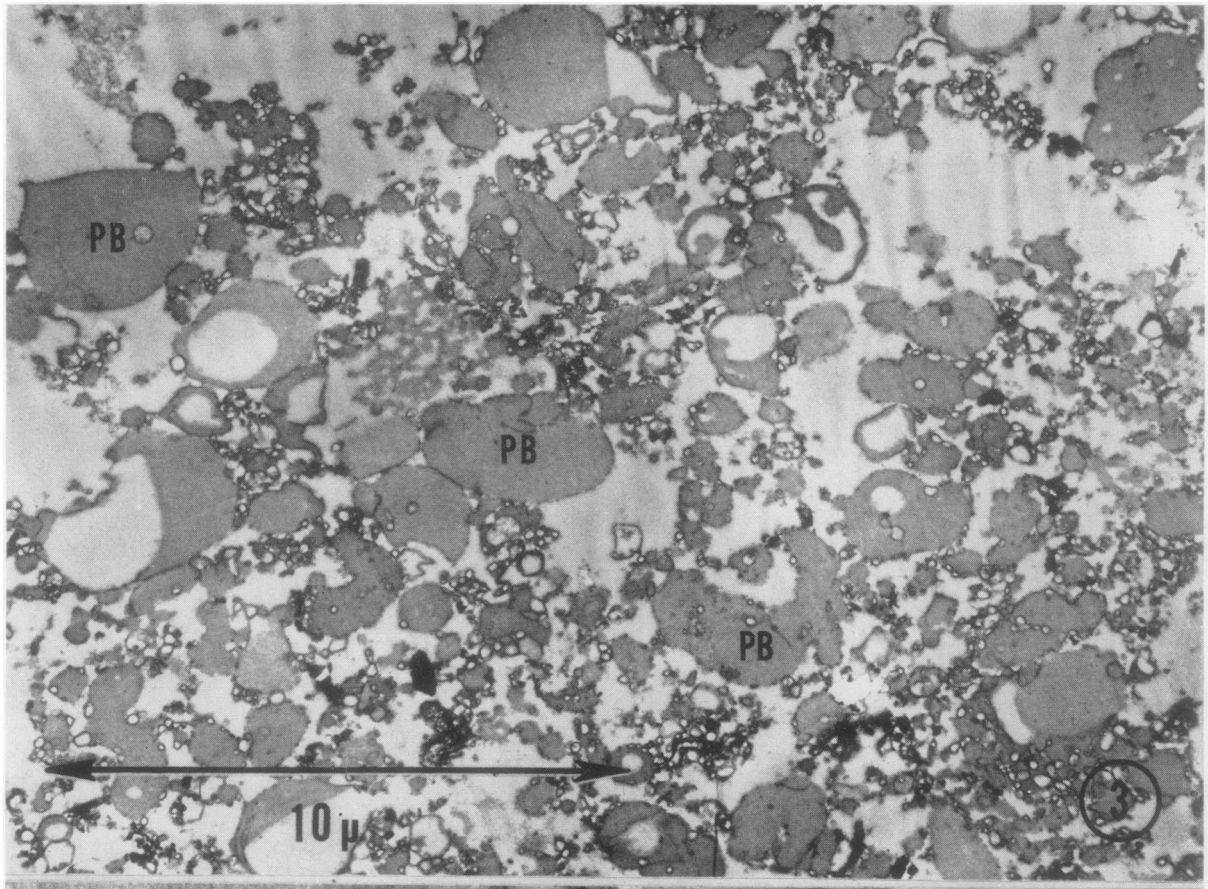
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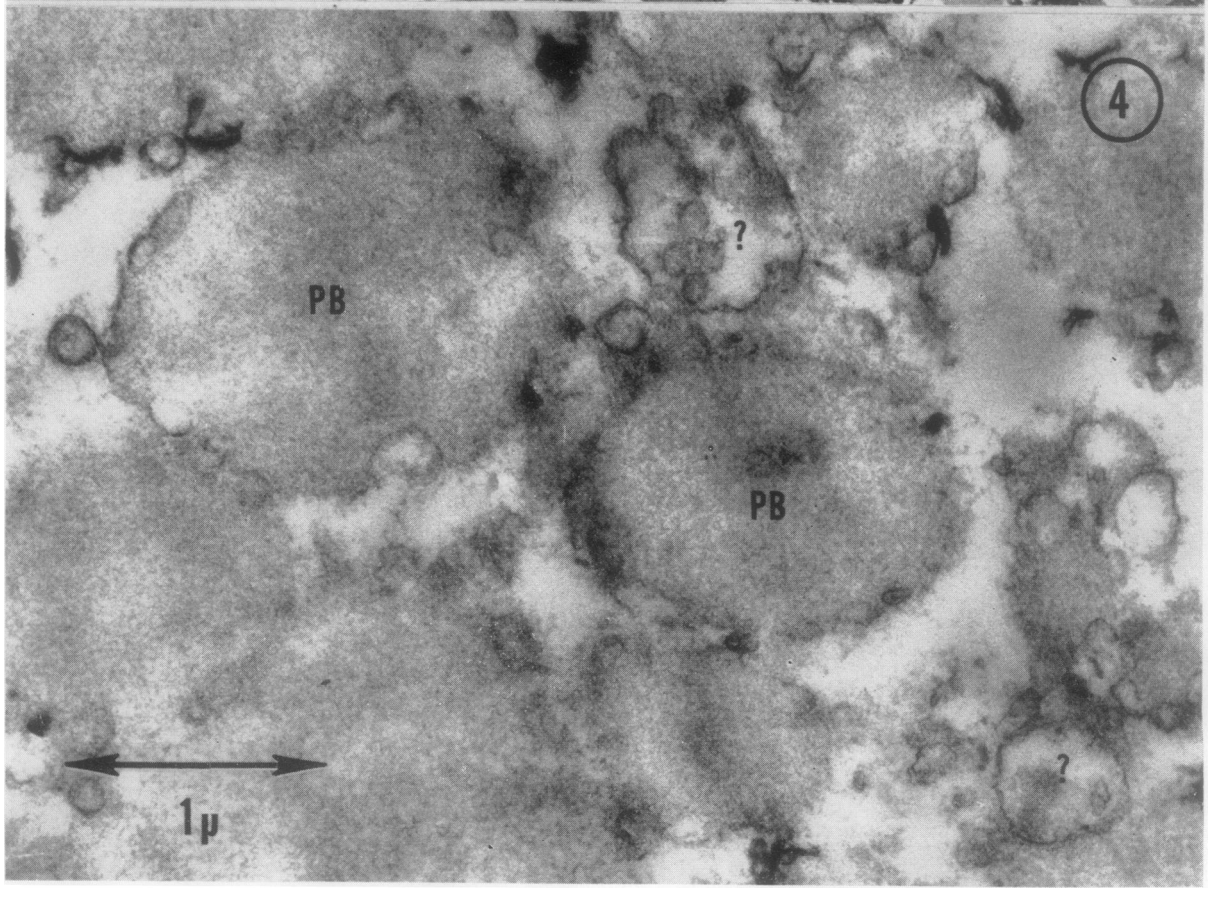
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FIG. 1. Electronmicrograph of pea cotyledon, incubated in moist sand overnight (approximately 18 hrs). The section is cut perpendicularly to the epidermal surface (EP) underlying the seed coat. The starch (ST) containing cells are typical of those found in the bulk of the cotyledon away from the axis tissue. The protein bodies (PB) occupy a large part of the cytoplasmic volume. They are usually roughly spherical and occasionally elongated or irregular in outline. They average approximately two microns in diameter. Because they are only rarely sectioned along their equatorial plane, many appear smaller. Their distribution is even throughout the cytoplasm and they do not appear to be associated with any other particular cytoplasmic component. Some strands of endoplasmic reticulum may be distinguished at the unmarked arrows and other areas. Potassium permanganate-uranyl nitrate fixation. Epoxy resin embedding. Magnification : 3,900 x.

FIG. 2. A higher magnification of an area in the lower left hand corner of figure 1 depicts strands of endoplasmic reticulum (ER), portion of a starch grain (ST), a mitochondrion (M), and several protein bodies (PB). The diameter of the latter varies partly because of intrinsic size variations and partly because they were sectioned somewhere away from their equatorial plane. The possibility of a membrane surrounding the protein bodies cannot yet be established or rejected. The generally mottled appearance of the section is a surface effect resulting from sectioning artifacts. Magnification : 22,000 x.

FIG. 3. Section through a pellet fraction. Relatively intact protein bodies (PB) form the bulk of the pellet. They now appear darker than the background due to the lack of dense cytoplasm surrounding them in whole tissue. Some contamination in the form of small, electron transparent, and irregularly shaped vesicles can be recognized. Potassium permanganate-uranyl nitrate fixation. Methacrylate embedding. Magnification : 7,500 x.

FIG. 4. A higher magnification of protein bodies (PB) as found in sections of the pellet fraction. No continuous membrane can be recognized with certainty around the protein bodies, although artifacts of preparation have to be expected. The irregular membrane bound vesicles (designated by question marks) are, as yet, of undetermined origin. Potassium permanganate-uranyl nitrate fixation. Epoxy resin embedding. Magnification : 34,500 x.

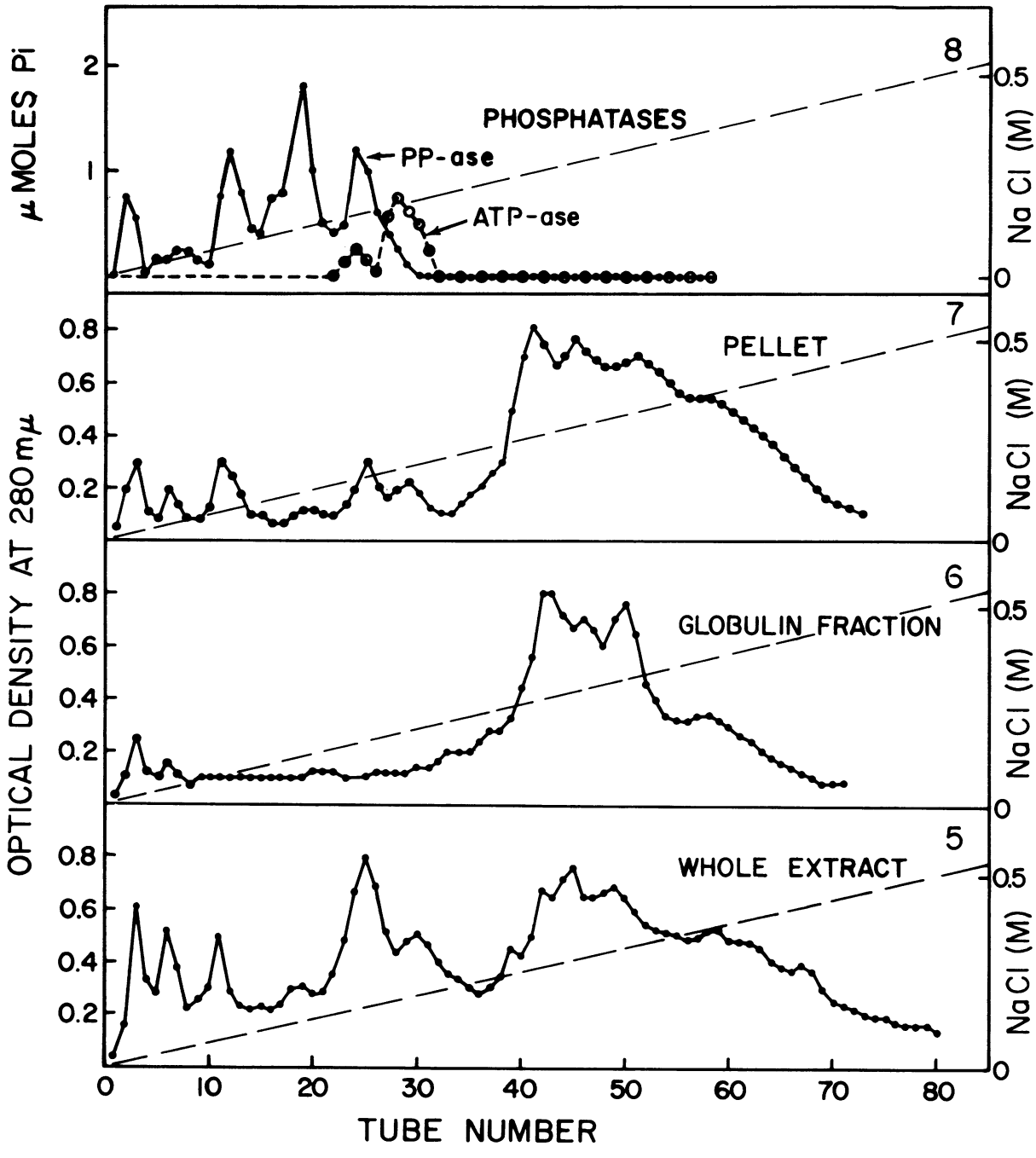

FIG. 5 to 8. DEAE cellulose chromatograms of various cotyledon materials. The preparation of the materials is described in Methods. In each case, elution was accomplished by a gradient of NaCl (in 0.01 M Tris, pH 7.8) increasing linearly from 0 to 0.5 M. Fractions were 6 ml each. The column was 1.1 × 20 cm. In figures 5, 6, and 7, protein concentration was followed by measuring optical density at 280 mμ. Figure 8 represents adenosine triphosphatase (ATP-ase) and pyrophosphatase (PP-ase) activity in the various fractions. Enzymic activity was measured as μ moles of P_i formed from potassium pyrophosphate and ATP as described under Methods. Figure 5. Whole extract obtained from 24 hour germinated peas. Figure 6. Globulin fraction from 24 hour germinated peas. Figure 7. Pellet fraction from 24 hour germinated peas. Figure 8. Supernatant fraction (144,000 × *g*) from peas germinated for 72 hours.