

DISTRIBUTION OF SOLUBLE PROTEINS AND ENZYMES DURING EARLY DEVELOPMENT OF *PISUM SATIVUM*

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Summary

Changes in soluble protein and in peroxidase, esterase, and amylase enzymes during development of the pea plant (*P. sativum*) have been investigated using a horizontal method of acrylamide gel electrophoresis. Both qualitative and quantitative variation between organs at different stages of physiological development are recorded.

I. INTRODUCTION

The existence of multiple forms of enzymes has been demonstrated in many organisms. These isoenzymes, all of which appear to be involved in normal growth and differentiation processes, often coexist in the one organ or tissue. Further, the relative abundance of each form may vary more or less independently during ontogenesis (Wilkinson 1965). Most investigations of isoenzyme systems have been made using various animal tissues. By comparison, there is little information available concerning similar systems in plants, especially with respect to ontogenetic variation. However, the study of multiple forms of plant enzymes has been approached with increasing vigour over the past few years. Peroxidase has been studied in corn (McCune 1961), in *Pisum* (Macnicol 1966; Siegel and Galston 1967), in *Nicotiana* (Pandey 1967), and in *Linum* (Tyson and Jui 1967). Esterase isoenzyme systems have been investigated in maize by Schwartz *et al.* (1965), and in *Solanum* by Schwartz *et al.* (1964) and Desborough and Peloquin (1967), whilst Macko, Honold and Stahmann (1967) have shown developmental variation in a number of isoenzyme systems in wheat during germination and the early growth phase.

This present paper reports an examination, using acrylamide gel electrophoresis, of synchronous changes in the activities of peroxidase, esterase, and amylase enzyme systems during growth and development of the pea plant *Pisum sativum*.

II. MATERIALS AND METHODS

Plant material used in this investigation was a dwarf, early-flowering variety of *P. sativum* (Line 22, Massey, from the collection of Mr. I. C. Murfet, Botany Department, University of Tasmania). Plants were grown during late summer in a glasshouse without supplementary lighting. Seeds were sown in a mixture of vermiculite-dolerite chips (1:1), and the young

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plants were supplied twice weekly with quarter-strength Hoagland's nutrient solution. Plantings were staggered to permit synchronous harvesting at all stages of development. The various organs were sampled as shown in Figure 1. Twenty-five plants were used in the preparation of each extract.

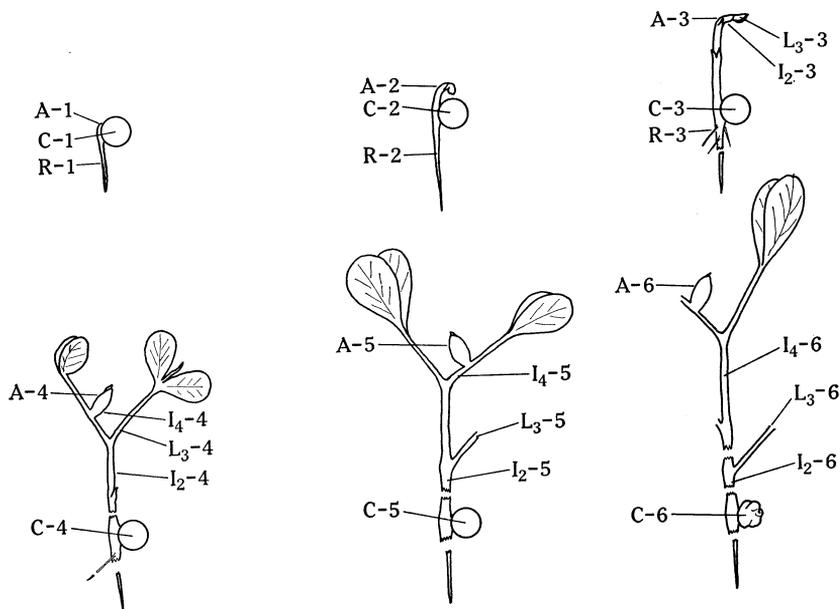


Fig. 1.—Diagrams showing various plant organs of *P. sativum* at six stages (1–6) of development. *A*, apex; *C*, cotyledon; *I*₂, internode 2; *I*₄, internode 4; *L*₃, leaf 3; *R*, root. Sampling took place at 2 (stage 1), 4 (stage 2), 7 (stage 3), 10 (stage 4), 12 (stage 5), and 16 (stage 6) days (approximately) after germination. This explanation of symbols also applies to Figures 2–9.

(a) Preparation of Extracts

All procedures were conducted at 2°C. The plant material was ground in a mortar with fine sand. In the case of samples of apices and undeveloped leaves and internodes it was necessary to add 0.2 ml of buffer [Tris (38 μM)–citric acid (2.5 μM), pH 8.7 at 20°C] during grinding to obtain sufficient volume of extract. The resultant slurry was centrifuged at 20,000 *g* for 20 min, and the supernatant assayed for protein content by the method of Lowry *et al.* (1951). All samples were then adjusted to give a protein concentration of about 2.8 mg/100 ml before electrophoresis.

(b) Electrophoresis

Horizontal electrophoresis was conducted in a Shandon apparatus, using an 8% acrylamide gel (Cyanogum 41, B.D.H.) after the method of Lund (1965). The gel was polymerized in a flat Perspex mould (10.0 by 18.0 by 0.65 cm) covered with a plate-glass lid, from which projected a row of 13 celluloid slot formers (7.0 by 6.0 by 0.5 mm). Each slot accepted 20 μl of extract. An initial potential of 8 V/cm was applied to the gel and a constant current of 35 mA maintained throughout. A run of about 5 cm was achieved in 3½–4 hr with this system. At the conclusion of the run, the gel was sliced horizontally into four using the Shandon "slicer". These slices were then separately stained to show the distribution of various enzymatic activities, and to demonstrate the variation in protein composition between the samples. This procedure has advantages over the more commonly employed tube method of disk electrophoresis in that it

allows for direct comparison of a large number of samples under strictly comparable conditions in a single gel preparation. Further, the method allows for the direct comparison of four different reactions of each sample by superimposition of the slices. After staining, the gel patterns were recorded photographically, and a quantitative comparison of the samples was made by densitometry. The stained patterns corresponding to individual samples were cut from the respective slices and examined in a microdensitometer (Unicam S.P. 590 attachment for the S.P. 500 spectrophotometer, modified for automatic drive and linear recording in the Botany Department workshop).

(c) Staining Reactions

All reactions were conducted at room temperature. All buffer solutions were prepared according to Gomori (1955).

Peroxidase activity was demonstrated by immersing a slice for 20 min in 50 ml of citrate-phosphate buffer, pH 4.4, containing *o*-dianisidine ($2.5 \times 10^{-3}M$) and H_2O_2 (0.5 ml of 30% w/v). Other peroxidase substrates were also tested, e.g. benzidine, guaiacol, and *p*-anisidine, but these gave a less intense and less permanent stain.

Esterase activity was shown by first incubating a slice for 15 min in 50 ml of phosphate buffer, pH 6.4, containing α -naphthyl acetate ($2 \times 10^{-3}M$). It was then transferred for 10–15 min to a fresh 50 ml of phosphate buffer containing, in addition to α -naphthyl acetate, 50 mg of diazo blue B salt (Michrome 250, E. Gurr Ltd.). The background colouring was removed from the gel by several water rinses. As most of the pea samples contain phenolic compounds which react with diazo blue B, blanks were conducted omitting α -naphthyl acetate from the assay in order to specify true esterase activity.

Amylase activity was shown by negative staining of a gel to which 0.6% soluble starch had been added during preparation. After electrophoresis a slice was incubated for 1 hr with 50 ml of phosphate buffer, pH 6.4, and then transferred to a fresh 50 ml of phosphate buffer containing 0.005% I_2 and 1.5% KI (w/v). Bands corresponding to amylase activity appeared as clear patches on a dark blue background.

Protein was located by immersing one slice for 1 hr in a solution of 0.7% amido black in 10% acetic acid, followed by numerous rinses in 10% acetic acid to clarify the background. Identification of bands was based on their relative electrophoretic mobilities. The bands were labelled in their numerical order, starting from the anodic end of the gel. The position of bands is also specified by their R_p values (after Fox, Thurman, and Boulter 1964) for anodic-migrating bands, and R'_p values for cathodic-migrating components, where

$$R_p \text{ or } R'_p = \frac{\text{distance from origin to leading edge of band}}{\text{distance from origin to the borate ion boundary}},$$

differences in sign being ignored.

III. RESULTS

Steward, Lyndon, and Barber (1965), in their original application of high-resolution disk electrophoresis to the analysis of protein distribution in the pea plant, showed clearly that different organs contain a varying complement of proteins. Figure 2 illustrates the complex and changing protein pattern which characterizes development of the various organs as shown by these present investigations. However, the amount of useful information to be gained from Figure 2 is limited by doubts about the precise homology of components in different samples when a non-specific reagent such as amido black is used to detect bands. Only in one or two cases can one be confident about homology. Thus, extracts of leaves show (Fig. 8) a characteristic heavy band of low electrophoretic mobility which is probably

the so-called "fraction 1" of the chloroplast protein (Laycock, Boulter, and Thurman 1966). This band also appears in extracts of other green tissues, particularly internodes, and possibly in apices. Secondly, electrophoretograms of cotyledon

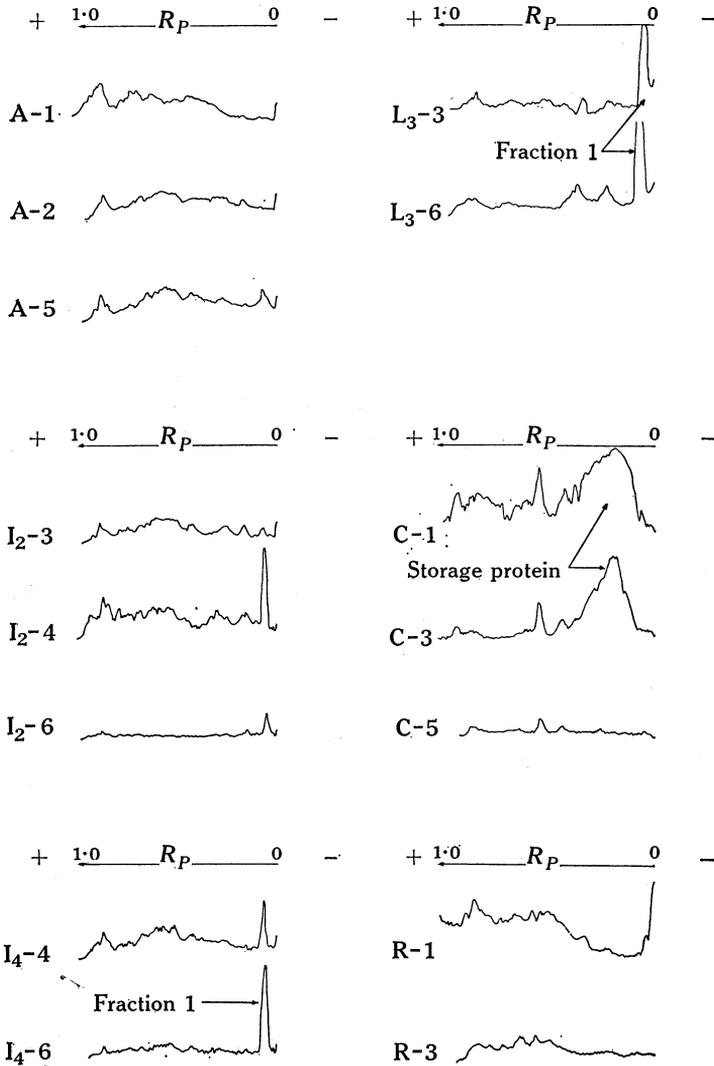


Fig. 2.—Densitometer traces illustrating the ontogenetic variation in soluble protein in organs of *P. sativum*.

extracts are characterized by heavy bands corresponding to the major storage globulins. The depletion of these globulins can be followed through successive samples (Fig. 8).

This problem of homology is overcome to a large extent when enzyme reagents are used as more specific locating stains. The results which are illustrated in Figures

3-5 show the transitional variations in the activities of peroxidase, esterase and amylase enzyme systems which occur during development of *P. sativum*. There is no evident congruence between the general protein pattern (amido black staining) and the patterns of these particular enzymes.

The investigation has revealed the presence of at least 17 components with peroxidase activity, 16 with esterase activity, and 9 amylases in the various organs of the pea plant. Many of the bands were seen in the electrophoretograms of all organs, though in varying amount, but a few bands were restricted in their distribution to a given organ at a particular phase of its development, e.g. peroxidase band 9 occurred only in root tissue. When mixed samples of extracts from different organs were examined, no additional bands could be resolved, suggesting that a high level of homology existed between the respective enzymic components in the different organs.

The patterns of activity shown by the different plant parts varied qualitatively as well as quantitatively during ontogenesis, but in general each organ tended towards a characteristic and constant pattern as it approached maturity. The most notable features revealed by the investigation are summarized below.

(a) *Peroxidase Activity (Figs. 3 and 9)*

There was considerable qualitative homology between organs, though quantitative variation was very marked. All organs showed a progressive intensification of peroxidase activity as development proceeded:

- (1) Bands 1 and 2 (R_p 0.94 and 0.92) were absent from apices but appeared in all other organs as they approached maturity. In this investigation the apex was taken to consist of the apical meristem and 4-5 rudimentary nodes and internodes.
- (2) Bands 3-5 (R_p 0.85, 0.82, and 0.77) all showed a marked increase in activity through successive samples of apices. In the roots, leaves, and cotyledons bands 3 and 5 were weakly represented, but band 4 increased during ontogenesis, whilst in internodes all three bands decreased with approaching maturity.
- (3) Band 6 (R_p 0.70) was seen only in the senescent cotyledons. This band may well be a contaminant, since these cotyledons showed signs of bacterial and fungal infection. Farkas and Stahmann (1966) have reported such an increase in peroxidase activity in infected bean.
- (4) Bands 7 and 8 (R_p 0.61 and 0.57) were absent from root tissue where instead a very strong component, band 9 (R_p 0.46), occurred.
- (5) Band 10 (R_p 0.39) was strongly represented in the aerial parts of the plant and appeared most active in apices with one or more flower primordia. The first flower primordium is visible in line 22 at 8-10 days from germination (Johnston 1966).
- (6) Bands 11 and 12 (R_p 0.22 and 0.14) showed very strong activity in cotyledons (but decreasing as germination progressed), and in mature

root tissue. However, other tissues showed only a diffuse zone of weak peroxidase activity extending from the origin.

- (7) Band 13 (R_p 0.05) was clearly evident only in root, internode, and leaf tissues as they became mature.

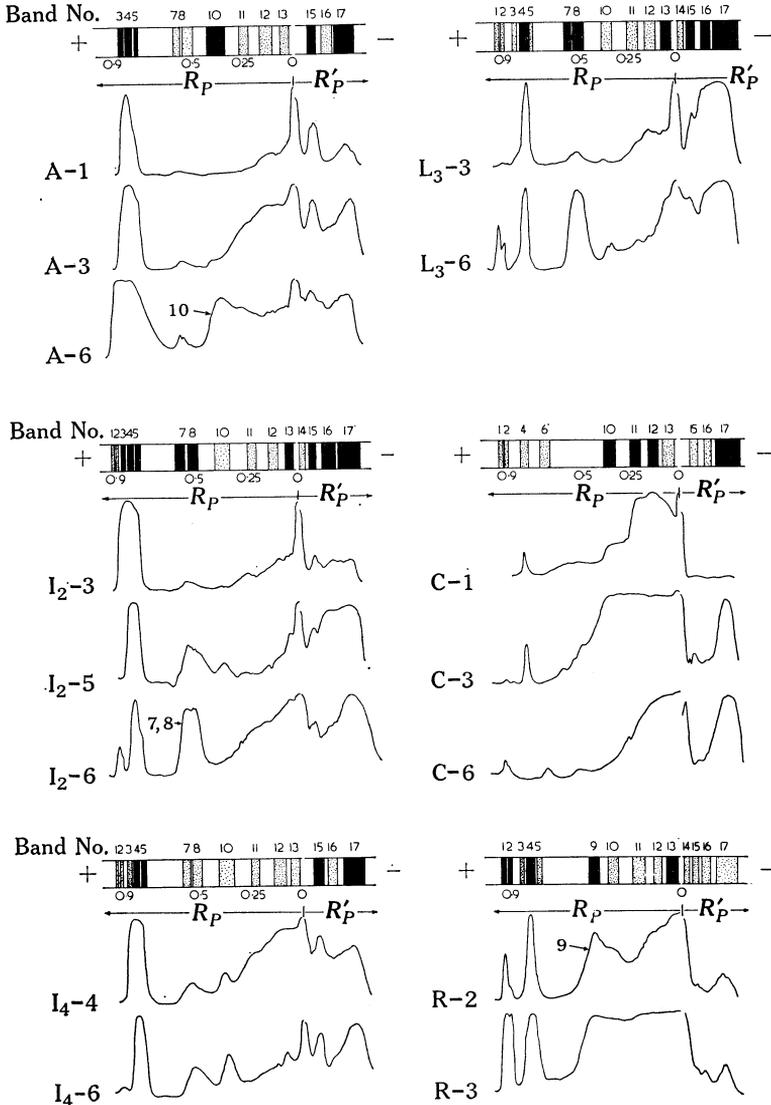


Fig. 3.—Interpretive diagrams and densitometer traces illustrating the ontogenetic variation in peroxidases in organs of *P. sativum*.

All the above components of the peroxidase complex showed anodic migration. A further group of 4 bands migrating towards the cathode were also present. In earlier investigations of peroxidase isoenzymes, both Moustafa (1963) and Siegel and Galston (1963) had demonstrated cathodic-migrating peroxidases. The enzymic

nature of these bands was confirmed in the present experiments, since all activity was lost on heating the extracts. These peroxidases were present in all organs, but were most strongly developed in leaf, internode, and cotyledon extracts. Band 17 (R'_P 0.29) was the most variable component.

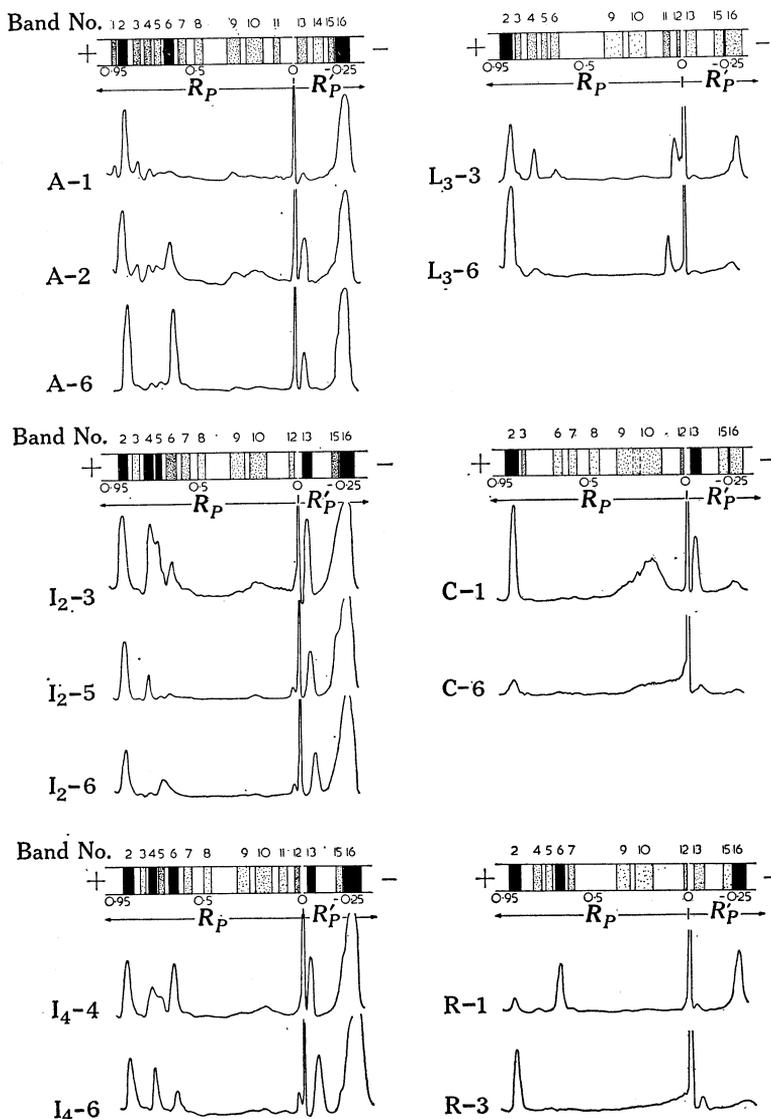


Fig. 4.—Interpretive diagrams and densitometer traces illustrating the ontogenetic variation in esterases in organs of *P. sativum*.

(b) Esterase Activity (Figs. 4 and 7)

As with the *P. sativum* peroxidases, both anodic- and cathodic-migrating esterase bands were demonstrated. Cathodic-migrating esterases were not reported in *Solanum* by Desborough and Peloquin (1967) or in wheat by Macko, Honold, and

Stahmann (1967). However, these authors all used the tube method of disk electrophoresis in their analysis of esterase distribution. With this technique any cathodic-migrating components are lost into the upper electrode chamber.

- (1) Band 1 (R_p 0.95) was observed only in the sample of 2-day apices. The presence of this band in other extracts may be obscured by phenolic compounds, which migrate near the borate ion boundary and react with the diazo blue B reagent.
- (2) Band 2 (R_p 0.92) was a regular and constant feature of all organs.
- (3) Bands 3-5 (R_p 0.83, 0.76, and 0.72) were present in young aerial organs but disappeared rapidly as development proceeded.
- (4) Band 6 (R_p 0.66) was present in all organs in variable amounts. It increased during ontogenesis of apices, but decreased in other organs as they matured.
- (5) Bands 7, 8, and 14 (R_p 0.55 and 0.50, and R'_p 0.12) were seen only in apices and internodes.
- (6) Bands 9 and 10 (R_p 0.31 and 0.24) were diffuse zones of esterase activity, present in apices, internodes, and cotyledons.
- (7) Bands 11 and 12 (R_p 0.09 and 0.04) were restricted to leaf tissue.
- (8) Band 13 (R'_p 0.04) was present in relatively high concentration in all organs except mature leaves and senescent cotyledons.
- (9) Bands 15 and 16 (R'_p 0.20 and 0.26) were present in all organs. The activity of these bands remained constant in all samples of apices and internodes, but decreased rapidly during development of leaf and root tissue, and the senescent cotyledon.

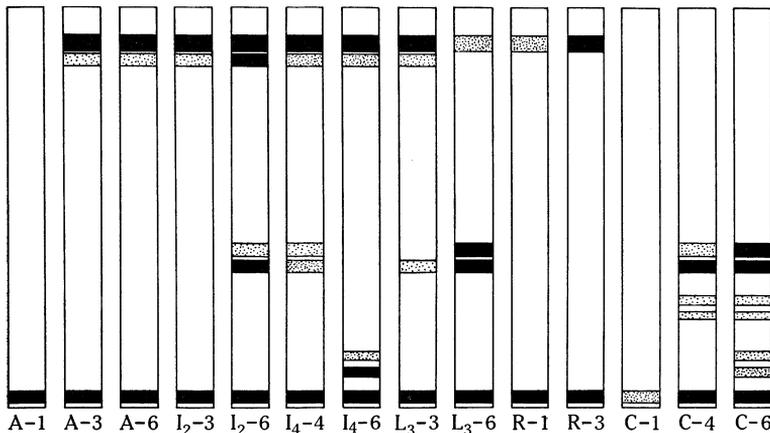
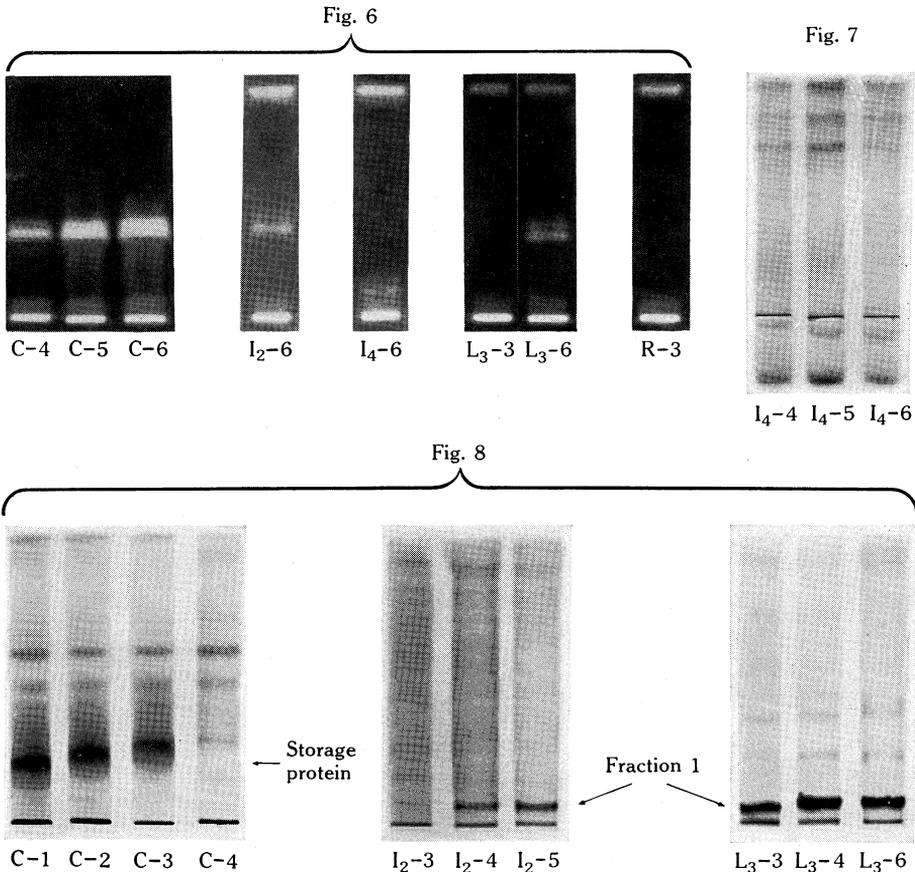


Fig. 5.—Interpretive diagram illustrating the ontogenetic variation in amylases in organs of *P. sativum*.

(c) *Amylase Activity (Figs. 5 and 6)*

A total of nine bands with amylase activity was demonstrated. All of these, except bands 1, 2, and 7 (R_p 0.93, 0.89, and 0.14) were present in cotyledon extracts. Bands 3 and 4 (R_p 0.42 and 0.37), which also appeared weakly in extracts of mature internodes and leaves, were the most active of the cotyledon amylases. Bands 5 and

6 (R_p 0.28 and 0.24) appeared to be restricted in distribution to cotyledons. Two other components of low electrophoretic mobility, bands 8 and 9 (R_p 0.10 and 0.05), were present in all extracts.



Figs. 6-8.—Photographs of gel slices stained to show variation in amylases (Fig. 6), esterases (Fig. 7), and soluble protein (Fig. 8) in organs of *P. sativum*.

IV. DISCUSSION

The precise role of isoenzyme systems in metabolism is as yet not clearly understood. However, the rapidly expanding documentation of enzyme polymorphism, with examples from all groups of living organisms, attests to the increasing importance which biochemists attach to these complexes, and suggests that they have considerable metabolic significance. Schwartz *et al.* (1965), investigating maize esterases, and Pandey (1967), studying *Nicotiana* peroxidases, have been able to demonstrate the genetic relationship in production of the respective isoenzymes. However, in each case the precise biochemical roles of these systems are not defined. Indeed, a broad spectrum of biochemical function including involvement in respiration, lignification (Brown 1966), and regulation of hormone levels (Ray 1958), as well as a possible photosynthetic function (Macnicol 1966), has been assigned to

the peroxidase complex. It may well be that more than one group of peroxidase isoenzymes is present in plant tissues which have distinctive functions *in vivo*, yet are not discriminated by the type of unselective reagent usually employed in assays *in vitro*. Thus Macnicol (1966) has demonstrated variable indoleacetic acid oxidase: peroxidase ratios in four fractions obtained from various *P. sativum* tissues, and

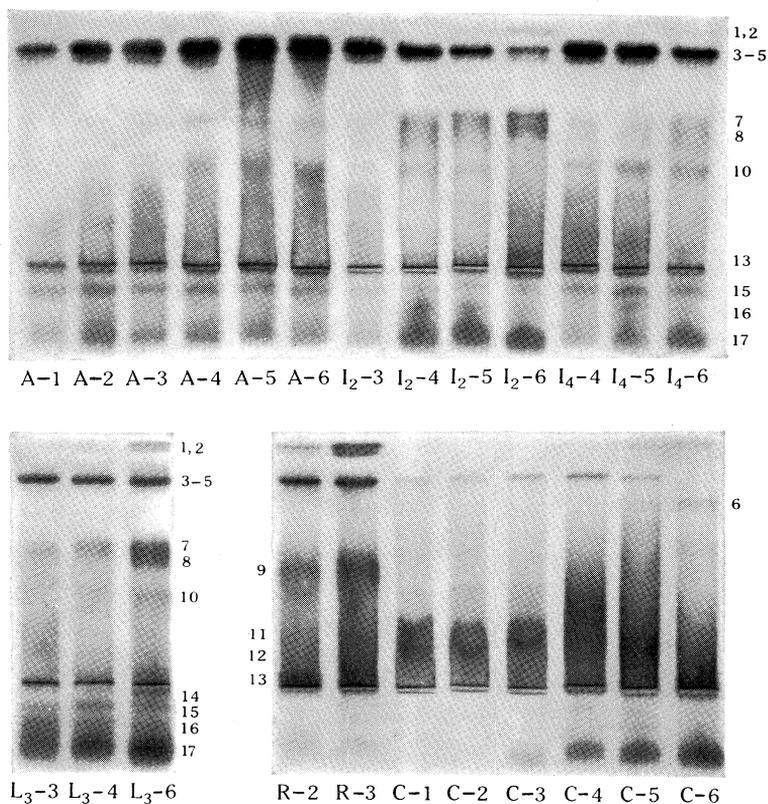


Fig. 9.—Photographs of gel slices stained to show variation in peroxidases in organs of *P. sativum*.

separated by ion-exchange chromatography. In our laboratory we have conducted some preliminary experiments which test the relative sensitivity of the peroxidase components to various inhibitors, and allow a measure of differentiation to be made between them (Mills and Crowden, unpublished data). As an illustration, we have shown that bands 3-5 are practically insensitive to 10^{-3}M potassium cyanide, and that band 13 is only partially inhibited by 10^{-3}M sodium azide. These same concentrations effect total inhibition of the remaining components.

A broad reaction spectrum is also characteristic of esterases. However, a provisional grouping of animal esterases has been made by Augustinsson (1961) on the basis of substrate and inhibitor specificities. Our preliminary experiments with inhibitors have shown a differential sensitivity of *P. sativum* esterases to eserine and *p*-chloromercuribenzoate within a concentration range of inhibitor of 10^{-4} – 10^{-6}M .

These results suggest that the multiple bands which are resolved by electrophoresis are not all isoenzymic, and that at least some of the bands are due to different esterases.

The method of acrylamide gel electrophoresis as used in this investigation provides a useful and rapid tool for the study of enzyme activities during differentiation. By the application of these techniques on a wider scale it may be possible to construct developmental profiles for various enzyme systems which in turn may be used as indices of differentiation for the particular plant under investigation.

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