**Appearance of Three Chloroplast Isoenzymes in Dark-grown Pea Plants and Pea Seeds**

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**ABSTRACT**

Activity peaks characteristic of the chloroplastic Calvin cycle enzymes triose-phosphate isomerase, ribose 5-phosphate isomerase, and fructose 1,6-diphosphate aldolase are found in isoelectric focusing patterns of dark-grown pea (*Pisum sativum*) seedlings and seeds. Apparently, in this higher plant these three chloroplastic isoenzymes can be formed in the absence of light and of chloroplast formation.

As in other angiosperms, light is required for chlorophyll biosynthesis and chloroplast formation in pea plants. The levels of the Calvin cycle specific enzymes ribulose-1,5-diP carboxylase (8), alkaline fructose-1,6-diP phosphatase (14), and TPN-linked glyceraldehyde-3-P dehydrogenase (9), and of chloroplast rRNA (13) are much lower in dark-grown pea seedlings than in normal leaves. Since the chloroplastic Calvin cycle forms of fructose-1,6-diP aldolase (5), triose-P isomerase (1), and ribose-5-P isomerase (2) can be separated from the cytoplasmic forms by isoelectric focusing, it seemed of interest to look for the three chloroplastic isoenzymes in etiolated seedlings. The experiments reported here clearly demonstrate that isoenzymes having isoelectric points characteristic of the chloroplastic forms of ribose-5-P isomerase, triose-P isomerase, and fructose-1,6-diP aldolase are present in dark-grown seedlings and in seeds. We conclude that light is not necessary for the formation of these three Calvin cycle enzymes.

**MATERIALS AND METHODS**

**Plant Material.** Pea (*Pisum sativum*, var. Little Marvel) plants were grown in vermiculite in the complete absence of light for 7 days or in the greenhouse as described previously (1).

**Isoelectric Focusing.** Extracts of dark-grown pea seedlings were prepared by homogenizing approximately 1 g of epicotyl tissue in 10 ml of 0.1% glycine, pH 7.4, in a glass tissue grinder in the dark room. Pea seeds were soaked overnight at 4°C in running tap water, the seed coats removed, and the seeds blended in 0.1% glycine, pH 7.4, in a Waring Blender. Green leaf chloroplast and cytoplasmic extracts were prepared as described previously (3). All extracts were centrifuged for 20 min at 40,000 g. Isoelectric focusing experiments were conducted as described in reference 5, using a 110-ml LKB isoelectric focusing column, and in the figure legends. Electrode solutions were dilute NaOH and H2PO4.

**Enzyme Assays and Protein Determination.** Fructose-1,6-diP aldolase activity was measured by the method of Wu and Racker (18), triose-P isomerase activity, as described by Gibbs and Turner (7), and ribose-5-P isomerase activity, using the assay of Axelrod and Jang (6). Protein concentrations were estimated by the biuret method as described previously (3).

**Reagents.** All reagents used were the highest quality commercially available. Pea seeds were obtained from Atlee Burpee Company.

**RESULTS**

Peaks characteristic of chloroplast and cytoplasmic aldolases are found when extracts of etiolated seedlings or seeds are subjected to isoelectric focusing (Fig. 1). Results with tissue from dark-grown plants are not entirely reproducible; in some instances only pl' 4.8 aldolase was found, in others, only pl' 4.2 aldolase. Since the cytoplasmic form of this enzyme is unstable to isoelectric focusing (5), it seems likely that both forms are present in the dark-grown plant.

Pea seed aldolase is remarkably stable to isoelectric focusing, which suggests that the seed isoenzymes are not as sensitive as the leaf isoenzymes to the molecular O2 generated during electrophoresis. Willard and Gibbs (17) found that although bean leaf aldolase is inhibited by p-chloromercuribenzoate, the seed aldolase is not. In spite of the similar focusing patterns and identical pl' values for the seed and leaf enzymes found in the present experiments, it seems likely that the seed enzymes do differ from the leaf enzymes, at least in the number of reactive thiol groups.

Takeo (16) found two bands of aldolase activity in the disc gel electrophoretic patterns of carrot and radish leaves, one of which in each case corresponded to a single band of activity from seed extracts. When spinach seeds and leaves were analyzed he found single, nonidentical activity bands. The distribution of aldolase activity in the pea seed and leaf apparently differs from that in carrot and radish, and in spinach.

Multiple peaks of triose-P isomerase activity are found when extracts of dark-grown shoots are focused (Fig. 2b). Clearly, a form of triose-P isomerase similar to, or identical with, the chloroplastic form is present in etiolated tissue. The minor peaks probably correspond to green leaf cytoplasmic isomerase. In another experiment (not shown), extracts of chloroplasts and of etiolated seedlings were mixed together and focused; the major peaks did not separate.

A large triose-P isomerase peak, which would overlap both

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2 A preliminary report of these findings was presented at the American Society of Plant Physiology Meetings, Minneapolis, Minn., 1972 (10).
Fig. 1. a: Isoelectric focusing patterns of pea leaf chloroplast (●) and cytoplasmic (■) aldolases from reference 5. The anode was at bottom of column, cathode at top, and 4–6 ampholyte was used in all experiments in this figure. pH values (abscissa) are the best straight-line fit to the actual values. Fraction size, as plotted, is therefore consistent within runs but not between runs. b: Isoelectric focusing pattern of etiolated pea shoot aldolases. Left hand ordinate corresponds to plot on left, right hand ordinate to plot on right. Extract containing 10 mg of protein was focused. In two experiments cytoplasmic-type aldolase on left was found, in two experiments chloroplast pattern was found, and in one experiment (elec-

trode position reverse of other runs) both peaks were found. c: Isoelectric focusing pattern of pea seed aldolases. Extract containing 10 mg of protein was focused. Similar results were obtained in three experiments.

Fig. 2. a: Isoelectric focusing patterns of pea leaf chloroplast (●) and cytoplasmic (■) triose-P isomerases. The cathode was at bottom of column, anode at top, and 4–6 ampholyte was used in all experiments in this figure. One mg of protein was focused in each experiment. pH values as in Figure 1. b: Isoelectric focusing pattern of etiolated pea shoot triose-P isomerase. Similar results were obtained in three different experiments. c: Isoelectric focusing pattern of pea seed triose-P isomerase. Similar results were obtained in three experiments.
green leaf isoenzymes, is found when extracts of pea seeds are focused (Fig. 2c). We were not able to resolve this isomerase activity into discrete peaks in four experiments. The width of the peak makes it seem possible that two or more forms with similar isoelectric points are present.

A peak of ribose-5-P isomerase activity corresponding to chloroplast isomerase is found in focusing patterns of seeds and of etiolated seedlings (Fig. 3). There is also a very minor peak which corresponds to cytoplasmic ribose-5-P isomerase.

**DISCUSSION**

Although in certain cereal plants levels of Calvin cycle enzymes are almost as high in dark-grown as in normal light-grown plants (15), in dark-grown pea plants the levels of activity of the Calvin cycle-specific enzymes ribulose-1,5-diP carboxylase, fructose-1,6-diP phosphatase, and TPN-linked glyceraldehyde-3-P dehydrogenase are quite low (8). Nevertheless, the three Calvin cycle enzymes included in this study are present in etiolated tissue at levels which, at least after isoelectric focusing, appear to be similar to green leaf levels. Although this technique is not quantitative, 10-fold differences in levels of enzyme activity would certainly have been detectable in these experiments. Clearly chloroplastic aldolase, triose-P isomerase and ribose-5-P isomerase are formed in the absence of light in tissue which is incapable of photosynthetic carbon dioxide fixation, not only because of the lack of chlorophyll but also because of the low levels of other Calvin cycle enzymes.

It seems likely that these three chloroplastic isoenzymes are functional in etiolated tissue, possibly participating in carbohydrate metabolism within the etioplast. A similar suggestion was made by Schnarrenberger et al. (12), who found high levels of triose-P isomerase and DPN-linked glyceraldehyde-3-P dehydrogenase activity in etioplasts isolated from sunflower cotyledons. Since plastids are found in etiolated tissues and in seeds, the chloroplast isoenzymes are probably actually plastid rather than chloroplast specific. Further experiments are needed to localize these isoenzymes and to determine whether they are actually structurally identical with the chloroplastic isoenzymes.

Dark-grown pea seedlings contain low levels of chloroplastic 70S rRNA (13), and etioplasts from such seedlings are not as active in amino acid incorporation as are chloroplasts from light-grown plants (11). The chloroplast form of aldolase may be coded by a nuclear gene, since the isoelectric point of this isoenzyme is under Mendelian gene control (4). The presence of these three chloroplastic isoenzymes in tissue with low levels of chloroplastic rRNA and low levels of plastid-localized protein synthetic activity suggests that they may be transcribed and translated outside of the chloroplast or proplastid. Additional work is needed to determine the site of synthesis of these Calvin cycle enzymes.

**LITERATURE CITED**


