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Isozymes of the Glycolytic Enzymes in Endosperm from Developing Castor Oil Seeds[1]

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JAN A. MIERNYK AND DAVID T. DENNIS
Biology Department, Queen's University, Kingston, Ontario Canada K7L 3N6

ABSTRACT

Ion filtration chromatography on diethylaminoethyl-Sephadex A-25 has been used to separate two isozymes each of triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, glycerate 3-phosphate kinase, enolase, and phosphoglycerate mutase from homogenates of developing castor oil (*Ricinus communis* L. cv. Baker 296) seeds. Crude plastid fractions, prepared by differential centrifugation, were enriched in one of the isozymes, whereas the cytosolic fractions were enriched in the other. These data (and data published previously) indicate that plastids from developing castor oil seeds have a complete glycolytic pathway and are capable of conversion of hexose phosphate to pyruvate for fatty acid synthesis. The enzymes of this pathway in the plastids are isozymes of the corresponding enzymes located in the cytosol.

Plastids are the site of long-chain fatty acid synthesis in developing castor oil seeds (19, 30). These plastids, separated from other organelles on sucrose gradients, contain: the pyruvate dehydrogenase complex (22); acetyl-CoA carboxylase (23); acyl carrier protein; and fatty acid synthetase (4). Pyruvate is probably produced from hexose phosphates within the plastids, since a crude plastid preparation from developing castor oil seeds contains the activities of all the glycolytic and pentose phosphate cycle enzymes with the exception of glucose-6-P dehydrogenase (24). The activities of pyruvate kinase (2), gluconate-6-P dehydrogenase, glucose phosphate isomerase and aldolase (25), and phosphofructokinase (7, 8) associated with the plastids are due to distinct isozymes.

Several isotope incorporation studies support the concept of plastid glycolysis and subsequent fatty acid synthesis from pyruvate (17, 18, 29). However, this does not appear to be universal. Insignificant phosphoglycerate mutase or enolase activities were found in particulate preparations from germinating *Cucurbita pepo* cotyledons (1), and phosphoglycerate mutase activity was not detected in isolated pea chloroplasts (27). It has been suggested that hexokinase activity is located only on the outer surface of pea chloroplasts and might represent an artifact associated with tissue disruption (28). In some cases, plastid-cytosol shuttles have been suggested to bypass missing enzymic steps (28). Reports that various plastids might lack specific enzymes of the complete glycolytic pathway prompted us to examine further the plastids from developing castor oil seeds. We now report that the association of triose-P isomerase, glyceraldehyde-3-P dehydrogenase, glycerate-3-P kinase, phosphoglycerate mutase, and enolase activities with plastids from castor oil seeds is due to distinct isozymes.

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MATERIALS AND METHODS

Castor oil plants, *Ricinus communis* L. cv. Baker 296, were grown in a greenhouse as described previously (26). To obtain the total enzyme complement, developing seeds were homogenized for 60 s with a Polytron (Brinkman Instruments) at a setting of 6, in two volumes of 100 mM K-phosphate (pH 6.9) containing 5 mM $MgCl_2$, and centrifuged at 27,000*g* for 20 min. The supernatant, after removal of the floating lipid layer, was then used directly. Crude plastid fractions were obtained by differential centrifugation, as described by Simcox *et al.* (24), using the homogenization medium of Miernyk and Trelease (16). The 10,000*g* supernatant fraction from plastid isolation was used as the crude cytosolic fraction.

Ion filtration chromatography (14) on Sephadex A-25 was done essentially as described by Simcox and Dennis (25). Columns were equilibrated with 20 mM imidazole buffer containing 5 mM $MgCl_2$ and, in some cases, 7 mM mercaptoethanol. The pH of the buffer was different for each enzyme separation and is given in the figure legends. After sample application (15 ml), the activities were eluted by application of 500 ml 0.5 M KCl in the equilibration buffer. Columns were run at 90 ml h^{-1}; 4.5 ml fractions were collected. In some cases, where enzyme activity was low, large samples were concentrated by precipitation with 80% (w/w) $(NH_4)_2SO_4$ before loading onto the column.

All *A* readings and enzyme assays were conducted at room temperature (approximately 21°C) using a Cary 210 spectrophotometer. All assays were linear with respect to time and sample concentration. Glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) was assayed according to Duggleby and Dennis (6), and triose-P isomerase (EC 5.3.1.1) was assayed according to Dennis and Green (3), except that 100 mM Tes (pH 7.8) was used. Assays for glycerate-3-P kinase (EC 2.7.2.3) contained 86 mM Tes (pH 7.5), 10 mM $MgCl_2$, 0.05 mM NADH, 2.7 mM ATP, 2 mM DTT, 6 units glyceraldehyde-3-P dehydrogenase, 9 units triose-P isomerase, and sample, in a final volume of 1.0 ml. Assays were initiated with 20 μl 135 mM glycerate-3-P. Assays for enolase (EC 4.2.1.11) contained 88 mM Tes (pH 7.5), 10 mM $MgCl_2$ 0.05 mM NADH, 2.7 mM ADP, 5 units pyruvate kinase, 6 units lactate dehydrogenase, and sample in a final volume of 1.0 ml. Reaction was initiated by addition of 20 μl of 25 mM glycerate-2-P. Assays for phosphoglycerate mutase (EC 5.4.2.1) were identical to those for enolase with the addition of 1 unit of rabbit muscle enolase. Reactions were initiated with 20 μl 25 mM glycerate-3-P.

All buffers, substrates, etc., were from Sigma, and coupling enzymes were either from Sigma or from Boehringer-Mannheim. Imidazole was recrystallized three times from ethyl acetate before use. DEAE Sephadex A-25 is a product of Pharmacia. All pH readings were made at room temperature, and conductivity was measured at room temperature with a Radiometer conductivity meter.

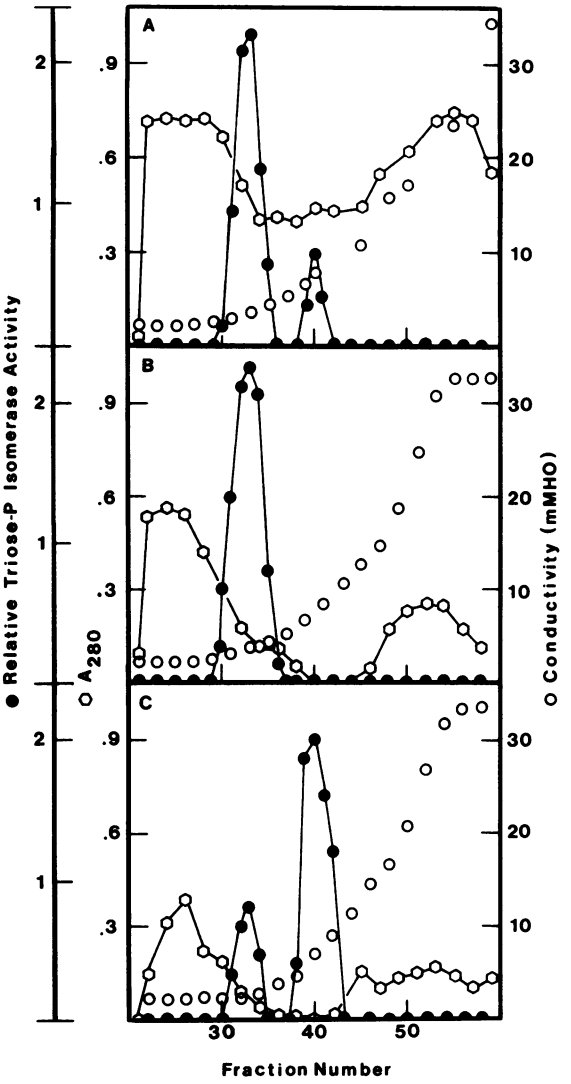

FIG. 1. Ion filtration chromatography of an S_{27} fraction (A); a crude cytosol fraction (B); and a plastid-enriched fraction (C); each assayed for triose-P isomerase activity. The column equilibration buffer was 20 mM imidazole (pH 7.1) containing 5 mM $MgCl_2$. ●, Enzyme activity; ○, conductivity; , A_{280}.

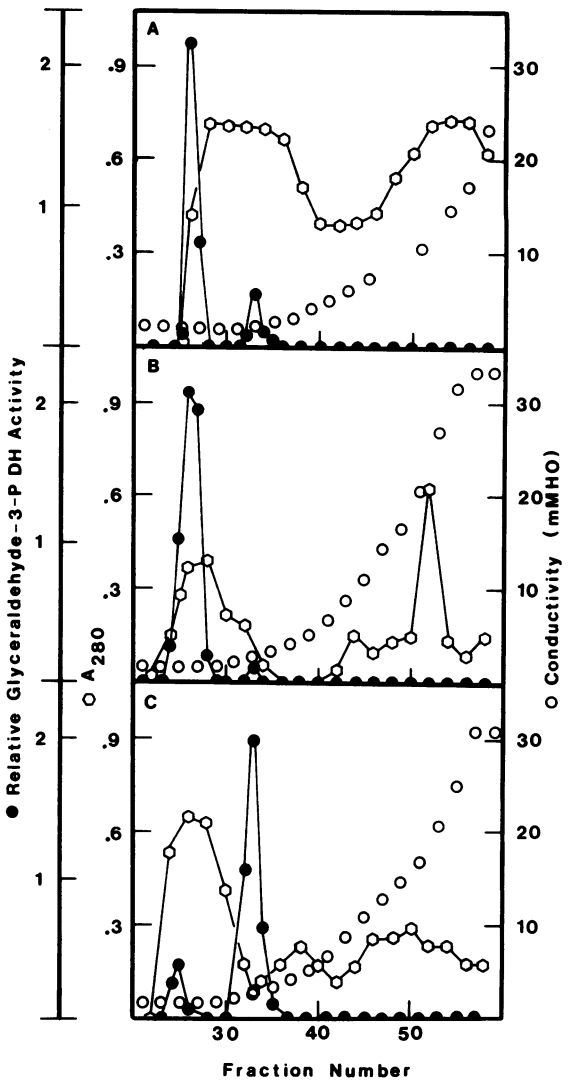

FIG. 2. Ion filtration chromatography of an S_{27} fraction (A); a crude cytosol fraction (B); and a plastid-enriched fraction (C); each assayed for glyceraldehyde-3-P dehydrogenase activity. Column equilibration buffer was 20 mM imidazole (pH 7.1) containing 5 mM $MgCl_2$ and 7 mM 2-mercaptoethanol. Symbols are as for Figure 1.

RESULTS AND DISCUSSION

Ion filtration chromatography is a powerful tool for enzyme separations, and, additionally, it allows a column run to be eluted in 4 h, an important consideration if an enzyme is unstable (25). For each of the enzymes studied, the equilibration pH was adjusted so that one isozyme did not bind to the column and the other eluted in the sieving range. Further details are given in the figure legends. In each case, this method yielded baseline separations between isozymes. Figures 1A, 2A, 3A, 4A, and 5A clearly show the presence of two isozymes of triose-P isomerase, glyceraldehyde-3-P dehydrogenase, glycerate-3-P kinase, phosphoglycerate mutase, and enolase, respectively, in a 27,000*g* supernatant from developing castor oil seeds. In each case, panel B is a column run under identical conditions but loaded with the cytosolic fraction; panel C is loaded with a crude plastid fraction. In some cases (Figs. 1C, 2C, and 3C), the plastid fraction shows contamination with the cytosolic isozyme.

Contamination of the cytosolic fraction with the plastid isozyme might also be expected, since it was reported previously that the plastid isolation procedure results in 50% breakage of this organelle (24). However, the amount of breakage of the plastids is variable and, in some cases, is not more than 20%. The plastid isozymes reported here represent, at most, 20% of the total cellular activity, so that, in some cases, it is to be expected that they are below the level of detection after column chromatography of the cytosolic fraction.

The separation of castor seed phosphoglycerate mutase isozymes is very pH-sensitive. Equilibration of columns at pH 6.6 gave excellent separations, while pH 6.5 resulted in all of the activity eluting in the column void. Elution at pH 6.7 resulted in a single symmetrical peak towards the top of the sieving range. The other isozyme pairs are less sensitive to small pH changes.

Initial observations (2, 25) showed that the plastid isozymes of glucose-P isomerase, aldolase, gluconate-6-P dehydrogenase, and pyruvate kinase from developing castor oil seeds are each more negatively charged than the corresponding cytosolic isozyme. This led to the proposal that the greater negative charge on the plastid isozymes might serve to regulate recognition or transport of the proteins into the plastids from the site of synthesis in the cytosol. Ireland and Dennis (12) obtained similar results in a survey of the isozymes of glucose-P isomerase, pyruvate kinase, and gluconate-

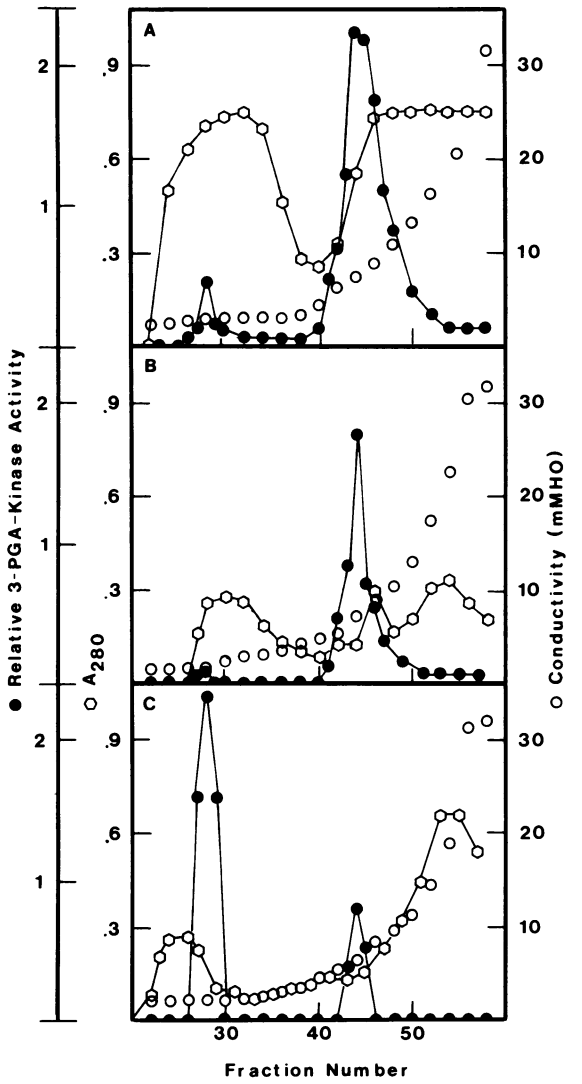

FIG. 3. Ion filtration chromatography of an S_{27} fraction (A); a crude cytosol fraction (B); and a plastid-enriched fraction (C); each assayed for glycerate-3-P kinase activity. Column equilibration buffer was 20 mM imidazole (pH 6.7) containing 5 mM $MgCl_2$. Symbols are as for Figure 1.

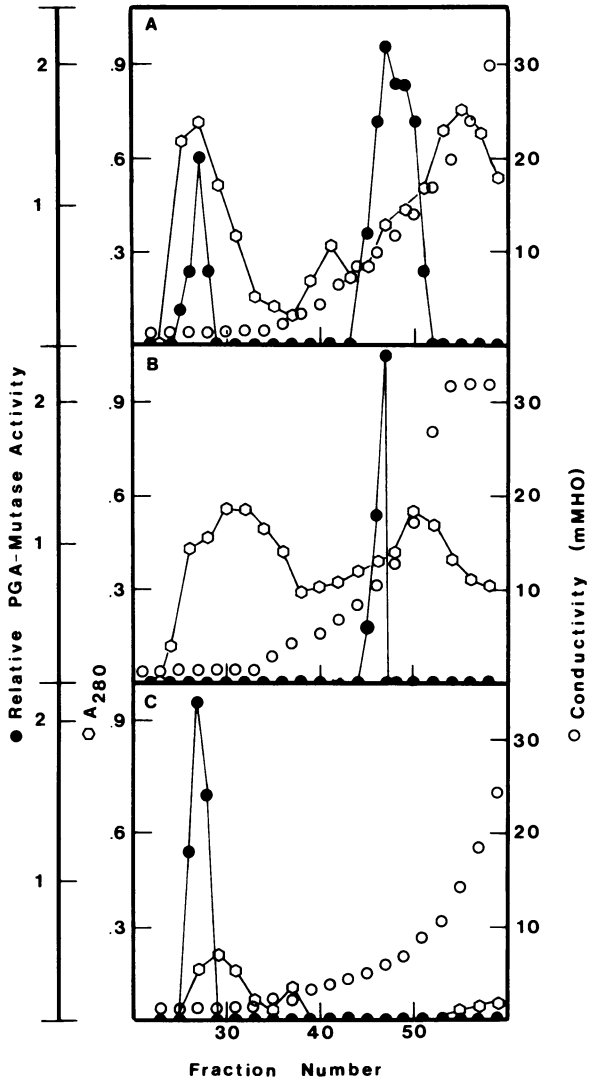

FIG. 4. Ion filtration chromatography of an S_{27} fraction (A); a crude cytosol fraction (B); and a plastid-enriched fraction (C); each assayed for phosphoglycerate mutase activity. Column equilibration buffer was 20 mM imidazole (pH 6.6) containing 5 mM $MgCl_2$. Symbols are as for Figure 1.

6-P dehydrogenase from developing soybean, safflower, and sunflower seeds. In this report, the plastid isozymes of triose-P isomerase and glyceraldehyde-3-P dehydrogenase also are more negatively charged than are the cytosolic isozymes. However, in contrast, the plastid isozymes of glycerate-3-P kinase, enolase, and phosphoglycerate mutase are the more positively charged. Additionally, the plastid isozyme of hexokinase is also more positively charged than is the cytosolic form but less positively charged than is the mitochondrial isozyme (J. A. Miernyk, D. T. Dennis, unpublished). Thus, while it remains a possibility that charge plays some role in the determination of final location of the isozymes within the cell, it is not as simple as previously proposed.

The activities of each of the plastid isozymes is adequate to account for the *in vivo* rates of fatty acid synthesis (24). These values should not, however, be regarded as absolute measures of the contribution of the plastids to total glycolytic activity. Ireland and Dennis (13) have reported that activities of plastid pyruvate kinase, glucose-P isomerase, and gluconate-6-P dehydrogenase followed a distinct developmental pattern correlated with lipid accumulation but not directly correlated to the development of the cytosolic activities. In this and previous studies (24), seeds of various development stages were used, so that the plastid activity would not be at the maximum value. Additionally, while detailed analyses of the isoforms of gluconate-6-P dehydrogenase (25), glucose-P isomerase (R. J. Ireland, D. T. Dennis, unpublished), and enolase (J. A. Miernyk, D. T. Dennis, unpublished) showed great similarity among the isozymes, major differences in the pH optima, K_m values, stability, and regulatory properties were found for pyruvate kinase (12) and phosphofructokinase (8). When assaying pyruvate kinase, for example, a standard assay as described by Duggleby and Dennis (5) would probably reveal no contribution of plastid pyruvate kinase to total activity.

From this report (in addition to those previously described), it is evident that the plastids from developing castor oil seeds are fully autonomous in their capability for synthesis of fatty acids from hexose phosphates. Although the evidence is as yet fragmentary, it seems that the situation is similar in other developing oilseeds (at least sunflower, safflower, soybean, and cotton seeds) and in some chloroplasts (10) and chromoplasts (15) as well. Nishimura and Beevers (21) recently reported that germinating castor oil seeds contain plastid and cytosolic isozymes of the enzymes of sugar-phosphate metabolism. Pea shoot chloroplasts have been reported to contain all of the enzymes of the glycolytic pathway apart from phosphoglycerate mutase (27), and a shuttle

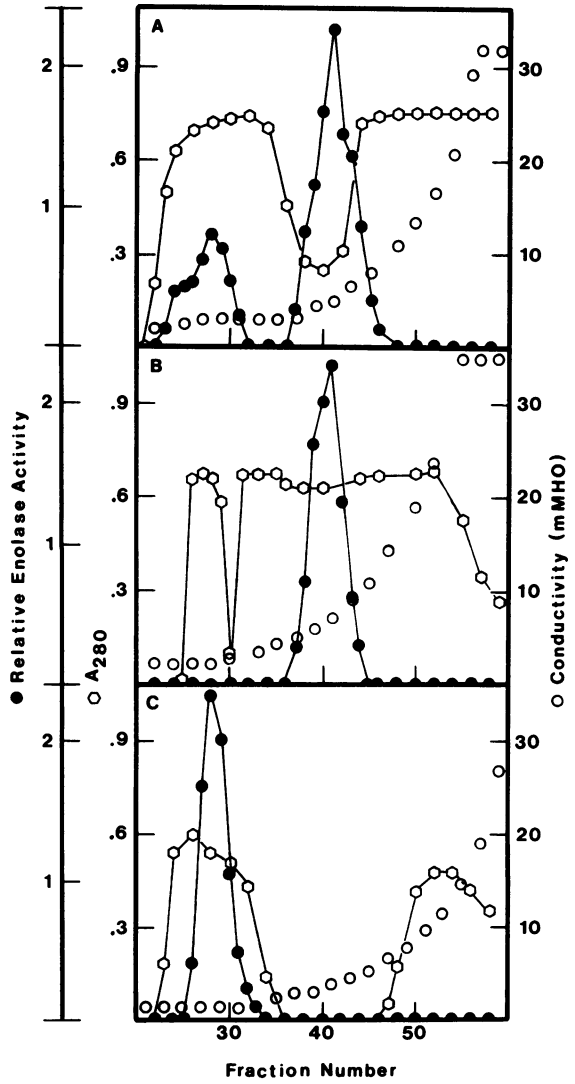

FIG. 5. Ion filtration chromatography of an S_{27} fraction (A); a crude cytosol fraction (B); and a plastid-enriched fraction (C); each assayed for enolase activity. Column equilibration buffer was 20 mM imidazole (pH 6.7) containing 5 mM $MgCl_2$. Symbols are as for Figure 1.

has been proposed to transport glycerate-3-P out of the chloroplast, and glycerate-2-P in, to bypass this step (28). It is possible that, in mature chloroplasts, the loss of this enzyme prevents removal of substrates from the reductive pentose phosphate cycle. It is known that a single enzyme of a pathway can be missing from plastids, as is the case with glucose-6-P dehydrogenase in castor oil seed plastids (20, 25), germinating hazel seed (9), and soybean amyloplasts (T. ap Rees, personal communication). The possibility exists, therefore, that, although the complete pathway may be present in some plastids, individual enzymes may be missing in others.

LITERATURE CITED

1. AP REES T, SM THOMAS, WH FULLER, B CHAPMAN 1975 Location of gluconeogenesis from phosphoenolpyruvate in cotyledons of *Cucurbita pepo*. Biochim Biophys Acta 385: 145–156
2. DELUCA V, DT DENNIS 1978 Isoenzyme of pyruvate kinase in proplastids from developing castor bean endosperm. Plant Physiol 61: 1037–1039
3. DENNIS DT, TR GREEN 1975 Soluble and particulate glycolysis in developing castor bean endosperm. Biochem Biophys Res Commun 64: 970–975
4. DRENNAN CH 1971 The subcellular localization, purification and partial characterization of acyl carrier protein from developing castor oil seeds. PhD thesis. Queen's University, Kingston
5. DUGGLEBY RG, DT DENNIS 1973 The characterization and regulatory properties of pyruvate kinase from cotton seeds. Arch Biochem Biophys 155: 270–277
6. DUGGLEBY RG, DT DENNIS 1974 Nicotinamide adenine dinucleotide—specific glyceraldehyde-3-phosphate dehydrogenase from *Pisum sativum*. J Biol Chem 249: 167–174
7. GARLAND WJ, DT DENNIS 1980 Plastid and cytosolic phosphofructokinases from developing endosperm of *Ricinus communis*. I. Separation, purification and initial characterization of the isozymes. Arch Biochem Biophys 204: 302–309
8. GARLAND WJ, DT DENNIS 1980 Plastid and cytosolic phosphofructokinases from developing endosperm of *Ricinus communis*. II. Comparison of the kinetic and regulatory properties of the isoenzymes. Arch Biochem Biophys 204: 310–317
9. GOSLING PG, JD ROSS 1981 Pentose phosphate metabolism during dormancy breakage in *Corylus avellana* L. Planta 148: 362–366
10. IRELAND RJ, V DELUCA, DT DENNIS 1979 Isoenzymes of pyruvate kinase in etioplasts and chloroplasts. Plant Physiol 63: 903–907
11. IRELAND RJ, V DELUCA, DT DENNIS 1980 Characterization and kinetics of isozymes of pyruvate kinase from developing castor bean endosperm. Plant Physiol 65: 1188–1193
12. IRELAND RJ, DT DENNIS 1980 Isozymes of the glycolytic and pentose-phosphate pathways in storage tissues of different oilseeds. Planta 149: 476–479
13. IRELAND RJ, DT DENNIS 1981 Isozymes of the glycolytic and pentose-phosphate pathways during the development of the castor oil seed. Can J Bot 59: 1423–1425
14. KIRKEGAARD LE, TJA JOHNSON, RM BOCK 1972 Ion filtration chromatography: a powerful new technique for enzyme purification applied to *E. coli* alkaline phosphatase. Anal Biochem 50: 122–138
15. KLEINIG H, B LIEDVOGEL 1980 Fatty acid synthesis by isolated chromoplasts from the daffodil. Energy sources and distribution patterns of the acids. Planta 150: 166–169
16. MIERNYK JA, RN TRELEASE 1981 Control of enzyme activities in cotton cotyledons during maturation and germination. IV. β-oxidation. Plant Physiol 67: 341–346
17. MURPHY DJ, RM LEECH 1977 Lipid biosynthesis from (^{14}C) bicarbonate, (2-^{14}C) pyruvate and (1-^{14}C) acetate during photosynthesis by isolated chloroplasts. FEBS Lett 77: 164–168
18. MURPHY DJ, RM LEECH 1978 The pathway of (^{14}C) bicarbonate incorporation into lipids in isolated photosynthesizing spinach chloroplasts. FEBS Lett 88: 192–196
19. NAKAMURA Y, M YAMADA 1974 Long chain fatty acid synthesis in developing castor bean seeds. I. The operation of the path from acetate to long chain fatty acids in a subcellular particulate system. Plant Cell Physiol 15: 37–48
20. NISHIMURA M, H BEEVERS 1979 Subcellular distribution of gluconeogenic enzymes in germinating castor bean endosperm. Plant Physiol 64: 31–39
21. NISHIMURA M, H BEEVERS 1981 Isoenzymes of sugar phosphate metabolism in endosperm of germinating castor beans. Plant Physiol 67: 1255–1258
22. REID EE, P THOMPSON, CR LYTTLE, DT DENNIS 1975 Pyruvate dehydrogenase complex from higher plants mitochondria and proplastids. Plant Physiol 59: 842–848
23. SCOTT-BURDEN T, DT CANVIN 1975 The effect of Mg^{+2} on density of proplastids from the developing castor bean and the use of acetyl-CoA carboxylase activity for their cytochemical identification. Can J Bot 53: 1371–1376
24. SIMCOX PD, EE REID, DT CANVIN, DT DENNIS 1977 Enzymes of the glycolytic and pentose phosphate pathway in proplastids from the developing endosperm of *Ricinus communis* L. Plant Physiol 59: 1128–1132
25. SIMCOX PD, DT DENNIS 1978 Isoenzymes of the glycolytic and pentose phosphate pathways in proplastids from the developing endosperm of *Ricinus communis* L. Plant Physiol 61: 871–977
26. SIMCOX PD, W GARLAND, V DELUCA, DT CANVIN, DT DENNIS 1979 Respiratory pathways and fat synthesis in the developing castor oil seed. Can J Bot 57: 1008–1014
27. STITT M, T AP REES 1979 Capacities of pea chloroplasts to catalyse the oxidative pentose phosphate pathway and glycolysis. Phytochemistry 18: 1905–1911
28. STITT M, T AP REES 1980 Carbohydrate breakdown by chloroplasts of *Pisum sativum*. Biochim Biophys Acta 627: 131–143
29. WEAIRE PJ, GO KEKWICK 1975 The synthesis of fatty acids in avocado mesocarp and cauliflower bud tissue. Biochem J 146: 425–437
30. ZILKEY BF, DT CANVIN 1972 Localization of oleic acid biosynthesis enzymes in the proplastids of developing castor bean endosperm. Can J Bot 50: 323–326