THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

INTRACELLULAR LOCATION AND ROLE OF ISOCITRATE LYASE IN NONGLUCONEOGENIC PLANT TISSUE

A DISSERTATION

SUBMITTED TO THE GRADUATE COLLEGE

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

By

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Norman, Oklahoma

1977

INTRACELLULAR LOCATION AND ROLE OF ISOCITRATE

LYASE IN NONGLUCONEOGENIC PLANT TISSUE

By

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Dr. John S. Fletcher for his guidance and advice during the course of this study and in the preparation of this manuscript. Appreciation is also expressed to the members of my advisory committee, Dr. Elroy L. Rice, Dr. Lois Pfiester, Dr. T. H. Milby, Dr. John H. Lancaster, and Dr. Simon H. Wender.

I also wish to thank my wife Bonnie, for her encouragement, assistance, and understanding, without which this study would not have been possible.

ABSTRACT

Density gradient centrifugation and electron microscopy were used to establish that isocitrate lyase, present in the leaves of garden peas and in Paul's Scarlet rose cells, was located in the mitochondrion and not other membrane fractions. Labeling studies, where various radioactive intermediates of the Krebs cycle were provided to suspension cultures of rose cells, indicated isocitrate lyase provided, at least in part, the carbon skeleton of the amino acids glycine and serine. In this same tissue, during the logarithmic phase of growth, we found that the isocitrate lyase activity was 11-fold greater than was needed to account for all of the carbon assimilated in protein-bound glycine and serine.

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INTRACELLULAR LOCATION AND ROLE OF ISOCITRATE LYASE IN NONGLUCONEOGENIC PLANT TISSUE

INTRODUCTION

The role of isocitrate lyase in the conversion of fats to sugars and its location in the glyoxysome have been well documented in fatty seeds. This enzyme has been reported to be present in tissues where gluconeogenesis is not occurring however. The purpose of this investigation was to determine the intracellular location and role of isocitrate lyase in tissues other than those of fatty seeds.

The results of this study are presented in three separate papers. Paper I was prepared according to the instructions for contributors to Plant Science Letters. Papers II and III were prepared according to the instructions for contributors to Plant Physiology.

PAPER I

INTRACELLULAR LOCATION OF ISOCITRATE LYASE IN LEAF TISSUE

SUMMARY

Density gradient centrifugation techniques were used to establish that the isocitrate lyase present in pea leaves was compartmented in the mitochondria and not the microbodies.

INTRODUCTION

Isocitrate lyase (EC 4.1.3.1), which catalyzes the conversion of isocitrate to succinate and glyoxylate has been recognized to play an important role in gluconeogenesis occurring in the fatty tissues of germinating seeds [1]. Furthermore it has been shown that the isocitrate lyase present in such tissues is compartmented in the glyoxysome [2]. However, isocitrate lyase has also been reported to be present in green leaves [3,4] where fats are not converted to sugar and glyoxysomes have not been identified. The present study examined the compartmentation of isocitrate lyase in leaf tissue.

METHODS

Pea seedlings (Pisum sativum L. var. Burpeeana) were grown in Vermiculite in a growth chamber at 3,000 ft-c during a 16-hr photoperiod at a day temperature of 23 C and a night temperature of 13 C. Leaves, removed from 10 day old seedlings, were rinsed with distilled water, suspended in grinding medium (w/v ratio of 3:1), and chopped for 1.5 min with a razor blade chopper. The grinding medium, modified from that of Moore and Beevers [5], contained 0.63 M sucrose, 0.15 M Tricine buffer (pH 7.5), 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol.

The crude homogenate was passed through Miracloth and then centrifuged for 10 min at 1,000 x g to remove cell debris, nuclei, and intact chloroplasts. The supernatant was then centrifuged for 15 min at 10,000 x g. The resulting crude particulate pellet was resuspended in grinding medium and assayed for isocitrate lyase activity, or 4 ml of this suspension was layered on top of a linear sucrose gradient consisting of a 1 ml 60 % (w/w) sucrose pad and a 34 ml linear sucrose gradient extending from 20 to 60 % sucrose (w/w). All sucrose solutions contained 1 mM EDTA and were adjusted to a pH of 7.5. The gradients were placed in a Spinco SW-27 rotor and centrifuged for 5 hr at 25,000 rpm in a L2-65B Beckman ultracentrifuge after which 1.2 ml fractions were collected.

The supernatant from the 10,000 x g centrifugation was filtered through a Sephadex G-50 column (3 cm x 28 cm) to remove low molecular weight compounds which interfere with the assay for isocitrate lyase (3). The solution used to pre-equilibrate the column and elute the sample off the column contained 0.15 M Tricine buffer (pH 7.5), 1 mM EDTA, 0.33 M sucrose, and 10 mM β-mercaptoethanol. Five ml aliquots of the effluent were collected and assayed.

All enzymes were assayed spectrophotometrically using a Beckman DBG spectrophotometer with the exception of catalase which was assayed with a Yellow Springs Instrument model 53 oxygen monitor [6]. Isocitrate lyase activity was determined by the method of Dixon and Kornberg [7]; cytochrome c oxidase by the method of Hodges and Leonard [8]; glycolic acid oxidase by the method of Feierabend and Beevers [9]; and malate synthetase by the method of Cooper and Beevers [10]. Chlorophyll was determined by the method of Arnon [11]. Sucrose concentrations were

determined with a Bausch Lomb refractometer. All of the enzyme assays and chlorophyll determinations were carried out on fractions recovered from the same sucrose gradient. Various modifications of the experiment reported in this paper were performed on 4 different tissue preparations and each experiment gave compartmentation results comparable to those reported in this paper.

RESULTS

Assays run on fractions resulting from differential centrifugation (Table 1) showed no activity in the chloroplasts (1,000 x g pellet), 26 % was in the mitochondria-microbody fraction (10,000 x g pellet) and 74 % was in the soluble fraction (10,000 x g supernatant). This distribution was comparable to that reported by Hock and Beevers [14] for the supernatant and 10,000 x g particulate fractions of watermelon cotyledons, another photosynthetic tissue.

Figure 1A shows the protein profile across the gradient and the sucrose concentration of the successive fractions. The protein and enzyme activities of fractions 1 to 8 represent soluble proteins, proteins released when the tissue was homogenized, and material solubilized from organelles as the 10,000 x g pellet was resuspended prior to its being layered on top of the gradient. The major protein peak found within the gradient (Fig. 1A) was the broken chloroplasts since it coincided with the chlorophyll band (Fig. 1B) present at a mean density of 1.17 g/cm^3. This value corresponds closely to the equilibrium density of 1.18 g/cm^3 reported by Miflin and Beevers [13] for broken chloroplasts. Intact chloroplasts, pelleted during the 1000 x g centrifugation, showed no isocitrate lyase activity.

The mitochondrial band, identified through the use of cytochrome c oxidase as a marker (Fig. 1C), was found at a density of 1.18 g/cm^3 as compared to a reported equilibrium density for mitochondria isolated from pea leaves of 1.20 g/cm^3 [13]. This density coincides with the second largest protein peak found within the gradient (Fig. 1A). Isocitrate lyase was confined to a narrow band which coincided with the mitochondrial fraction (Fig. 1D).

Catalase (Fig. 1C) and glycolic acid oxidase (Fig. 1B), both commonly used markers for microbodies, were found at a density of 1.24 g/cm^3, the same as the equilibrium density reported for microbodies from pea leaves [13]. Both of these enzymes were also present at the top of the gradient.

DISCUSSION

Prior to this study isocitrate lyase had not been reported to occur in the mitochondria of higher plants, but Szabo and Charlotte [14] showed isocitrate lyase activity in the mitochondria of yeast cells and Rubin and Trelease [15] recently reported both isocitrate lyase and malate synthetase located in the mitochondria of Ascaris suum larvae. Malate synthetase, an enzyme accompanying isocitrate lyase in gluconeogenesis, was not detected in our work with pea leaves. Although glycolic acid arising from the Calvin cycle has been demonstrated to be the precursor of glyoxylate formed by glycolic acid oxidase [2], we believe that the isocitrate lyase present in mitochondria of green leaves may serve as a second source of glyoxylate. Presumably this glyoxylate would be available for glycine and serine synthesis. If so it would explain

the data of Mahon, Fock, and Canvin [16] where $^{14}CO_2$ labeling studies showed that not all of the carbon entering glycine and serine came from Calvin cycle intermediates.

The amount of isocitrate lyase activity in pea leaves (1 μmole substrate.h^{-1}.g^{-1} fr wt) is much lower than that reported for tissues where gluconeogenesis occurs. The reported activity for castor bean endosperm is 408 μmole substrate.hr^{-1}.g fr wt [10]. This 400 fold difference in activity is perhaps not too surprising in light of the drastically different roles which the enzyme may play in the two tissues. In leaf tissue we propose that the enzyme functions to supply a portion of the carbon entering a restricted number of metabolites, perhaps only glycine and serine; whereas, in germinating castor bean seeds it is involved in the large scale conversion of fat to sugar, a process that involves an estimated 38 % of the seed's dry wt [17,18].

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Table 1. Distribution of isocitrate lyase activity following differential centrifugation.

Fraction	$\mu moles \cdot hr^{-1} \cdot g^{-1}$ fr wt	% of total activity
1,000 x g pellet	____[1]	0
10,000 x g pellet	0.28	26
10,000 x g supernatant	0.82	74
Total	1.10	100

[1] not detectable

FIGURE LEGEND

Fig. 1. (A-D) Separation of organelles on linear sucrose gradients and their identification through the use of marker enzymes.

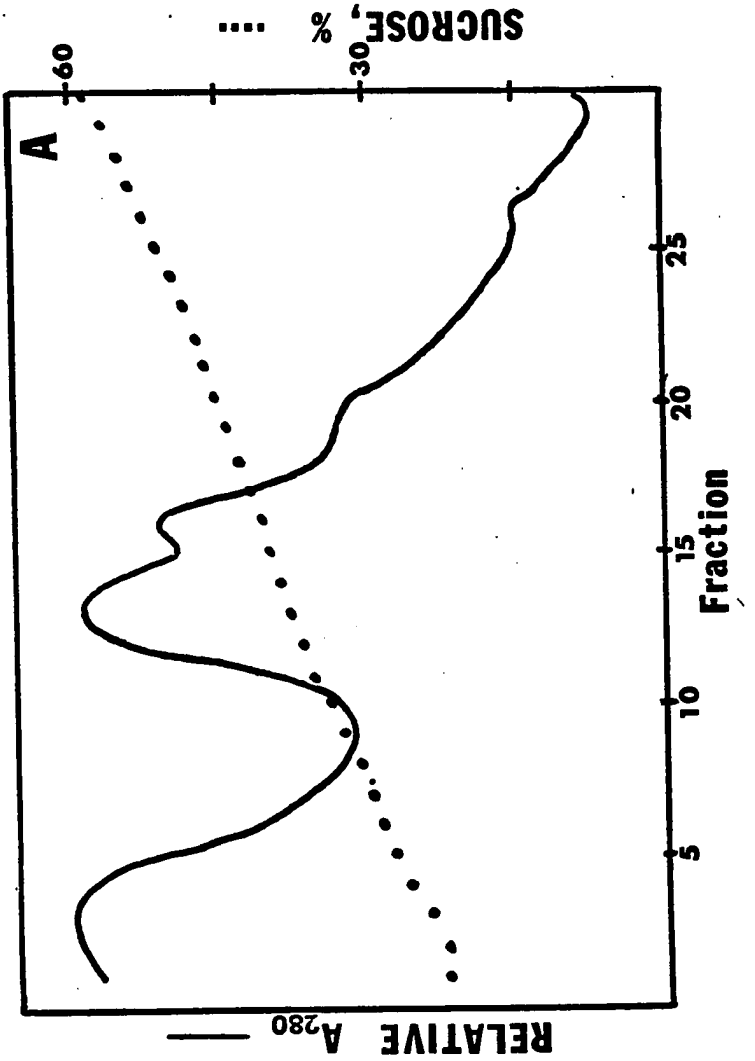

Figure 1-A

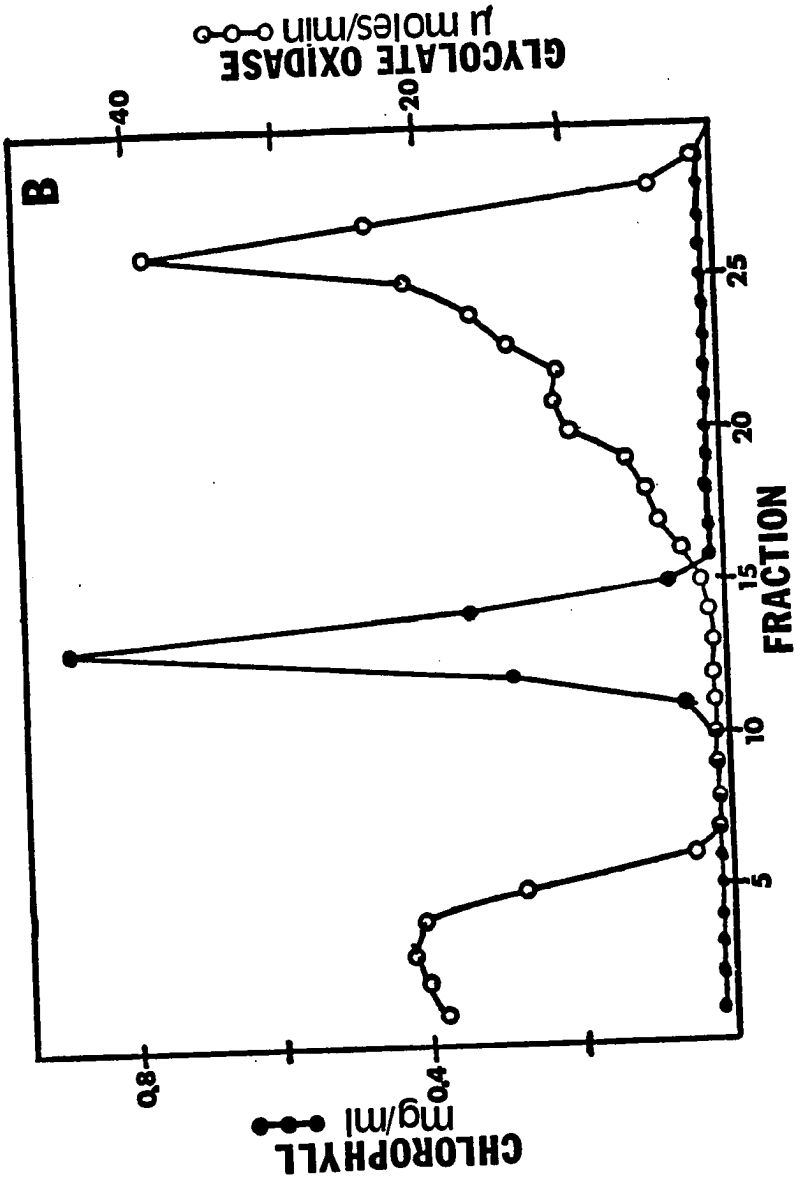
B
GLYCOLATE OXIDASE
μ moles/min
CHLOROPHYLL
mg/ml
FRACTION
40
20
0.8
0.4
5
10
15
20
25

Figure 1-B

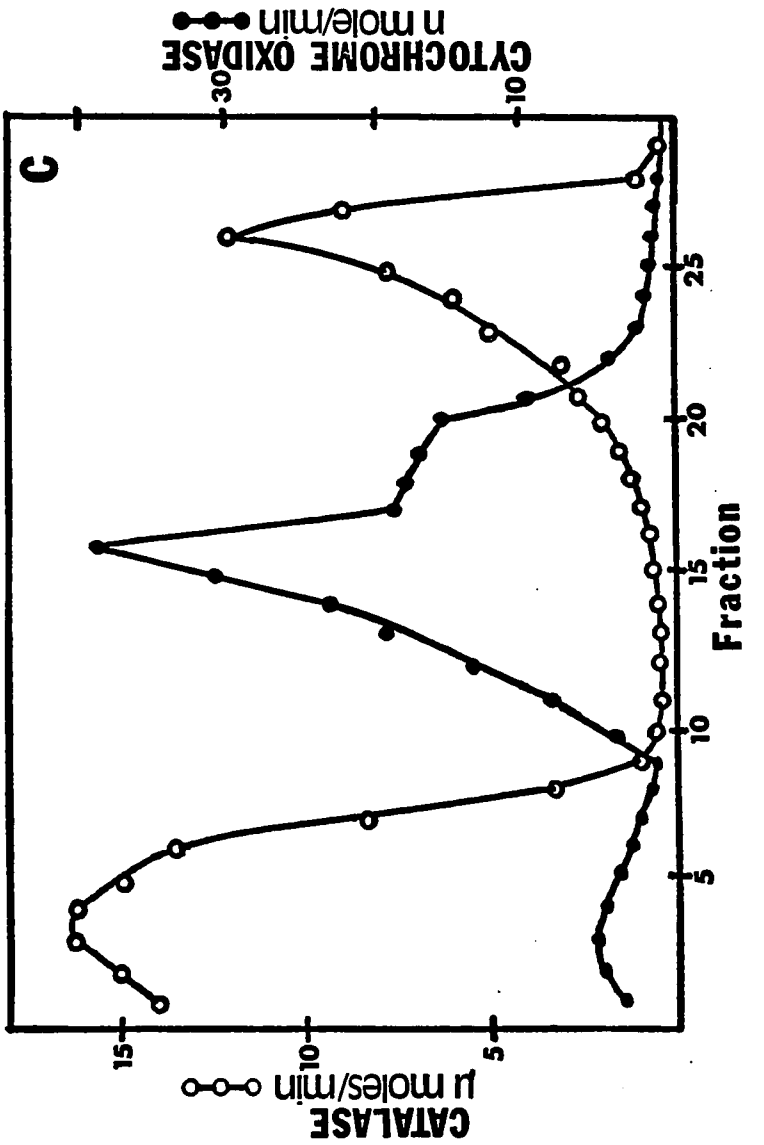
CYTOCHROME OXIDASE
n mole/min
CATALASE
μ moles/min
C
Fraction
5
10
15
20
25
30

Figure 1-C

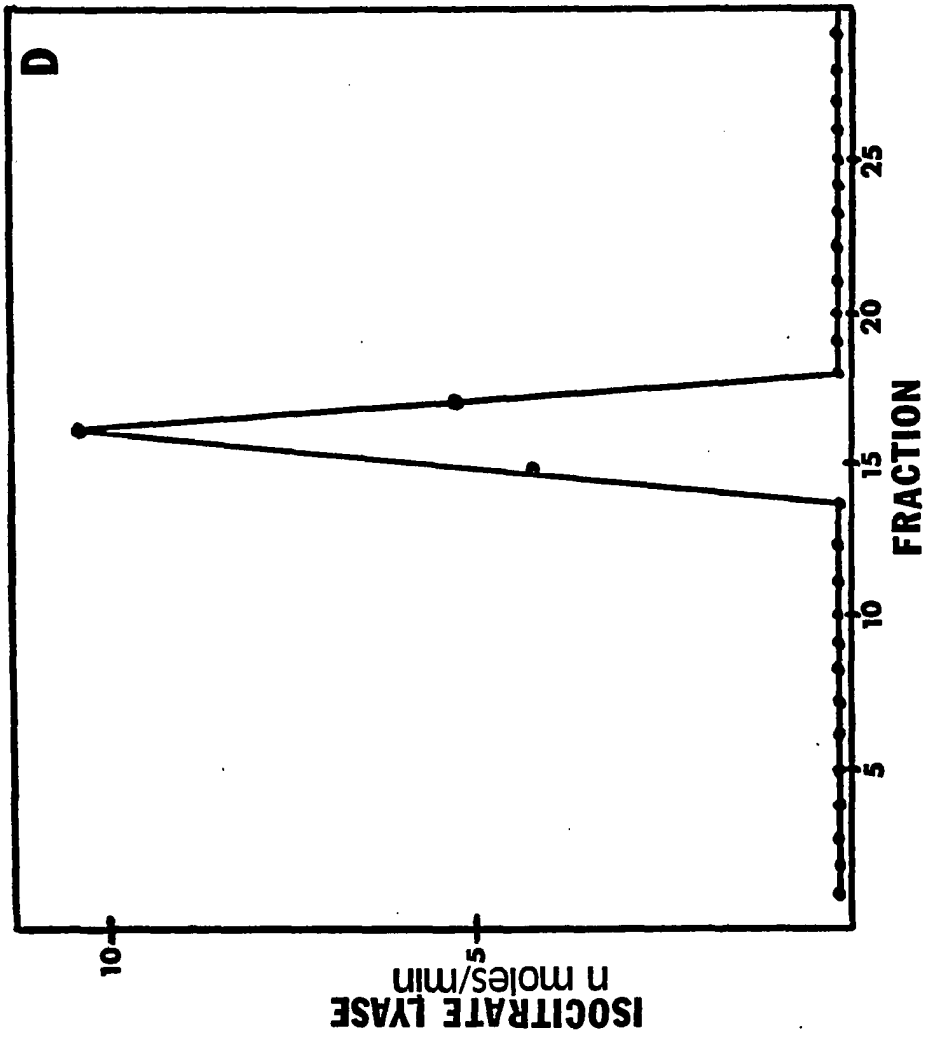

Figure 1-D

PAPER II

SUBCELLULAR LOCALIZATION OF ISOCITRATE LYASE IN NON-GREEN TISSUE CULTURE CELLS

ABSTRACT

Density gradient centrifugation and electron microscopy were used to establish that isocitrate lyase present in Paul's Scarlet rose cells was located in the mitochondria and not other membrane fractions. We propose that the enzyme may be important in glycine and serine synthesis. A comparison between the enzymatic activity of isocitrate lyase and the amount of glycine and serine synthesized during logarithmic growth indicated that the activity is great enough to account for all of the carbon entering these amino acids during that stage of growth.

INTRODUCTION

It has been well documented that isocitrate lyase (EC4.1.3.1.), which converts isocitrate to succinate and glyoxylate, is compartmented in the glyoxysomes found in the fatty tissues of germinating seeds (26). However, isocitrate lyase has been reported to be present in other plant tissues (6, 14, and 17). We recently showed that isocitrate lyase present in pea leaves was compartmented in the mitochondria, not the microbodies (10). The present study was conducted to determine if similar compartmentation occurred in non-photosynthetic tissue in which gluconeogenesis was not occurring.

MATERIALS AND METHODS

Cell Suspension Cultures. Suspension cultures of Paul's scarlet rose cells were grown in the dark in 250 ml Erlenmeyer flasks containing 80 ml of MPR medium (pH 5.5) (15) on a rotary shaker operating at 180 rpm. Cultures were propagated by transferring approximately 0.5 g of cells to fresh media every 14 days.

Tissue Preparation. Cells, collected on a Miracloth filter (Calbiochem) held in a Buchner funnel, were suspended in cold grinding medium and homogenized in a cold mortar and pestle (1 g of cells per 1 ml grinding medium) or a Dounce homogenizer (1 g of cells per 4 ml grinding medium). The Dounce homogenizer was used to grind tissue for the determination of particulate isocitrate lyase at various times during the growth cycle. The grinding medium, modified from that of Moore and Beevers (13), consisted of 0.63 M sucrose, 0.15 M Tricine buffer (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 10 mM β-mercaptoethanol. The crude homogenate was filtered through Miracloth and centrifuged for 10 min at 270 x g to remove whole cells, cell debris, and nuclei. The supernatant from this low speed spin was then centrifuged at 10,000 x g for 15 min. The 10,000 x g pellet was resuspended in grinding medium and 4 ml of this suspension was carefully layered on top of a linear sucrose gradient or pelleted and resuspended a second time to remove low molecular weight compounds which interfered with the assay for isocitrate lyase (6). Gel filtration was used to remove low molecular weight compounds from the 10,000 x g supernatant prior to determining soluble isocitrate lyase activity (10).

The gradients used consisted of a 1 ml 60 % (w/w) sucrose pad and a 34 ml linear sucrose gradient extending from 20 % to 60 % (w/w) sucrose. All sucrose solutions contained 1 mM EDTA and 10 mM β-mercaptoethanol and were adjusted to a pH of 7.5. The gradients were centrifuged for 4 hr at 25,000 rpm in a L2-65B Beckman ultra centrifuge (Spinco SW-27 rotor) after which 1.2 ml fractions were collected.

Analytical Methods. All enzymes were assayed spectrophotometrically using a Beckman DBG spectrophotometer except for catalase which was assayed with a Yellow Springs Instrument model 53 oxygen monitor (20). Isocitrate lyase activity was determined by the method of Dixon and Kornberg (2); cytochrome c oxidase by the method of Hodges and Leonard (7); fumarase by the method of Racker (18); malate dehydrogenase by the method of Ting (25); triose phosphate isomerase by the method of Gibbs and Turner (5); glycolate oxidase and hydroxypyruvate reductase by the method of Feierabend and Beevers (4); and malate synthetase by the method of Cooper and Beevers (1). Protein was determined by the Lowry procedure (11). A Bausch Lomb refractometer was used to determine sucrose concentrations.

Electron Microscopy. Appropriate gradient fractions were pooled and fixed for 1.5 hr in a final concentration of 2.5 % (v/v) phosphate buffered glutaraldehyde (pH 7.0). Immediately after adding the glutaraldehyde, the organelle preparation was transferred to a cellulose nitrate tube, placed in a Spinco SW41 head, and centrifuged for 30 min at 30,000 rpm to pellet the organelles present. After fixation the pellet was then rinsed 3 times with cold 100 mM phosphate buffer (pH 7.0)

and post fixed with 4 % phosphate buffered osmium tetroxide (pH 7.0) for 1 1/2 hr. The pellet was then dehydrated in a graded series of acetone concentrations (30 %, 50 %, 70 %, and 90 %) at 30 min intervals and in 100 % acetone over night. The tissue was then embedded in a mixture of low viscosity resins consisting of 24 % vinylcyclohexene dioxide, 14 % diglycidy ether of polypropylene, 61 % nonenylsuccinic anhydride, and 1 % dimethyl aminoethanol (w/w, all purchased from Polysciences, Inc.). Whole cells were collected on a Miracloth filter and were fixed and embedded in the same manner as were the isolated organelles. A Sorvall MT-2 ultramicrotome was used to thin-section the tissue which was then examined on a Phillips model 200 transmission electron microscope after post-staining with saturated aqueous uranyl acetate and lead citrate.

Protein Analysis. The protein content of cells on days 4 and 6 of the growth cycle was determined and this information was used to calculate the amount of glycine and serine synthesized. Cells were rinsed with water, extracted twice with 80 % (v/v) ethyl alcohol, and homogenized with a Williams polytron. The amount of protein, recovered from this crude homogenate by the Osborn method (16) was determined by the Lowry method (11).

RESULTS

Figure 1 shows changes in the activity of isocitrate lyase associated with the 10,000 x g pellet during the 14 day growth cycle of Paul's Scarlet rose cells. The activity increased rapidly from day 3 to day 5 of the growth cycle followed by a rapid decrease through day 8. After day 8 the activity remained constant through the remainder of the growth cycle. All differential and density gradient centrifugation studies were conducted using 5 day old cells, the age when isocitrate lyase activity was highest.

Table 1 shows the distribution of isocitrate lyase activity between the soluble and particulate fractions of 5 day old cells. The total activity present in these cells was 1.1 μmole of substrate used per hr per g fr wt of tissue. Sixty-four % of this activity was associated with the 10,000 x g pellet and 36 % was soluble.

Isolation of Organelles On Sucrose Gradients. Figure 2E shows the sucrose concentration of the successive gradient fractions and the protein profile across the gradient. The major protein peak found within the gradient was the mitochondrial fraction at a mean density of 1.18 g/cm^3 (41 % sucrose, w/w). The protein peaks, located at a mean density of 1.12 g/cm^3 (28 % sucrose, w/w) and 1.13 g/cm^3 (31 % sucrose, w/w), were not identified by marker enzymes; however these correspond to reported densities for endoplasmic reticulum and golgi membranes respectively (13). Neither of these fractions exhibited isocitrate lyase activity. No protein peak corresponding to the microbody fraction, located at 49 % sucrose (w/w), was observed when the gradient was scanned at A_{280}. The lack of a protein peak coinciding with the microbody

fraction has been reported for several other tissues (8). The relatively low amount of soluble protein located at the top of the gradient indicated the methods employed in tissue preparation yielded a high population of intact organelles.

Isocitrate lyase activity (Fig. 2A) was confined to a narrow band with the peak activity located at a mean density of 1.18 g/cm^3. This coincided with the peak activity of the commonly used mitochondria markers cytochrome c oxidase (Fig. 2B), fumarase (Fig. 2C) and malate dehydrogenase (Fig. 2C).

The microbody fraction, identified through the use of the marker enzyme catalase (Fig. 2B), was located at a mean density of 1.22 g/cm^3 (49 % sucrose, w/w). No glycolate oxidase, hydroxypyruvate reductase, or malate synthetase activity was detected in this tissue. Plant tissues have been shown to contain isoenzymes of malate dehydrogenase associated with the mitochondria and microbodies as well as soluble activity (19). This would account for the malate dehydrogenase activity associated with the microbody fraction (Fig. 2C).

The distribution of triosephosphate isomerase, a commonly used marker for proplastids, is shown in Figure 2D. Activity was found at the top of the gradient and associated with the mitochondrial fraction. There was also a large peak of activity present at a mean density of 1.20 g/cm^3 (44 % sucrose, w/w) and 1.24 g/cm^3 (51 % sucrose, w/w). The peak activity at a density of 1.20 g/cm^3 corresponds to reported densities for the proplastid fraction from other tissues (12). Based on the wide distribution of triosephosphate isomerase, it appears that the proplastids were not recovered intact as were the mitochondria and

microbodies. Moore and Beevers (13) experienced this same problem in their work with soybean cell cultures.

Electron Microscopy. The fine structure of Paul's Scarlet Rose cells 5 days after transfer to new media was similar to that observed in suspension cultures of sycamore cells of a comparable age (23, 27). The cells contained a large central vacuole and a very thin peripheral layer of cytoplasm (Fig. 3). Analysis of numerous electron micrographs showed that the mitochondria were by far the most plentiful organelle. Figure 3 shows the homogeneity of the mitochondrial fraction isolated on a linear sucrose gradient. The mitochondria in this fraction have the same morphology as that observed in thin sections of whole cells.

Comparsion of isocitrate lyase activity with glycine and serine synthesis. The μmoles of glycine and serine incorporated into protein during the two day interval between day 4 and 6 of the growth cycle was determined according to the sample calculation shown in Table II. The second column in the table shows the moles percentage of amino acids in the protein of suspension cultures of Paul's Scarlet rose as reported by Dougall (3). The weight % (column 3) was calculated in the same manner as previously reported (19). The milligrams of glycine and serine incorporated into protein (column 4) during this two day period was calculated by multiplying the weight % by the mg of protein (4.7 mg) synthesized during the same 2 day period. In column 5 the μmoles of each individual amino acid were calculated by dividing the mg of each of these amino acids by its molecular weight. Column 7 shows the μmoles of glyoxylate required to produce the corresponding amount of glycine and serine. This calculation showed that a total of 7.2 μmoles of

glyoxylate was required for the synthesis of glycine and serine incorporated into protein during the two day interval from day 4 to 6 of the growth cycle.

The potential production of glyoxylate during this same two day period was calculated by multiplying the activity of isocitrate lyase in 5 day old rose cells of 1.1 μmoles per hr per g fresh weight (Table I) by the average fresh weight of 4 and 6 day old cultures of rose cells (9). This calculation showed a total potential for the production of glyoxylate of 80 μmoles during this same 48 hour period. Thus the potential for production of glyoxylate by isocitrate lyase is 11 times that required to synthesize glycine and serine incorporated into protein during days 4 to 6 of the growth cycle.

DISCUSSION

Results from this study indicated that isocitrate lyase present in nonphotosynthetic and nongluconeogenic plant tissue, such as rose cells, is located in the mitochondria not the microbody.

This conclusion was based on the enzymatic distribution of selected enzymes among organelle fractions separated on sucrose gradients. The identity of the mitochondrial fraction was confirmed by electron microscopy. The compartmentation of isocitrate lyase in the mitochondria of rose cells is consistent with the recent work of Szabo and Charlotte with yeast cells (24); and Rubin and Trelease with Ascaris suum larvae (21) where both research groups reported isocitrate lyase to be present in the mitochondria.

Regarding the function of isocitrate lyase in nongluconeogenic tissue we speculate that if the endogenous level of glyoxylate were high and the level of isocitrate low, then isocitrate lyase may function to generate isocitrate from succinate and glyoxylate. This proposition is in keeping with in vitro work on isocitrate lyase isolated from bacteria (22), but contradicts the normal operation of the enzyme in gluconeogenic tissue where in vivo studies have shown that isocitrate lyase cleaves isocitrate into succinate and glyoxylate. This is probably the more likely event in rose cells since we are not aware of a pathway in this nonphotosynthetic tissue which would generate large amounts of glyoxylate and thereby drive the reaction in the direction of isocitrate. With this in mind, we propose that in nongluconeogenic tissue isocitrate lyase may function to produce glyoxylate which in turn is used for glycine and serine synthesis.

The level of isocitrate lyase in rose cells as well as in other non-fatty tissues where it has been reported (6, 14, and 17) is extremely low in comparison to that observed in gluconeogenic tissues such as castor bean endosperm (1). In the case of rose cells it is only 1/400 of that present in endosperm tissue. The question arises as to whether or not the small amount of isocitrate lyase present in rose cells is sufficient to be of any physiological importance. To examine this question we compared the enzymatic potential of isocitrate lyase with the actual amount of glycine and serine which was synthesized during a 2 day period of logarithmic growth. Such a calculation was possible since the cells were grown on a defined medium containing no amino acids and only sucrose and inositol serving as carbon sources. These calculations indicated that the level of isocitrate lyase was 11-fold greater than would be necessary to account for all of the glycine and serine incorporated into protein during logarithmic growth of rose cells. This supports the hypothesis that isocitrate lyase supplies carbon skeletons for the synthesis of glycine and serine in nonphotosynthetic tissue.

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Table 1. Total Isocitrate Lyase Activity and Its Distribution in 5 Day Old Cells.

	$\mu Moles \cdot hr^{-1} \cdot g$ fresh $weight^{-1}$	%
Soluble	0.4	36
Particulate	0.7	64
Total	1.1	100

Table II. Calculation of the Amount of Glycine and Serine Incorporated into Protein Between Day 4 and 6 of Growth Cycle.

Amino Acid	Mole %[1]	Weight %	Protein mg[2]	Protein Amino Acids μmoles	μMoles of Glyoxylate Required[3]
Gly	7.9	5.0	.24	3.2	3.2
Ser	5.3	4.5	.21	2.0	4.0
Total					7.2

[1]Data taken from Ref. 3.

[2]Net protein synthesized day 4 to 6 = 4.7 mg.

[3]Each μmole of glycine requires 1 μmole of glyoxylate, each μmole of serine synthesized requires 2 μmoles of glyoxylate.

LEGENDS FOR FIGURES

Figure 1. Changes in isocitrate lyase activity during the 14 day growth cycle of Paul's Scarlet rose cells.

Figure 2. Separation of organelles on linear sucrose gradients.

Figure 3. Electron micrograph of intact cells (A) and the mitochondrial fraction isolated on a linear sucrose gradient (B).

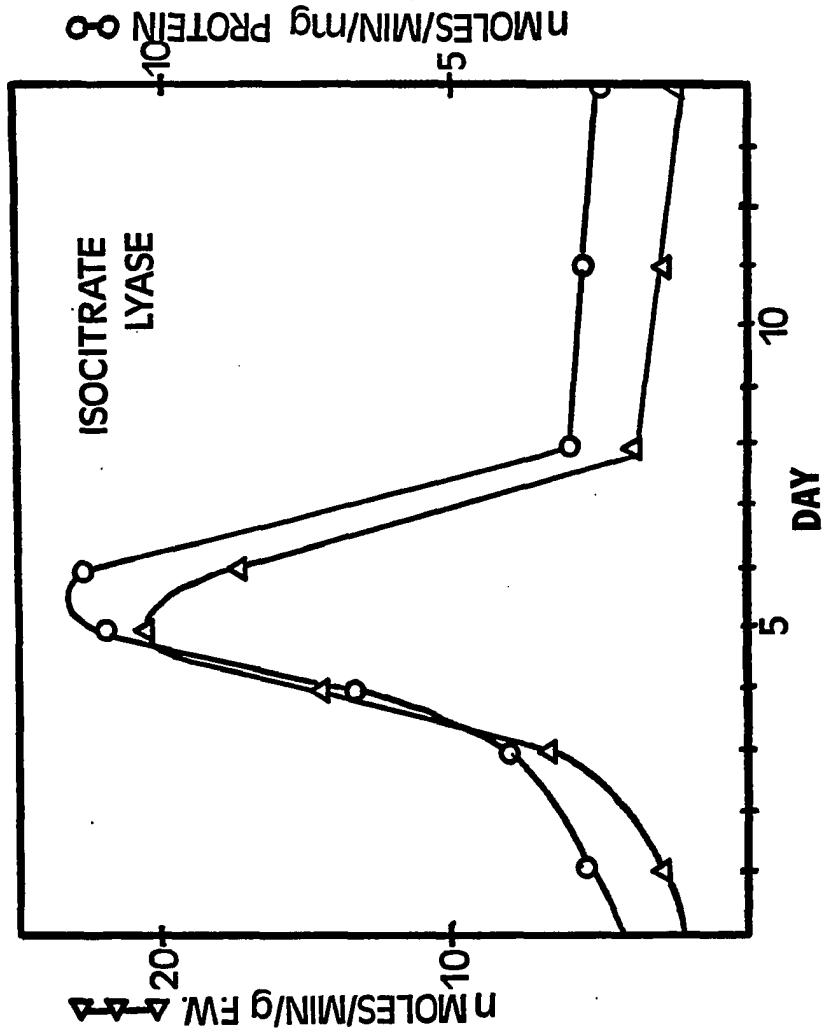
n MOLES/MIN/mg PROTEIN
10
5
ISOCITRATE
LYASE
DAY
10
5
n MOLES/MIN/g F.W.
20
10

Figure 1

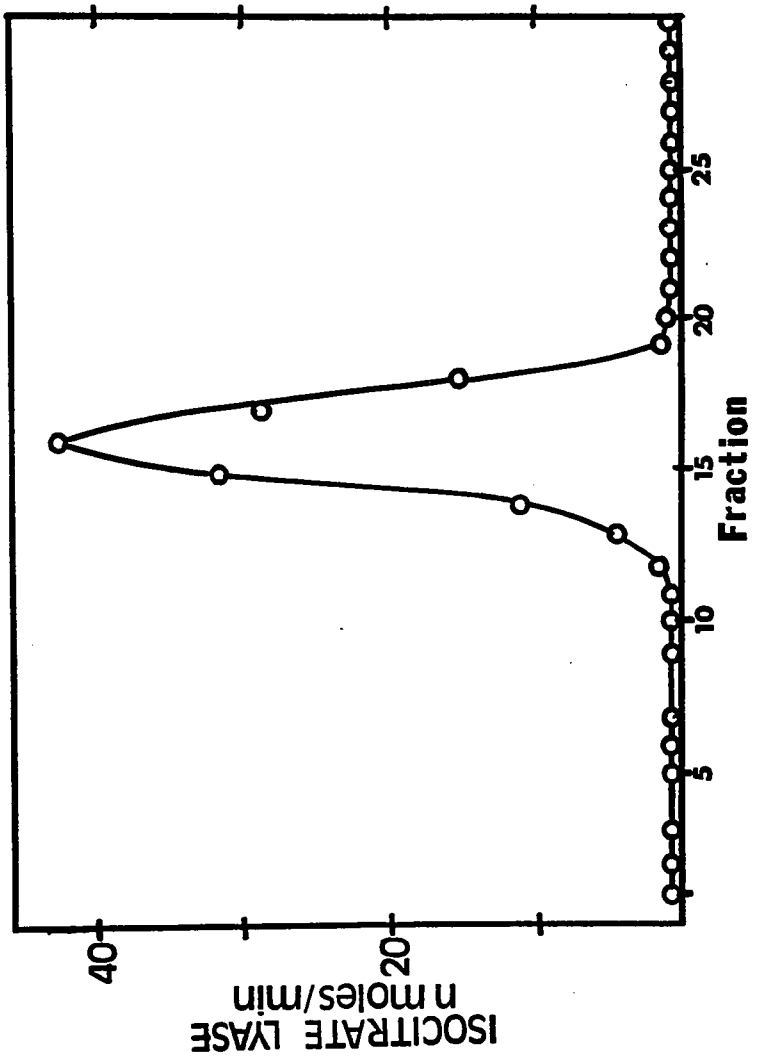

Figure 2-A

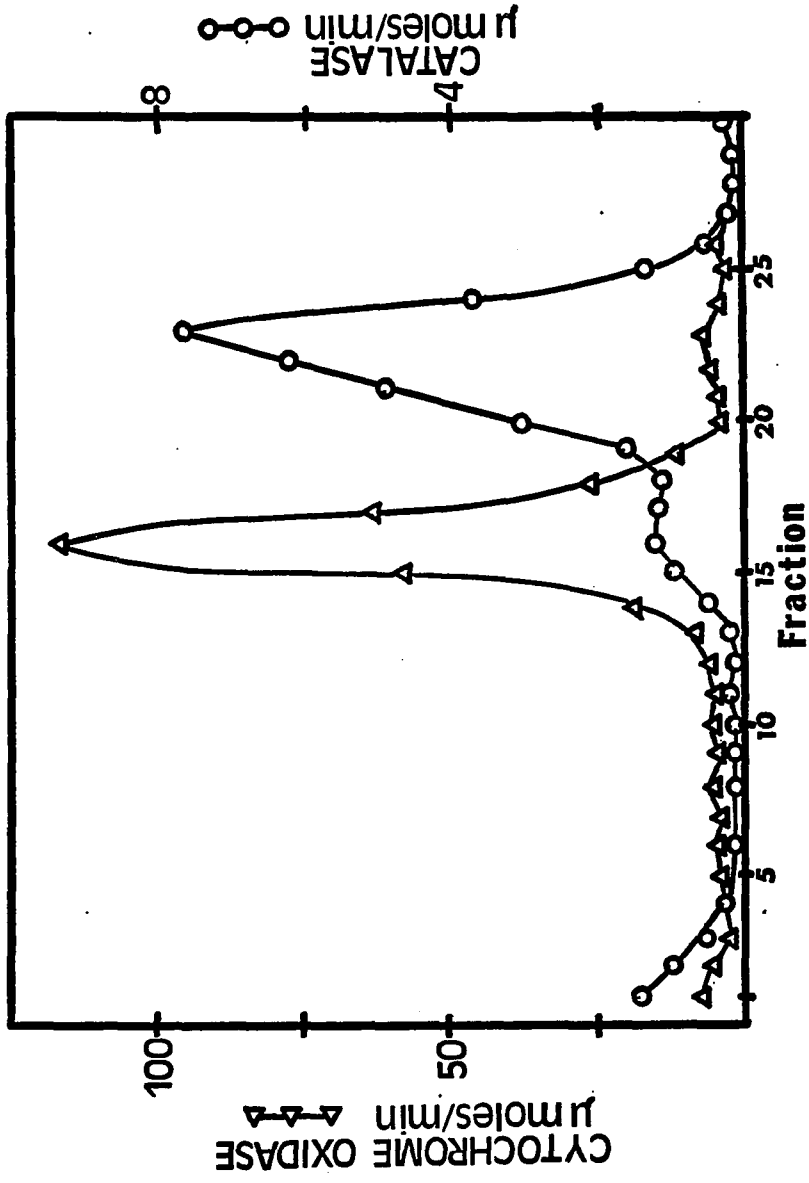
CATALASE
μ moles/min
8
4
100
50
CYTOCHROME OXIDASE
μ moles/min
5
10
15
20
25
Fraction

Figure 2-B

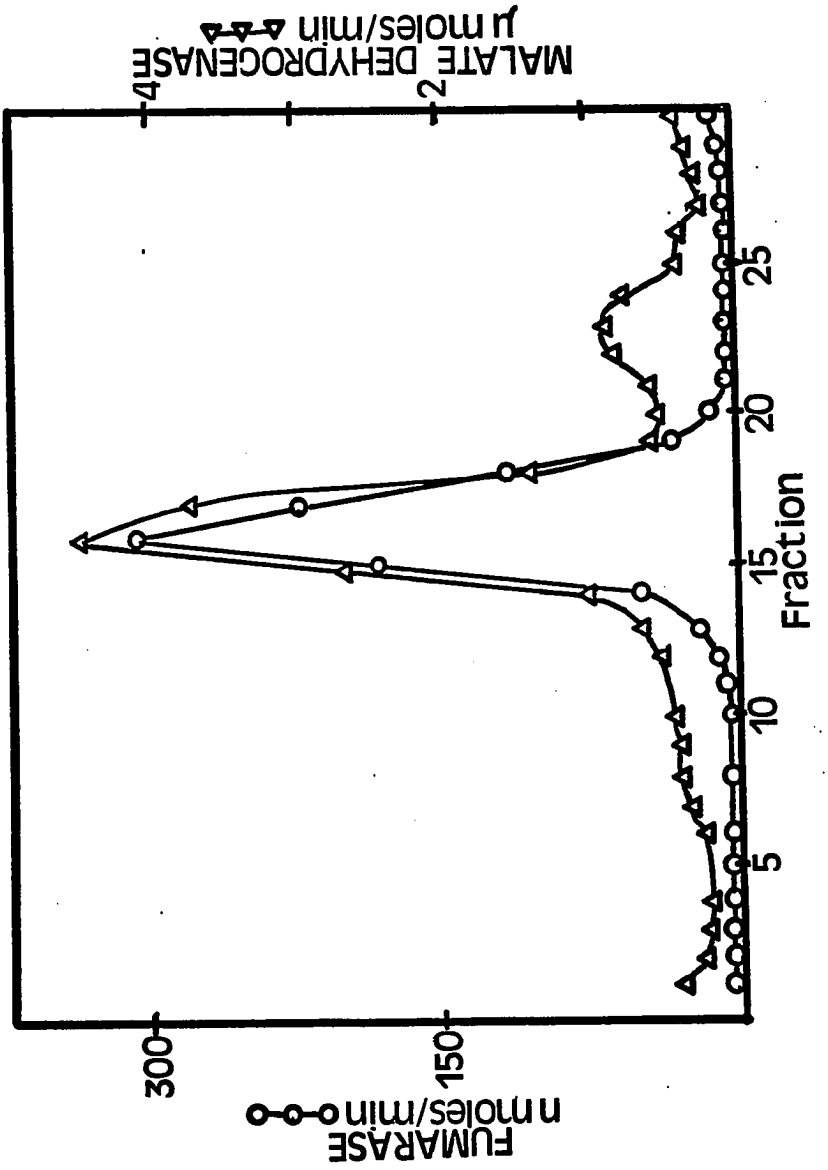
MALATE DEHYDROGENASE
μ moles/min
FUMARASE
n moles/min
Fraction
4
2
300
150
5
10
15
20
25

Figure 2-C

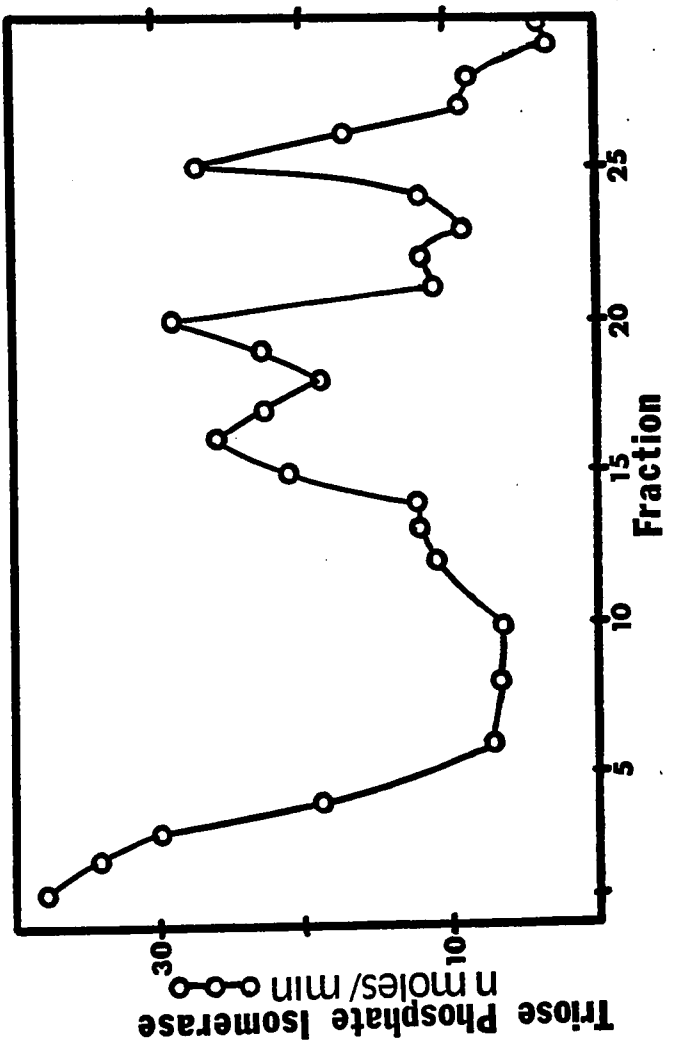
Triose Phosphate Isomerase
n moles/ min
30
10
5
10
15
20
25
Fraction

Figure 2-D

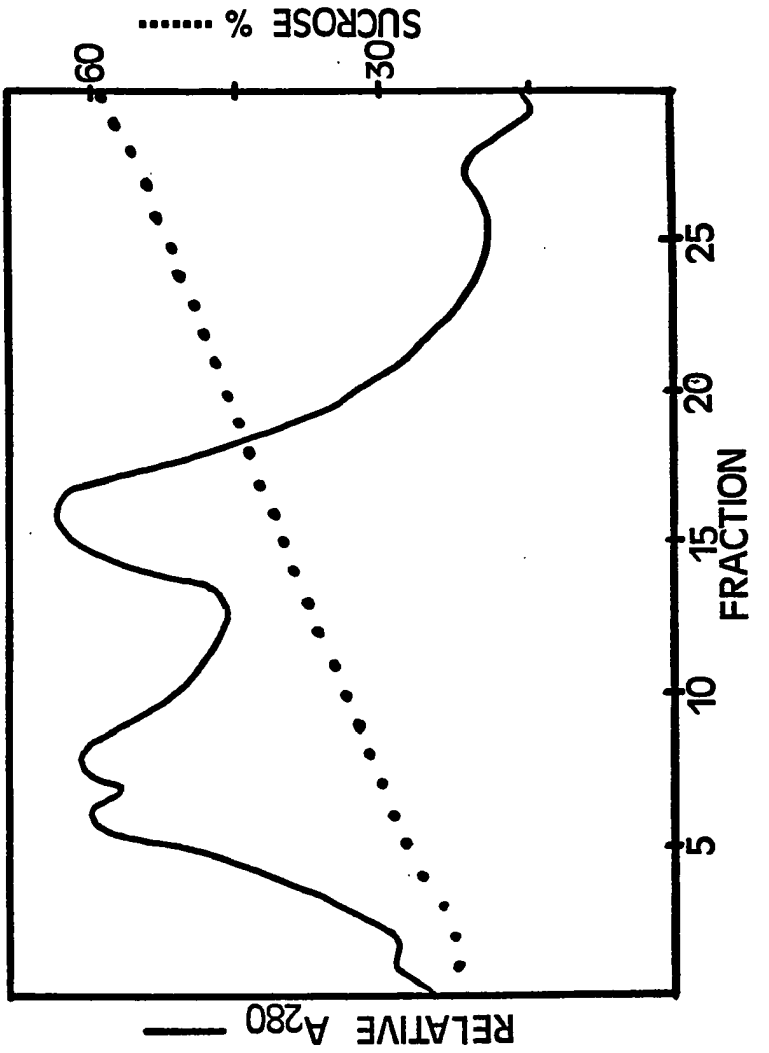

Figure 2-E

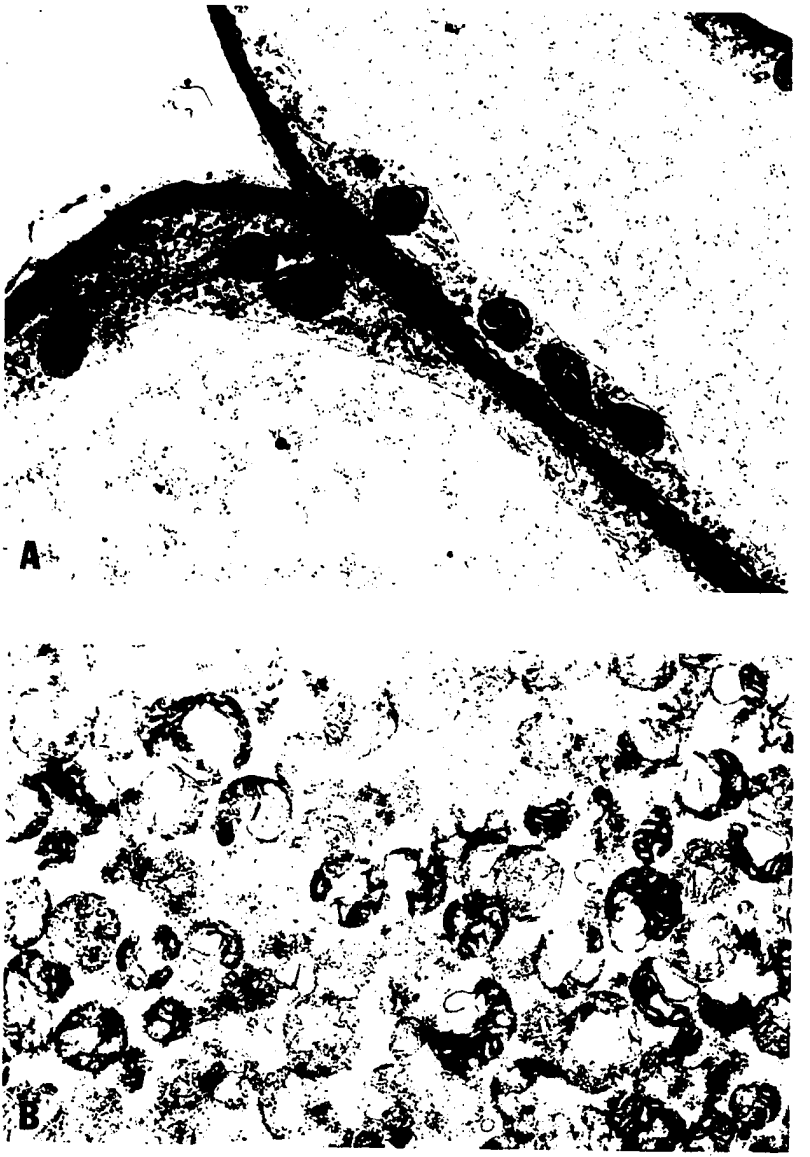

Figure 3

PAPER III

ROLE OF ISOCITRATE LYASE

IN NONGLUCONEOGENIC PLANT TISSUE

ABSTRACT

Labeling studies where Glyoxylate-U-^{14}C was provided to suspension cultures of Paul's Scarlet rose cells indicated that glyoxylate was assimilated to the amino acids glycine and serine. Studies where various Krebs cycle intermediates were provided to this same tissue indicated isocitrate lyase located in the mitochondria did generate an endogenous pool of glyoxylate which was available for the synthesis of glycine and serine. These studies also suggested that other pathways leading to the production of glyoxylate were present in these cells however. Glyoxylate-glutamate amino transferase was also demonstrated to be present in the mitochondria of rose cells.

INTRODUCTION

In earlier work from this laboratory (4,5) we showed that isocitrate lyase present in nongluconeogenic tissue was compartmented in the mitochondrion rather than the glyoxysome, its accepted location in gluconeogenic tissue. Furthermore, we suggested that isocitrate lyase in nongluconeogenic tissue may serve as a source of glyoxylate for glycine and serine synthesis. In suspension cultures of rose cells we were able to show that the isocitrate lyase activity was 11-fold greater than was needed to account for all of the carbon assimilated into protein-bound glycine and serine during logarithmic growth. The present investigation was conducted to determine if radioactive intermediates of the TCA were metabolized into glycine and serine in a manner consistent with the hypothesis that isocitrate lyase operating in conjunction with the TCA cycle is a source of glyoxylate for glycine and serine synthesis.

MATERIALS AND METHODS

Suspension cultures of Paul's Scarlet rose cells were grown in the same manner as previously reported (6).

Continuous feeding experiments were performed by incubating 1 g of cells for 2 or 3 hr in 5 ml of medium (pH 5.0) containing from 4 to 10 μCi of ^{14}C labeled substrate. Millipore filters, connected to air lines, served as incubation vessels (2). The medium used was recovered from 3 or 5-day-old cultures and its sterility was established by plating and aliquot on nutrient agar and incubating for 72 hr at 25 C. In some experiments carbon dioxide released by the cells was trapped and assayed for ^{14}C as previously described (4). At the end of the incubation period the cells were rinsed thoroughly with water and homogenized in boiling 80 % (v/v) ethyl alcohol with a Virtis grinder.

The ethyl alcohol homogenate was fractionated into the chloroform soluble material (lipids), soluble amino acids, organic acids, soluble carbohydrates, and the ethyl alcohol insoluble residue by the method of Fletcher and Beevers (2). Their method was also used for the hydrolysis of proteins present in the ethyl alcohol insoluble material. Amino acids were separated by paper chromatography according to the methods of Morris and Thompson (9). Organic acids were separated by column chromatography using Dowex 1 formate resin (2).

The mitochondria and microbody fractions used in the assay for glutamate-glyoxylate amino transferase were isolated from 5 day old cells on discontinuous sucrose gradients. The gradients consisted of a 14 ml 50 % (w/w) sucrose pad, 10 ml of 43 % (w/w) sucrose, and 10 ml of 35 % (w/w) sucrose. The design of these gradients was based on the

densities of mitochondria and microbodies isolated on linear sucrose gradients. All sucrose solutions contained 1 mM EDTA, 10 mM β-mercaptoethanol, and were adjusted to a pH of 7.5. A 10,000 x g pellet was prepared as described previously (6), resuspended and gently layed on top of the gradient. The gradient was placed in a L2-65B Beckman ultracentrifuge (Spinco SW-27 rotor) and centrifuged at 25,000 rpm for 2 hr. After centrifugation the mitochondria band, located at the 35 % - 43 % sucrose interface, and the microbody band, located at the 44 % - 50 % sucrose interface, were removed and assayed for glutamate-glyoxylate amino transferase. The assay used, modified from that of Kisaki and Tolbert (7), involved the incubation of isolated organelles in a test tube which contained, in a final volume of 1.25 ml, 0.5 μCi glyoxylate U-^{14}C, 25 μ M·glutamate, 0.1 μ M pyridoxal phosphate, and 200 mM phosphate buffer (pH 7.5). At the end of the incubation period hot ethanol was added to stop the reaction. Amino acids were then separated as previously described.

A Beckman model LS-100 liquid scintillation counter was used to determine the amount of ^{14}C present in the various fractions examined. Ten ml of scintillation fluid, consisting of 6 g PPO, 100 g naphthalene, and 1 liter of 1,4-dioxane was added to each vial containing the sample to be counted.

Radioactive substrates used included: acetate-1-^{14}C (9 mCi/mmole); citrate-1,5-^{14}C (8 mCi/mmole); glyoxylate-U-^{14}C (5.8 mCi/mmole), and succinate-2,3-^{14}C (22 mCi/mmole).

RESULTS

Table I shows the distribution of ^{14}C in cell constituents after the provision of glyoxylate-U-^{14}C to 3 day old rose cells, the period of peak protein synthesis (4). The majority of the label was recovered in protein-bound and soluble amino acids. The $^{14}CO_2$ that was released was thought to arise primarily from the decarboxylation that would occur as two glycine molecules condense to form one serine molecule (15). The low percentage of label recovered in the organic acid fraction is consistent with our earlier finding that malate synthetase was not present in this tissue (6). The lack of ^{14}C in the organic acids, largely TCA cycle intermediates, also indicated that isocitrate lyase did not lead to the formation of isocitrate, but instead appeared to operate in the direction of glyoxylate and succinate formation as we proposed.

Table II shows the distribution of ^{14}C in individual protein-bound and soluble amino acids when glyoxylate U-^{14}C was provided to 3 and 5 day old rose cells. Day 5 of the growth cycle is the peak period of isocitrate lyase activity in these cells (6). In both 3 and 5 day old tissue, glyoxylate was incorporated almost exclusively into the carbon structure of the amino acids glycine and serine.

Having demonstrated that exogenously provided glyoxylate was assimilated to glycine and serine in the predicted fashion, the next series of experiments was designed to determine if isocitrate lyase, previously shown to be in the mitochondrion (5 and 6), was instrumental in generating an endogenous pool of glyoxylate which was available for the synthesis of these two amino acids. Three day-old cells were provided with various radioactive metabolites which had been previously shown to be accessible to the TCA cycle following uptake by these cells (4).

Table III compares the incorporation of ^{14}C into glycine and serine to that of other amino acids when citrate-1,5-^{14}C was provided to 3 day old rose cells. The amount of ^{14}C incorporated into these two amino acids was greater than that incorporated into amino acids whose carbon skeleton is not derived directly from the TCA cycle. Acetate-1-^{14}C (Table IV) and succinate-2,3-^{14}C (Table V) also contributed a significant amount of ^{14}C to glycine and serine. These three TCA cycle intermediates contributed more label to protein-bound glycine than serine. This labeling pattern was also observed when ^{14}C glyoxylate was provided to rose cells (Table II).

Table VI reports the results of the radioactive assay for the enzyme glutamate-glyoxylate amino transferase as ^{14}C in glutamate and glycine. This was necessary due to the poor separation of these amino acids by paper chromatography. The selection of glutamate as the amino donor for glycine synthesis was based on the work by Kisaki and Tolbert which showed glutamate was approximately 2 times more effective as an amino donor than any other amino acid (7).

We feel that the majority of the ^{14}C was in glycine, not glutamate. Isocitrate lyase would be required to operate in the direction of isocitrate formation for the synthesis of glutamate and none of our in vivo results indicated that this occurs. Furthermore we do not believe that sufficient amounts of NAD would be present in the isolated mitochondria to permit isocitrate dehydrogenase to catalyze the conversion of isocitrate to α-ketoglutarate. The mitochondrial fraction showed more total ^{14}C incorporated into glycine and glutamate than did the microbody fraction while the microbody fraction exhibited a higher specific activity of the enzyme.

DISCUSSION

When various ^{14}C labeled metabolites were provided to rose cells, the carbon skeletons of glycine and serine were produced. We believe that each of the metabolites provided (acetate-1-^{14}C, citrate-1,5-^{14}C, and succinate-2,3-^{14}C) entered the TCA cycle where they were converted to radioactive isocitrate. Furthermore we feel that isocitrate lyase, located in the mitochondrion, was responsible for converting the ^{14}C labeled isocitrate to ^{14}C glyoxylate and succinate. The labeled glyoxylate was then converted to glycine by glutamate-glyoxylate aminotransferase which we also showed to be compartmented in the mitochondrion.

The scheme we have proposed may also explain some of the inconsistencies noted in regard to the synthesis of glycine, serine, and glycolate in photosynthetic tissue. Work by Mahon, Fock, and Canvin (8) showed that, when sunflower leaf disks were exposed to $^{14}CO_2$ at a O_2 concentration of 21 %, glycine, serine, and alanine changed specific activity more slowly than 3-PGA. At 1 % O_2 levels the specific activity of these compounds was lower than at 21 % O_2 levels thus indicating that these amino acids were not derived solely from Calvin cycle intermediates. Work by Zelitch (16) showed that when acetate-2-^{14}C or pyruvate-3-^{14}C was provided to maize and tobacco leaf tissues, glycolate-2-^{14}C was produced. If these labeled compounds entered and completed one turn of the TCA cycle, the ^{14}C labeled carbon atoms would be in the correct position, the #2 carbon of isocitrate, for the production of glyoxylate-2-^{14}C by isocitrate lyase. Zelitch also demonstrated glyoxylate-2-^{14}C was readily converted to glycolate-2-^{14}C by glyoxylate reductase in these two tissues (16). The glyoxylate pathway which we have proposed as an

alternate to those starting from ribulose diphosphate (15) or 3-phosphoglycerate (10) could account for this labeling pattern.

An alternative explanation for the entry of ^{14}C into glycine and serine from the metabolites provided in this study would be that the metabolites gave rise to $^{14}CO_2$ which was refixed in the form of carbon-4 acids. If these acids were converted to glycerate or a derivative thereof, they could then serve as the starting point for one of the accepted pathways of glycine and serine synthesis (10). If this happened, then one would predict that a ^{14}C-metabolite which readily produced $^{14}CO_2$ would lead to the formation of more radioactive glycine and serine than from one which was less likely to give rise to $^{14}CO_2$. The position of the ^{14}C in acetate-1-^{14}C and citrate-1,5-^{14}C make them vulnerable to decarboxylation when they enter the TCA cycle whereas this is not true for succinate-2,3-^{14}C. Therefore, in the present study, if ^{14}C entered glycine and serine following decarboxylation and refixation, then acetate-1-^{14}C and citrate-1,5-^{14}C should be a better source than succinate 2,3-^{14}C. It is not possible to make direct comparisons of radioactivity recovered in glycine and serine upon provision of different substrates since the substrates do not enter the cells in equal amounts. However, another form of analysis is to compare the ratio of ^{14}C in glycine and serine to that of an amino acid such as glutamate whose carbon skeleton is believed to arise only from the TCA cycle. When this was done, the ratios of ^{14}C in glutamate to that in serine plus glycine were 9.1, 9.4, and 22.7 for acetate, citrate, and succinate respectively. Thus it would appear that refixation of $^{14}CO_2$ may serve as a source of carbon for glycine and serine synthesis in this nonphotosynthetic tissue.

It is certain that several pathways exist for the assimilation of carbon into glycine and serine. Those arising from ribulose diphosphate (15) and 3-phosphoglycerate have been well documented (10). We believe that isocitrate serves as still an additional source. The presence of isocitrate lyase in the mitochondrion and the labeling data presented in this paper support this hypothesis. Labeling studies with isolated mitochondria are underway to give more definitive proof of this proposed pathway leading to glycine and serine.

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Table I. Distribution of ^{14}C in Cell Constituents after a 3 hr Incubation in 10 μCi of Glyoxylate-U-^{14}C.

	cpm	%
CO_2	212,007	11
Soluble Amino Acids	447,660	23
Protein Amino Acids	908,780	46
Lipids	335,440	17
Carbohydrates	18,500	1
Organic Acids[1]	40,905	2

[1]Other than glyoxylate.

Table II. Distribution of ^{14}C in Soluble and Protein-Bound Amino Acids after a 2 hr Incubation in 4 μCi of Glyoxylate-U-^{14}C.

	cpm			
	Soluble		Protein	
Amino Acid	Day 3	Day 5	Day 3	Day 5
Aspartate	1,565	745	800	490
Serine	98,100	53,625	39,730	24,280
Glycine	22,715	14,205	162,225	63,300
Glutamate	1,785	1,760	20,160	2,000
Threonine	465	185	320	160
Methionine Sulfoxide	555	730	941	582
*Alanine	865	570	660	520
*Tyrosine	230	140	120	51
Proline	1,560	350	740	452
*Valine	550	720	730	370
*Phenylalanine	230	360	190	80
Leucine & Isoleucine	115	85	930	520
Asparagine	1,615	550	-	-
Glutamine	730	410	-	-
Homoserine	1,125	395	-	-
γ-Aminobutyrate	295	210	-	-

*Amino acid whose carbon structure is not derived directly from the TCA cycle.

Table III. Distribution of ^{14}C in Soluble and Protein-Bound Amino Acids after a 3 hr Incubation in 10 μCi of Citrate-1,5-^{14}C.

	cpm		
	Soluble	Protein	Total
Aspartate	850	2,000	2,850
Serine	520	340	860
Glycine	365	860	1,225
Glutamate	10,650	9,020	19,670
Threonine	210	590	800
Methionine Sulfoxide	205	640	845
*Alanine	1,040	370	1,410
*Tyrosine	270	80	350
Proline	650	2,170	2,820
*Valine	270	350	620
*Phenylalanine	235	220	455
Leucine & Isoleucine	410	1,770	2,180
Asparagine	320	-	-
Glutamine	1,295	-	-
Homosine	50	-	-
γ-Aminobutyrate	6,775	-	-

*Amino acid whose carbon structure is not derived from the TCA cycle.

Table IV. Distribution of ^{14}C in Soluble and Protein-Bound Amino Acids after a 3 hr Incubation in 10 μCi of Acetate-1-^{14}C.

	cpm		
	Soluble	Protein	Total
Aspartate	5,050	60,100	65,150
Serine	6,485	5,030	11,515
Glycine	4,115	12,690	16,805
Glutamate	39,455	161,130	200,585
Threonine	2,615	16,920	19,535
Methionine Sulfoxide	1,050	2,560	3,610
*Alanine	4,500	4,210	8,710
*Tyrosine	665	130	795
Proline	6,420	7,850	14,270
*Valine	415	2,110	2,525
*Phenylalanine	155	2,100	2,255
Leucine & Isoleucine	2,645	118,320	120,965
Asparagine	2,455	-	-
Glutamine	3,910	-	-
Homoserine	885	-	-
γ-Aminobutyrate	16,810	-	-

*Amino acid whose carbon structure is not derived from the TCA cycle.

Table V. Distribution of ^{14}C in Soluble and Protein-Bound Amino Acids after a 3 hr Incubation in 10 μCi of Succinate-2,3-^{14}C.

	cpm		
	Soluble	Protein	Total
Aspartate	35,115	176,830	211,945
Serine	1,640	2,120	3,750
Glycine	1,765	9,470	11,235
Glutamate	125,515	215,330	340,845
Threonine	8,555	49,640	58,195
Methionine Sulfoxide	1,785	4,310	6,095
*Alanine	1,005	29,890	30,895
*Tyrosine	2,175	620	2,795
Proline	6,195	47,600	53,795
*Valine	1,165	15,170	16,335
*Phenylalanine	315	1,470	1,785
Leucine & Isoleucine	3,155	59,350	62,505
Asparagine	5,020	-	-
Glutamine	17,160	-	-
Homoserine	2,365	-	-
γ-Aminobutyrate	21,440	-	-

*Amino acid whose carbon structure is not derived from the TCA cycle.

Table VI. Results ^{14}C Assay for Glutamate-Glyoxylate Amino Transferase in Isolated Mitochondria and Microbodies.

Fraction	Total cpm in Glu and Gly	cpm per μg Protein in Glu and Gly per hr
Mitochondria	1,835	43
Microbody	1,288	89