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THE UNIVERSITY OF ALBERTA

CO_2 FIXATION IN RHODOSPIRILLUM RUBRUM

by

KATHLEEN MARY CLIFF

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Abstract

RUBP carboxylase, the key enzyme of the Calvin cycle, has been purified as a 112,000 M.W. dimer from the photosynthetic bacteria *Rhodospirillum rubrum*. When DNA and RNA coding for this enzyme were examined in Southern and Northern hybridizations it was found that the enzyme is encoded by a single gene which is transcriptionally regulated by the light. Messenger-RNA coding for RUBP carboxylase was found in light-grown cultures but undetectable in dark-grown cultures of *R. rubrum*.

R. rubrum defective in CO_2 fixation were obtained from a $^{14}CO_2$ assimilating screen in which the bacterial cells were grown photoheterotrophically on malate and $^{14}CO_2$. Variants that failed to incorporate $^{14}CO_2$ at wild-type rates were assayed for RUBP carboxylase activity but none were found to be gentically modified for this enzyme. When the rate of $^{14}CO_2$ fixation was quantified at the point of maximum CO_2 fixation, in early log-phase, the variants incorporated $^{14}CO_2$ at rates ranging from 13.1-58.2% that of the wild-type control. The failure to obtain RUBP carboxylase mutants from the CO_2 assimilating screen not only suggests a low level of expression of this gene in malate and CO_2 grown bacteria but also that other CO_2 assimilating enzymes contribute to the photometabolism of malate in *R. rubrum*.

Two approaches have been taken to examine CO_2 assimilation in *R. rubrum* grown photoheterotrophically malate and CO_2. First, short-term $^{14}CO_2$ labelling experiments have been performed to elucidate the pathway(s) for CO_2 incorporation and second, CO_2 assimilating variants, obtained from *in vivo* mutagenesis, have been examined.

In short-term labelling experients, the rapid labelling of 3-PGA and the negative slope of isotope incorporation into the phosphorylated compounds suggested a functional Calvin cycle. However the rapid appearance of ^{14}C-label in α-ketoglutarate, malate and aspartate indicated that other carboxylases besides RUBP carboxylase contribute to the metabolism of *R. rubrum*.

When variant 12-1 was labelled with $^{14}CO_2$ for 15 min the quantity of ^{14}C-phosphorylated compounds and the specific activity of RUBP carboxylase were equivalent to that of wild-type yet the CO_2 fixation rate was reduced 85.4%. Since the

reduction in CO_2 fixation as measured by $^{14}CO_2$ incorportion cannot be accounted for by the elimination of RUBP carboxylase, the residual CO_2 fixation of 14.6% may represent the maximum contribution that the Calvin cycle makes to CO_2 fixation in malate and CO_2 light-grown cultures of *R. rubrum*.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
BPB	Bromophenol blue
BSA	Bovine serum albumin
Cpm	Counts per minute
CTP	Cytosine 5'-triphosphate
DEAE	Diethylaminoethyl-cellulose
DTT	Dithiothreitol
Dpm	Disintegrations per minute
EDTA	Ethylenediaminetetracetate
GTP	Guanosine 5'-triphosphate
MW	Molecular weight
PPO	2,5, diphenyloxazole
SCTD	Sonicated calf thymus DNA
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane
A1	Acid 1 fraction
A2	Acid 2 fraction
A3	Acid 3 fraction
N	Neutral fraction
AA	Amino acid fraction

Chapter 1

Introduction

Photosynthesis is the process that harnesses light energy for the conversion of CO_2 into organic compounds. It occurs in all green plants and algae and in several genera of photosynthetic bacteria. There are two sets of reactions. The light reactions are concerned with converting light energy into ATP bond energy and producing reducing equivalents as NADPH (NADH). The *dark reactions* are those reactions in which CO_2 is reduced to organic compounds. The major difference between the various groups of photosynthetic organisms is that higher plants and blue-green algae produce ATP and NADPH in the light-dependent reactions and obtain their reducing equivalent from the photolysis of water whereas photosynthetic bacteria produce ATP and NADH (Frenkel 1970; Wraight *et al*. 1978; Prince and Dutton 1978) in the *light reactions*, using reduced compounds as the electron donor rather than water. Since oxygen is not evolved during bacterial photosynthesis the process is anoxygenic.

In plants the products of the *light reactions* are used for the assimilation of CO_2 via the Calvin cycle, elucidated in 1946 by Calvin and colleagues. In the more than 50 species of photosynthetic bacteria which fall into three classes - the purple non-sulfur (Rhodospirillaceae), the purple sulfur (Chromatiaceae), and the green sulfur (Chlorobiaceae) bacteria - few have been rigorously examined for CO_2 fixation and utilization (Clayton and Sistrom 1978; Kondratieva 1979). In most cases the presence of ribulose-1,5-biphosphate carboxylase is the only evidence for the functioning of the Calvin cycle.

Light Reactions

The *light reactions* are first recognized in 1937 by R. Hill who found that isolated chloroplasts split water and evolve oxygen continuously in the presence of light. The excited electrons from water are passed down an electron transport chain, in the inner membrane of the chloroplast, where the energy they lose is used to generate ATP. In photosynthetic bacteria the photochemical apparatus is present in widely divergent

structures ranging from invaginations of the cytoplasmic membranes (Holt and Marr 1965, Oelze and Drews 1972; Hurlbert *et al*. 1974; Golecki and Oelze 1975) to independent vesicles (Holt et al. 1966). The photosynthetic pigments and electron carriers, within these structures, are organized into a transport system (Gloe *et al*. 1975). The photosynthetic pigment bacteriochlorophyll absorbs light at a wavelength between 800-890 nm (Gloe *et al*. 1975) allowing bacteria to use light that contains less energy than that used by other phototrophs (400-700 nm).

Dark Reactions

The process by which carbon dioxide is incorporated into organic compounds was charted with two unicellular algae, *Chorella pyrenidosa* (Calvin and Benson 1949) and *Scenedesmus obliquus* (Benson *et al*. 1950). The methods used by this group to follow the path of carbon dioxide fixation involved adding $^{14}CO_2$ in the form of bicarbonate to an illuminated suspension of algae. After a few seconds of CO_2 fixation the organisms were killed by rapid immersion in hot methanol. The pathway was deduced by identifying the radioactively labelled compounds using paper chromatography and autoradiography. After 5 sec of $^{14}CO_2$ labelling there was just one prominent radioactive spot, corresponding to 3-phosphoglycerate (3-PGA). It was ultimately found that the first step of CO_2 reduction consists of the reaction of CO_2 with a phosphorylated 5-carbon sugar, ribulose-1,5-bisphosphate (RUBP), to form two molecules of 3-PGA. The two molecules of 3-PGA so formed are used in the formation of glucose and the regeneration of RUBP. The major steps in the Calvin pathway were later confirmed by demonstrating that cell-free extracts could carry out the individual reactions and later still by purifying the enzymes concerned.

RUBP carboxylase

The key enzyme of the Calvin cycle, RUBP carboxylase, has now been purified from a whole range of taxonomic sources, including higher plants (Badger *et al*. 1975; Goldstein *et al*. 1975), green algae (Lord and Brown 1975; Akazawa and Osmond 1976), and photosynthetic bacteria (Purohit *et al*. 1967; Takabe and Akazawa 1973; McFadden 1974; Ryan *et al*. 1974; Purohit and McFadden 1979). Although the subunit

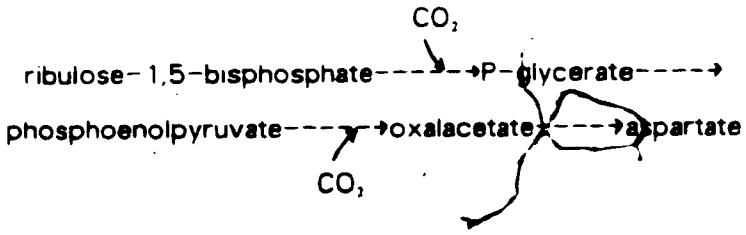
construction of RUBP carboxylase from the various sources is strikingly different, all the enzymes possess an oxygenase activity (Lorimer and Andrews 1981). Kinetic data of Jordan and Ogren (1981) has revealed that the bacterial enzymes have a higher affinity for oxygen than the higher plant enzymes. The utility of the oxygenase activity in bacterial photosynthesis is not understood because bacteria photosynthesize under anaerobic conditions. Lorimer and Andrews (1973) have proposed that the oxygenation reaction is an unavoidable consequence of the carboxylation mechanism where the enolated substrate, RUBP, is susceptible to attack by both oxygen and carbon dioxide. Since oxygen competitively inhibites CO_2 fixation at the enzyme's active site the bifunctional nature of RUBP carboxylase/oxygenase could be a property not only of the reaction mechanism but also a function of the enzyme's active site. Because photoautotrophic bacteria have the enzyme RUBP carboxylase, it should be possible to obtain bacterial mutants unable to assimilate CO_2 and therefore defective in RUBP carboxylase. A genetically modified enzyme lacking the carboxylase activity while maintaining its oxygenase activity would yield information about enzyme structure and function and disprove Lorimer's hypothesis of the inseparability of the two reactions. Bacteria defective in CO_2 assimilation would be useful not only for studying the bifunctional nature of RUBP carboxylase but also other enzymic defects would help clarify the flow of carbon in the *dark reactions* of bacterial photosynthesis.

Carbon Flow in Bacterial Photosynthesis

In 1967(b) Anderson and Fuller examined CO_2 assimilation in the purple non-sulfur bacterium *Rhodospirillum rubrum* using photoautotrophically grown cells and short-term $^{14}CO_2$ fixation experiments. After 1 sec of $^{14}CO_2$ labelling, 75% of the radioactivity appeared in 3-PGA. As the $^{14}CO_2$ labelling interval increased a negative slope of $^{14}CO_2$ incorporation was observed in this compound. Radioactivity was flowing from 3-PGA to the diphosphorylated compounds of the Calvin cycle. After 30 sec of $^{14}CO_2$ fixation 87% of the total assimilated radioactivity was associated with the phosphate esters. The remainder of the radioactivity was found in alanine, glutamate, fumarate and aspartate. Malate and α-ketoglutarate (α-kg) were not labelled until 1 min of $^{14}CO_2$ fixation and radioactive citrate and succinate did not appear until 3 min. A negative slope of isotope

incorporation into the phosphate esters, with 3-PGA as the first labelled product, and the diphosphates becoming secondarily labelled, is indicative of Calvin cycle CO_2 fixation. When the enzymes of the Calvin cycle were found to have high specific activities Anderson and Fuller (1967d) concluded that the Calvin cycle is the main pathway for CO_2 fixation in *R. rubrum*.

In the purple sulfur bacterium *Chromatium vinosum*, Fuller *et al*. (1961), using short-term $^{14}CO_2$ fixation experiments, also observed a decaying percentage of ^{14}C-label in 3-PGA, indicating a primary carboxylation of RUBP. The secondary products of CO_2 fixation, the diphosphates, had the usual increasing percentage of ^{14}C-carbon. However, in contrast to the findings in *R. rubrum*, where 87% of the radioactivity is found in the phosphorylated compounds after 30 sec of $^{14}CO_2$ labelling, in *C. vinosum* as much as 45% of the total fixed carbon after 30 sec appeared in aspartic acid. The negative slope into aspartic acid with time was believed to be the result of an active ancillary carboxylation of phosphoenolpyruvate (PEP) to form oxalacetate and its subsequent transamination to aspartate. Fuller *et al*. suggested that this organism uses a "double CO_2" fixation as a rapid method for incorporating carbon into organic acids for the production of new cellular material:



In the green sulfur bacterium *Chlorobium thiosulfatophilum* the first stable products of photosynthesis are not 3-PGA and sugar diphosphates but organic and amino acids (Evans *et al*. 1966). The short-term $^{14}CO_2$-labelling experiments in *C. thiosulfatophilum* showed that among the earliest labelled products appearing were the oxo acids: α-kg, pyruvate and oxalacetate (OAA). After 30 sec of $^{14}CO_2$ fixation in the light, glutamate contained 75% of the label, presumably formed from α-kg. Since RUBP carboxylase and phosphoribulokinase were not detectable in cell-free extracts, CO_2 fixation in this bacterium must occur by a different mechanism than it does in other

photoautotrophic organisms. Further research led Buchanan *et al.* (1967) to the discovery in these bacteria of enzymes which catalyse two new carboxylation reactions, namely pyruvate and α-kg synthase. These enzymes have been purified (Evans and Buchanan 1965; Buchanan 1979) and are found to catalyse the following reactions:

acetyl-CoA+CO_2+reduced ferredoxin $\dashrightarrow$pyruvate+CoA+oxidized ferredoxin

succinyl-CoA+CO_2+reduced ferredoxin$\dashrightarrow$ α-ketoglutarate+CoA+oxidized ferredoxin

These discoveries led to the postulation of a new cycle for the net carbon dioxide fixation which is referred to as the reductive carboxylic acid cycle. The cycle involves the reversal of several reactions of the TCA cycle and results in net synthesis of one molecule of oxalacetate from four molecules of carbon dioxide and a regeneration of the first carbon dioxide acceptor, acetyl-CoA (Buchanan 1972; Buchanan and Sirevag 1976, Sirevag 1974). These enzymes are present in numerous photosynthetic anaerobes (Buchanan *et al.* 1967) but despite the presence of ferredoxin in algae and higher plants these ferredoxin-dependent carboxylases have not been found in organisms that evolve oxygen during photosynthesis (Buchanan and Arnon 1970). A common feature of all organisms showing ferredoxin-linked carboxylation is a requirement for an anaerobic environment. The operation of the reductive carboxylic acid cycle (Evans *et al.* 1966; Buchanan *et al.* 1967) in bacterial photosynthesis rests on the identification of these enzymes, in cell free extracts of *C. thiosulfatophilum*. Other than Buchanan's measurements, made to establish the presence of the enzymes, there is no definitive information about their relative activities *in vivo*. Other evidence that may support the functioning of this cycle is that the equilibrium of the PEP synthase reaction lies far on the side of PEP formation (Buchanan 1973).

In later studies, Buchanan *et al.* (1972) found that the molarity of $^{14}CO_2$, $H^{14}CO_3^-$ and $^{14}CO_3^{2-}$ profoundly influenced the fixation products in *C. thiosulfatophilum*. Using cells grown on 40mM Na_2CO_3, the percentage of $^{14}CO_2$ fixed into glutamate, α-kg and glutamine was 50% with 28% of the ^{14}C-carbon in citrate and isocitrate after 5 sec of $^{14}CO_2$ labelling. Both percentages showed a negative slope when plotted against time, as would be expected if these compounds were early intermediates of the reductive carboxylic acid cycle. There was no incorporation of radioactivity into the phosphate esters until 25 sec of $^{14}CO_2$ labelling. This is in contrast to the labelling pattern that was observed when the

total concentration of carbon dioxide, bicarbonate, and carbonate was 0.8mM. In this case phosphoglycerate was the principal labelled product after 5 sec of CO_2 labelling. Moreover, the percentage of radioactivity incorporated into phosphoglycerate revealed the steepest negative slope with time. Thus it seems likely that *C. thiosulfatophilum* fixes CO_2 via the Calvin cycle, although this must be established more rigorously.

Carbon Dioxide Assimilation on Organic Compounds

Carbon dioxide fixation with participation of RUBP carboxylase often occurs in purple bacteria growing on medium containing carbon compounds other than CO_2. In the presence of methanol and formate *Rhodopseudomonas acidophilia* (Sahm *et. al.*, 1976) and *Rhodopseudomonas palustris* (Stokes and Hoare 1969), CO_2 generated from the decarboxylation of formate, is assimilated via the Calvin cycle. With other photosynthetic organisms the presence of organic compounds in the growth medium affects the amount of CO_2 incorporated via the Calvin cycle because, under these conditions, the specific activity of RUBP carboxylase is much lower than under photoautotrophic conditions (Fuller 1969; Cherniadiev *et al.* 1974). However this does not necessarily happen with all species of purple bacteria and it also depends on the nature of the organic compounds present (Tabita and McFadden 1974a; Zhukov 1976).

The rapid production of metabolites needed for the synthesis of new proteins, lipids and other substances is satisfied in photosynthetic bacteria by the existence of several carboxylases (Table 1.1). Although the presence of such enzymes varies with the organic substrate, the number of CO_2 assimilating enzymes in photosynthetic bacteria is greater than that found in other phototrophs. The synthesis of phenylpyruvate as a result of phenylacetyl-CoA carboxylation seems to be involved in the formation of phenylalanine. Also, the action of α-ketobutyrate synthase allows the production of isoleucine by a new pathway without participation of threonine (Buchanan 1972, 1973). The metabolism of propionate in the purple bacteria *R. rubrum*, (Knight 1962) and *C. thiosulfatophilum* (Olsen and Merrick 1968) is connected with the participation of another carboxylation enzyme propionyl-CoA; Buchanan and colleagues have also found pyruvate and α-kg synthase in many species of purple and green bacteria.

Table 1.1

Carboxylases of Photosynthetic Bacteria[1]

Enzyme	Purple nonsulfur bacteria	Purple sulfur bacteria	Green bacteria
Ribulose-1,5-bisphosphate carboxylase	*R. rubrum*; *R. palustris*; *R. sphaeroides*; *R. vannielii*; *R. sulfoviridia*	*C. Okenii*; *C. vinosum*; *T. roseopersicina*; *Thiopedia sp.*; *Thiospirillum spp.*; *E. shaposhnikovii*	*C. thiosulfatophilum* (?)
Phosphoenolpyruvate carboxylase	*R. rubrum*; *R. palustris*	*C. vinosum*; *C. okenii*; *E. shaposhnikovii*; *T. roseopersicina*	*C. thiosulfatophilum*
Phosphoenolpyruvate carboxykinase	*R. rubrum*; *R. palustris*; *R. capsulata*; *R. gelatinosa*	*C. vinosum*; *C. okenii*; *E. shaposhnikovii*; *T. roseopersicina*	
Phosphoenolpyruvate carboxytransphosphorylase	*R. palustris*; *R. rubrum*	*E. shaposhnikovii* (?)	
Pyruvate carboxylase	*R. sphaeroides*; *R. rubrum*	*C. vinosum*; *C. okenii*; *T. roseopersicina*	
Propionyl-CoA carboxylase	*R. rubrum*	*E. shaposhnikovii*	*C. thiosulfatophilum* (?)
Malate dehydrogenase (decarboxylating) (NAD(P)) "malic enzyme"		*C. vinosum*	
Pyruvate synthase	*E. shaposhnikovii*; *T. roseopersicina*; *R. rubrum*	*C. vinosum*	*C. thiosulfatophilum*
α-Ketobutyrate synthase		*C. vinosum*	
α-Ketoglutarate synthase	*R. rubrum*; *E. shaposhnikovii*	*C. vinosum*	*C. thiosulfatophilum*
Phenylpyruvate synthase	*R. rubrum*	*C. vinosum*	*C. thiosulfatophilum*

[1]Adapted from Kondratieva (1979)

Although the enzymes listed in Table 1.1 may not all operate at one time or in the forward direction it is increasingly apparent that individual carboxylations and even additional cycles may function for cellular biosynthesis in photosynthetic bacteria. However, the establishment of any metabolic pathway or cycle that functions *in vivo* depends on evidence or observations obtained from several diverse experimental approaches among which are: reasonable amounts of the required enzymes, kinetic analysis of the series of reactions, and the regulation of the enzymes concerned. Photosynthetic bacteria would serve as excellent experimental material for these approaches, especially the use of $^{14}CO_2$ labelling and the *switch* mechanisms using light and growth conditions as regulators.

This research originally had one objective: to mutagenise *R. rubrum* cells in order to obtain RUBP carboxylase mutants. Under anaerobic light conditions with malate and CO_2 as the carbon sources, *R. rubrum* was believed to express RUBP carboxylase and to assimilate CO_2 via the Calvin cycle. The screen for CO_2 fixing mutants was based on the assumption that during the screening process the bacterial cells were expressing RUBP carboxylase and would incorporate $^{14}CO_2$ via the Calvin cycle. Bacteria unable to assimilate $^{14}CO_2$ (as detected by autoradiography) would either be defective in the carboxylation or regeneration of RUBP, as were the CO_2 assimilating mutants in *Alcaligenes eutrophus* (Anderson *et al.* 1978) and *Chlamydomonas reinhardi* (Spreitzer and Mets 1980). The carbon requiring (Moll and Levine 1970) CO_2 fixing mutants in *R. rubrum* would then be recovered from dark aerobic cultures in which an organic compound was provided as a carbon source. It would be of particular interest to obtain RUBP carboxylase deficient bacteria (RUBP carb$^-$) in order to study the bifunctional nature of RUBP carboxylase. A genetically modified enzyme lacking the carboxylase activity would reveal information about enzyme structure and function.

During the course of this work certain discrepencies in the literature have been noted. For instance, the contribution of the Calvin cycle to the photometabolism of *R. rubrum* grown on CO_2 and malate has not clearly been established. Slater and Morris (1973b) claim that under chemostatic conditions the Calvin cycle is the main route for CO_2 assimilation whereas Anderson and Fuller (1967c) found limited evidence for the functioning of the Calvin cycle *in vivo*. Since such differences in the mode of CO_2

assimilation jeopardize the effectiveness of the above screen, the decision was made to examine CO_2 assimilation in photoheterotrophically grown cultures of *R. rubrum*.

Chapter 2

Rhodospirillum rubrum has One Gene for RUBP Carboxylase as Demonstrated by DNA and Protein Analysis

INTRODUCTION

Ribulose-1, 5-bisphosphate carboxylase catalyses the hydrolytic carboxylation of ribulose-1,5-bisphosphate (RUBP) to yield two molecules of 3-phosphoglycerate. This reaction is present in photosynthetic organisms and constitutes the primary step of CO_2 fixation via the Calvin cycle.

RUBP carboxylase has three distinct molecular forms. In higher plants (Paulsen and Lane 1966; Ridley *et al*. 1967; Wilson and McCalla 1968; Kawashima and Wildman 1971), green algae (Sugiyama *et al*. 1971; Givan and Griddle 1972; Tabita *et al*. 1976) and some prokaryotic organisms (McFadden and Denend 1972; McCarty and Charles 1975; Anderson 1979) it is a 550,000 M.W. protein containing eight large (Mr 56,000) catalytic and eight small (Mr 12,000) regulatory subunits (Rutner 1970; Akazawa *et al*. 1978). In the purple nonsulfur bacterium *Rhodospirillum rubrum* there is a 120,000 M.W. homodimer (Akazawa *et al*. 1970; and Tabita and McFadden 1974a) whereas in *Rhodopseudomonas sphaeroides* Gibson and Tabita (1977a) have purified two species of RUBP carboxylase. One form of the enzyme is similar to the higher plant carboxylase whereas the other is a 360,000 M.W. intermediate form of six large subunits.

Although there is a striking difference in molecular architecture, RUBP carboxylase from all sources exhibits an oxygenase activity (Bowes and Ogren 1972; McFadden 1974; Lorimer 1981). In higher plants the oxygenase reaction initiates photorespiration, an energy wasteful pathway that evolves previously fixed CO_2 (Lorimer and Andrews 1973; Asami *et al*. 1977) whereas in the photosynthetic bacteria *Chromatium* (Asami and Akazawa 1974; Fogg 1976) and *R. rubrum* (Codd and Smith 1974; Anderson and Fuller 1976a; Storro and McFadden 1981) the oxygenase reaction results in the excretion of carbon as glycolate. The carboxylase and oxygenase enzyme activities reside in the catalytic subunit and require activation by Mg^{+2} and CO_2 before binding of the substrate RUBP (Badger and Lorimer 1976). Although all forms of the enzyme are immunologically distinct, affinity labelling experiments indicate that two lysine residues, at positions 175

and 334, are involved in binding RUBP in both spinach (Schloss *et al.* 1978) and bacterial enzymes (Shinozaki *et al.* 1983).

In higher plants the mode of inheritance of RUBP carboxylase has been largely deduced from the results of interspecific hybridization in the genus *Nicotiana*. The approach is based on the fact that different species of *Nicotiana* exhibit heterogeneity for the small (SS) and large (LS) subunits. Wildman and his coworkers used the SS and LS types as phenotypic markers to show that the LS is maternally inherited and that the SS is biparentally inherited (Kawashima and Wildman 1972; Chan and Wildman 1972). Recently these studies have been confirmed by cloning the LS gene from chloroplast DNA (Coen *et al.* 1977; Bedbrook *et al.* 1979; Zurawaski *et al.* 1981) and the SS gene from nuclear DNA (Bedbrook *et al.* 1980). Thus in all plants and green algae the two subunits of RUBP carboxylase are encoded separately in chloroplast and nuclear DNAs.

In photosynthetic bacteria that lack organelles (chloroplasts) the genes for RUBP carboxylase must be encoded on the bacterial chromosome (Curtis and Haselkorn 1983). The experiments described in this paper were designed to examine the genomic DNA from *R.rubrum* to determine whether the bacterial enzyme (containing just the catalytic large subunit) is encoded by a single gene. The higher plant carboxylase clone was not used as a probe in the Southern analysis because it was considered unlikely that bacterial and plant genes have sufficient homology to allow DNA-DNA hybridization. This assumption was based on the fact that there is no amino acid sequence homology between cysteine-containing tryptic peptides of *R. rubrum* with *Zea mays* or the spinach enzyme, as well as no immunological cross reactivity (Lorimer 1978).

I now report that unlike other members of the **Rhodospirillaceae** family (*R. sphaeroides, Rhodopseudomonas capsulata*) (Gibson and Tabita 1977a,b), *R. rubrum* has one form of RUBP carboxylase encoded by a single gene. RUBP carboxylase from *R.rubrum* is thus the simplest of any known carboxylase: a homodimer of 112,000 M.W.

METHODS

Growth of Escherichia coli

Escherichia coli HB101 (hsdR hsdM recA13 supE44 lacZ44 leuB6 proA2 thi-1 rpsL) harboring pRR116 was grown in a 2 liter erlenmeyer flask containing 500 ml of Luria broth (LB). The flasks were vigorously aerated in an orbital shaker at 37°C for 18 hours.

Plasmid pRR116

Plasmid pRR116 was constructed by C.R. Somerville (in press) by lygating Eco RI cut *R. rubrum* DNA with pBR325 and transforming *E. coli*. *E. coli* cells carrying pBR325 with a cloned RUBP carboxylase gene were ampicillin-resistant, chloramphenicol-sensitive, and showed RUBP dependent $^{14}CO_2$ fixation.

Extraction of Plasmid DNA

Plasmid DNA was extracted from a 500 ml culture of *E. coli* using a procedure similar to that of Thompson, Hughes, and Broda (1974). Late logarithmic cells were centrifuged at 4,000 xg for 5 min and resuspended in 12 ml of 0.73M sucrose, 0.05M Tris (pH=8.0). The resuspended cells were lysed by adding 17.5 mg of lysozyme dissolved in 1.75 ml of 0.25M Tris (pH 8.0) and then incubated for 5 min at 37°C. The cells were then returned to ice for 5 min and 6.5 ml of 0.25M EDTA was added. Membranes were disrupted with 13.5 ml of a 5% Triton solution (50 mM Tris, 62.5mM Na_2EDTA, and 5 ml of Triton X-100) and incubated at 37°C for 15 min. Bacterial DNA and debris were pelleted at 17,000 rpm in an SS-34 rotor for 30 min. The supernatant (31 ml) was layered on 3 ml of CsCl (refractive index 1.3856) and spun in a SW27 rotor at 23,000 rpm for 16 hr (15°C). The DNA solution (7 ml) was withdrawn from the bottom of the tube and added to 0.5 ml of ethidium bromide (10mg/ml) and 5.0 ml of CsCl (refractive index 1.3856). The bacterial and plasmid DNAs were separated in the CsCl equilibrium gradient by spinning the DNA solution in a Ti50 rotor for 20 hr at 226,000 xg (15°C). The upper bacterial and lower plasmid DNA bands were visualized under a short-wave UV lamp and the plasmid band removed with a 1 ml syringe. Ethidium bromide was removed by two n-butanol extractions and the DNA was dialysed against 10mM Tris,

1mM EDTA (pH=7.3).

Extraction of Bacterial DNA

R. rubrum DNA was extracted from 0.9 grams of malate grown cells by a procedure similar to Andrzej *et al.* (1965) and Marmur (1961). The pelleted cells were resuspended in 10 ml of TNE buffer containing 0.01M Tris, 0.15M NaCl, and 0.015M EDTA (pH=7.6). The cells were lysed by adding 4 ml of 10% SDS and incubating for 15 min at 40°C. Following incubation, 6 ml of pronase (10 mg/ml in TNE) was added and digestion was carried out at 37°C for 12 hr. The proteins were extracted from the lysate by gently agitating for 10 min with 40 ml of water-saturated phenol. The preparation was centrifuged at 7,700 xg for 10 min and the upper aqueous layer removed. An equal volume of cold, water-saturated ether was added and after gentle agitation the two phases were allowed to separate at 37°C. The ether layer was discarded and the wash repeated once more.

The washed nucleic acids were precipitated by layering on two volumes of cold 95% ethanol. The DNA was collected and dissolved in TE buffer (50mM Tris, 1mM EDTA (pH=7.7). RNAse was added to a final concentration of 0.05 mg/ml and incubated at 37°C for 1.5 hr. The nucleic acid was reprecipitated with 95% ethanol as described above. After spooling the DNA on a glass rod it was resuspended in a small volume of TE buffer and its concentration determined by the 260 nm absorbance (1 A_{260} = 50 mg/ml).

DNA Agarose Gel Electrophoresis

1% agarose in TEA (50mM Tris, 20mM Na Acetate, 2mM Na_2 EDTA and 18mM NaCl (pH=8.2)) was poured at 60°C into a horizontal Bio-Rad gel apparatus (15 cm x 10.5 cm). The bacterial restriction digests (1 ug DNA) containing blue dye (50% v/v glycerol, 0.25% w/v BPB, and 25mM EDTA) were loaded into the slot by displacing the electrophoresis buffer TEA. The gel was run at 25V for 18 hours and stained with ethidium bromide at 1 mg/l TEA for 30 min. Fluorescing DNA was visualized with a long wave UV transilluminator. Photographs were taken using a Polaroid HP4 camera with Polaroid type 57 film and a Kodak Wratten #9 filter.

Restriction Endonuclease Digests

Restriction endonucleases were purchased from Boehringer Mannheim and used according to their specifications for cleaving 1 ug of DNA. The digests were incubated at 37°C and enzyme reaction stopped after 3 hr by heating to 65°C for 5 min.

Nick Translation

pRR116 was nick translated at 14°C for 3 hr according to the procedure of Rigby *et al.* (1977). The 25 ul reaction mixture contained: 2.5 ul of 10X NT reaction buffer (0.5M Tris, 0.1M $MgSO_4$, 10mM DTT, and 500 ug/ml of BSA (pH=7.5); 1.5 ul of 0.33mM of ATP, GTP, TTP; 8 ul H_2O; 10 ul $dCT^{32}P$ (160 uCi); 1 ul pRR116 (0.5ug); 1 ul DNAse (200 ng/ml); and 1 ul *E. coli* DNA polymerase I (4.5 units). The nick translation reaction was stopped by adding 25 ul of 0.02M Na_3EDTA, 2 mg/ml sonicated calf thymus DNA, and 0.2% w/v SDS. Fractions were collected from a Sephadex G-50 column equilibrated with TE and ^{32}P-labelled DNA located with a hand monitor.

Southern Transfer

After agarose gel electrophoresis the bacterial DNA was depurinated, denatured, and neutralized before transfer to nitrocellulose using a procedure similar to that of Southern (1975). Depurination of the agarose bound DNA consisted of two 15 min washes in 0.25M HCl followed by alkaline denaturation in 0.5M NaOH and 1.5M NaCl.

For transfer to the Bio-Rad nitrocellulose paper the gel was neutralized by gentle agitation in 500 ml of 0.5M Tris-HCl (pH=7.5) and 1.5M NaCl. The DNA was transferred to the solid support by overlaying the gel with H_2O saturated Bio-Rad nitrocellulose paper (cut to the same size as the gel). Beneath the gel were five sheets of 3 mm Whatman paper saturated in 20X SCC (3M NaCl and 0.3M Nacitrate). The bottom sheet of Whatman paper served as a wick so that the 20X SCC could be absorbed by the 3 cm of paper toweling placed on the nitrocellulose filter. The paper towelling was cut to the same size as the nitrocellulose filter and weighted with a 1 kg plate. The DNA was transferred from the gel to the nitrocellulose filter for 12 hr. The nitrocellulose filter was then vacuum dried at 80°C for 2 hr.

Southern Hybridization Conditions

The filter was prehybridized in a heat-sealed bag containing 10.0 ml of 50% v/v formamide, 5X SCC, 50mM Na_2HPO_4 (pH=6.5), 2.5 mg SCTD, and 1X Denhardts (BSA, polyvinyl pyrrolidone, and Ficol 400, each 0.02%) for 4 hr at 42°C. After pretreatment the filter was then incubated at 42°C in a hybridization solution containing 50% formamide v/v, 5X SCC, 50mM Na_2HPO_4 (pH=6.5), 2.5 mg SCTD, 10% w/v sodium dextran sulfate 500, 1X Denhardts, and 10^6 dpm of ^{32}P-pRR116 denatured by heating to 95°C for 10 minutes. The hybridization was performed in a heat sealed bag for 12 hr at 42°C.

After hybridization the filters were washed four times with 250 ml of 2X SCC and 0.1% SDS at 22°C for 5 min. This was followed by two 50 ml washes of 0.1X SCC and 0.1% SDS at 50°C for 15 min. Filters were then air-dried and overlayed with Kodak X-Omat R film.

Growth of R. rubrum

R. rubrum (strain S1) was obtained from the American culture collection at Rockville, Maryland and maintained in in the dark on solid malate medium (Ormerod *et al.* 1961). These cells were used as an inoculum for photoheterotrophically grown cultures. Two liter erlenmeyer flasks were filled to the top with Ormerod medium in which the final concentration of $NaHCO_3$ was 10mM and 0.6% butyrate replaced malate as the carbon source. The cultures were stirred continuously under a panel of 100 Watt tungsten filament bulbs giving a light intensity of 3600 lux at the surface of the bottles. The cultures were maintained at 26°-28°C by circulating cold water around the flasks in 10 mm diameter tubing.

Cells were harvested during logarithmic growth by spinning at 6,000 xg for 10 min. The resultant cell paste was then weighed and stored at -20°C until needed.

RUBP carboxylase Assay

RUBP carboxylase activity was measured by RUBP dependent $^{14}CO_2$ fixation at pH 8.0. The enzyme was activated at 22°C in a sealed Eppendorff tube containing 50mM Tris, 20mM $MgCl_2$, 4mM EDTA, 4mM DTT, and 20mM $NaHCO_3$ (1.8 uCi/umole)

(Laing *et al*. 1975; Laing and Christeller 1976; Christeller and Laing 1978). After activating the enzyme for 30 min, 0.1M RUBP was added to the reaction mixture and CO_2 fixation was stopped after 1 min (Lorimer *et al*. 1977) by injecting 6N acetic acid. The reactions were oven-dried at 100°C and resuspended in 100mM HCl. Scintillation fluid was then added and the radioactivity determined in a Beckman LS7500 scintillation counter at an efficiency (for ^{14}C) of 80%. The scintillation mixture (Anderson and McClure 1973) contained Triton X-100 and xylene (1:4 v/v) with 6 g of PPO per liter of scintillant.

Purification of Ribulose-1,5-bisphosphate carboxylase

19 g of *R. rubrum* cells were resuspended in 25 ml of cold TEMM buffer containing 20mM Tris-SO_4, 1mM EDTA, 10mM $MgCl_2$, and 5mM 2-mercaptoethanol (pH=8.0). The cell suspension was passed three times through an Aminco French Press, lysing the cells at 15,000 psi. The unbroken cells were removed by centrifugation at 30,000 xg for 15 min in a Sorvall RC-5B centrifuge. The pellet was resuspended in 1/5 the original volume of TEMM and recentrifuged. The supernatant solutions were pooled and subjected to ultracentrifugation at 190,000 xg for 1 hr to remove chromatophores, ribosomes, and membrane fragments.

$MgCl_2$ was added to the high speed supernatant to a final concentration of 50mM. The temperature of the supernatant was rapidly raised to 55°C for 10 min and then quickly lowered to 0°C. Denatured proteins were removed by centrifugation and the resultant supernatant was subjected to streptomycin treatment as described by Tabita and McFadden (1974a).

The solution was slowly brought to 60% ammonium sulfate and allowed to stand for 60 min at 4°C. (Concentrations of NH_4SO_4 were calculated from solubility values at 25°C.) The precipitated protein was collected by centrifugation at 30,000 xg for 20 min. The protein was resuspended in and dialysed against TEMM.

The sample was then loaded on a DEAE cellulose anion exchange column equilibrated with TEMM. The proteins were eluted with a linear gradient of 0.1 to 0.3M KCl (in TEMM) and every second fraction assayed for RUBP carboxylase activity. Fractions with activity were pooled and the proteins precipitated with 70% NH_4SO_4.

The NH_4SO_4 was removed by TEMM dialysis before loading on an AcA-34 column. RUBP carboxylase was eluted from the gel filtration column with 100mM KCl (in TEMM). Active fractions were pooled and precipitated with NH_4SO_4. The protein was then dialysed against TEMM buffer and SDS-electrophoresis performed to assess its purity.

SDS-Polyacrylamide Electrophoresis

10% SDS-polyacrylamide electrophoresis was performed as described by Chua (1980) using a Bio-Rad vertical slab gel apparatus. The gel was stained with Coomassie Blue R250 (0.25 w/v in 50% methanol and 7% glacial acetic acid). The protein was then visualized by destaining with 40% methanol and 7% glacial acetic acid).

Molecular Weight Determination

The molecular weight of the RUBP carboxylase subunit was estimated from protein standards which when separated by electrophoresis migrated in proportion to the log of their molecular weights.

Protein Determinations

The protein was quantified using the assay of Spector (1978). The assay reagent was prepared by dissolving 100 mg of Coomassie brilliant blue in 100 ml of 88% phosphoric acid, adding 46.7 ml of 98% ethanol and bringing the final volume up to one liter. The colorometric reaction was performed by adding 900 ul of Spector reagent to 100 ul of protein, waiting 3 min, and reading the absorbance at 595 nm. A known amount of BSA was used to construct a standard curve.

RESULTS

One Form of RUBP carboxylase in R. rubrum

RUBP carboxylase was purified from a crude, high speed supernatant of lysed *R. rubrum* cells on a DEAE-cellulose column using a linear KCl gradient. When the fractionated eluate was assayed for RUBP carboxylase activity one symmetrical peak at 0.3M KCl was found with no RUBP carboxylase remaining bound to the column after a

0.5M KCl wash (Figure 2.1). It appears that on the basis of charge *R. rubrum* has one form of the enzyme.

Fractions containing RUBP carboxylase were then separated on the basis of molecular weight (pore size ranges from 20,000 to 360,000) by gel filtration. In the center of the 100mM KCl elution profile was one peak of RUBP carboxylase activity (Figure 2.2). The higher plant carboxylase of 550,000 daltons which would pass directly through the column was not detected in the void volume nor was the 330,000 dalton hexamer present in the final eluate fractions (Figure 2.2). Because the assay conditions are appropriate for detecting all three forms of the enzyme (Jorden and Ogren 1981) it appears that, on the basis of molecular weight and charge, *R. rubrum* has one functional RUBP carboxylase.

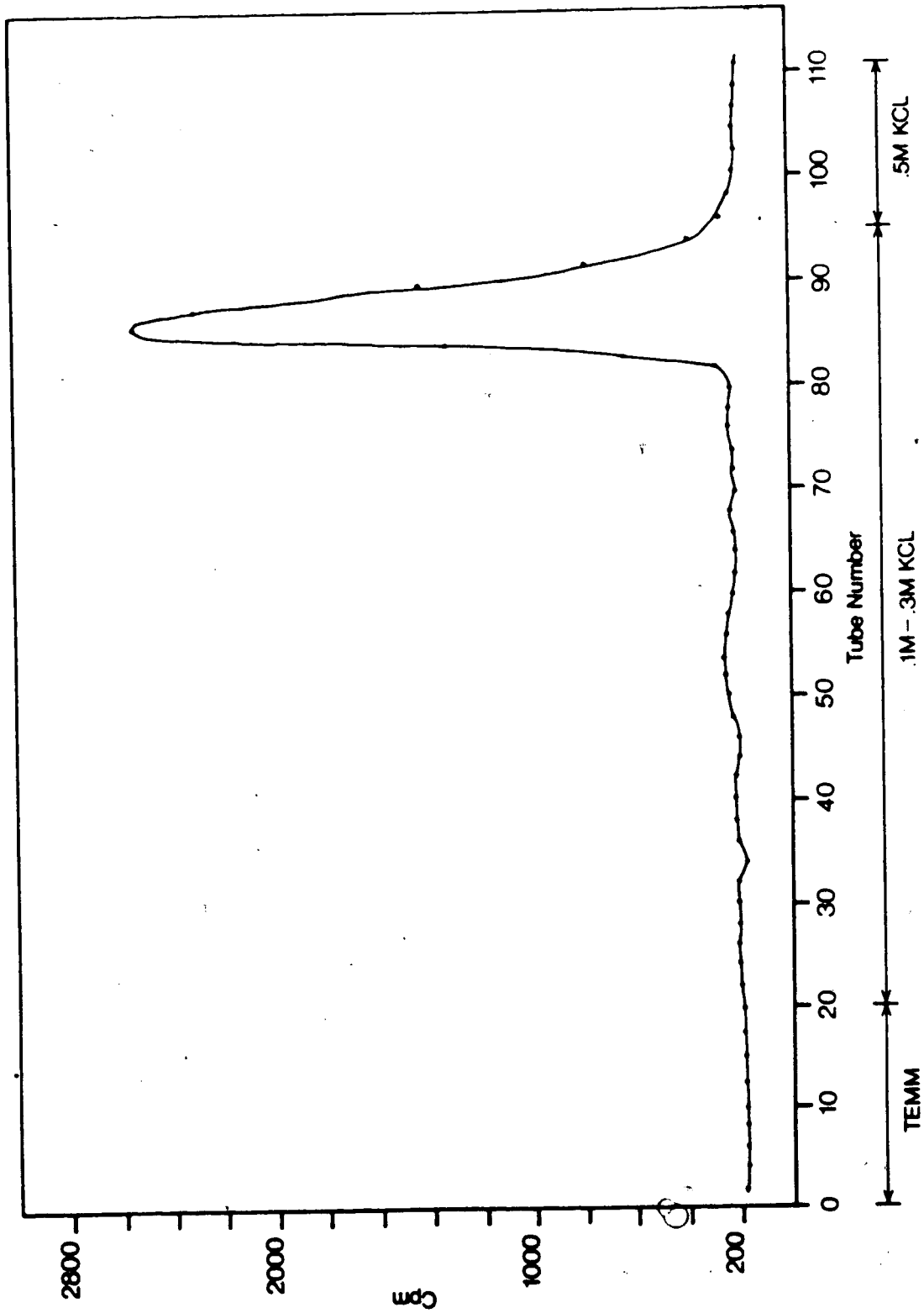
Subunit Composition and Molecular Weight

RUBP carboxylase was purified to a state of apparent homogeneity (~95%) by the criterion of SDS-polyacrylamide gel electrophoresis. The *R. rubrum* carboxylase was denatured and the electrophoretogram indicated the presence of one polypeptide chain (Plate 2.1). Subunit molecular weight was estimated from comparison with several known polypeptides as 56,000 daltons.

One Gene for RUBP carboxylase

In order to determine the number of genes coding for RUBP carboxylase in *R. rubrum* the previously cloned *R. rubrum* carboxylase gene (pRR116) was used to identify region(s) of genomic DNA that had homologous nucleotide sequence(s). (From DNA sequence information the probe (pRR116) is known to contain one gene for RUBP carboxylase (Somerville, in press). Restriction endonuclease fragments from *R. rubrum* were separated on the basis of molecular weight by agarose gel electrophoresis (Plate 2.2). When the DNA was transferred to nitrocellulose and hybridized with the cloned RUBP carboxylase gene (^{32}P-pRR116) a 6.6 kb Eco R1 fragment showed homology with the probe (Plate 2.3, lane 4). This 6.6 kb Eco R1 band represents the piece of genomic DNA that was cloned into pBR325. (From lane 2, Plate 2.3 both the vector (pBR325) and the cloned insert (6.6 kb Eco R1 fragment) are homologous to the ^{32}P-probe.)

Figure 2.1 DEAE-cellulose chromatography of ribulose-1,5-bisphosphate carboxylase from *R. rubrum* as described in the text. Ribulose-1,5-bisphosphate carboxylase activity (•) is expressed as counts of ($^{14}CO_2$) fixed during a 1 min assay. A 72 ul aliquot was taken from each fraction of 4.5 ml.


2800
2000
1000
200
CPM
0
10
20
30
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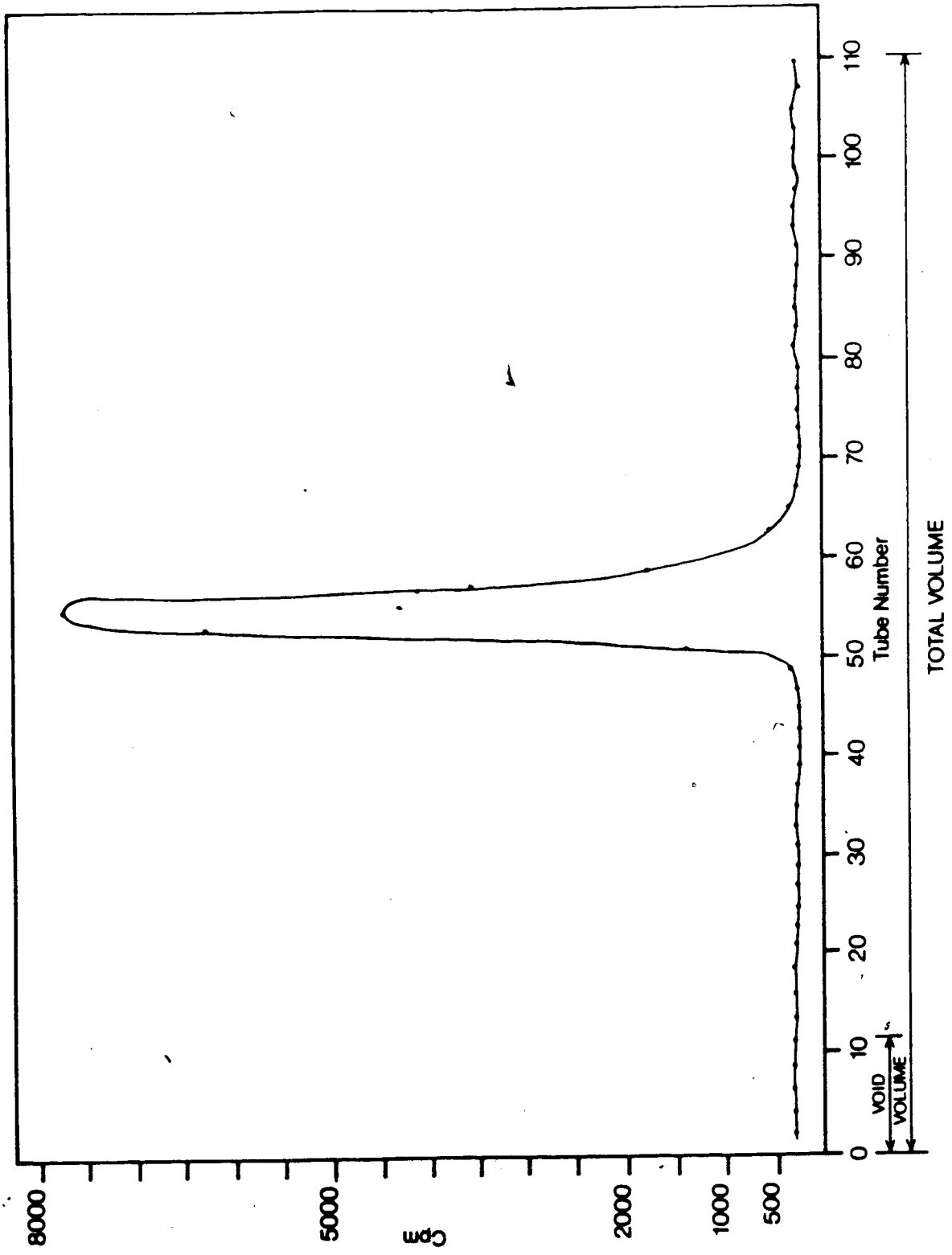
60
70
80
90
100
110
Tube Number
TEMM
.1M – .3M KCL
.5M KCL

Figure 2.2 AcA-34 Chromatography of ribulose-1,5-bisphosphate carboxylase from *R. rubrum* as desribed in the text. RUBP carboxylase activity (•) is expressed as counts ($^{14}CO_2$) fixed during a 1 min assay. A 72 ul aliquot was taken from each fraction of 1 ml


8000
5000
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cpm
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30
40
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60
70
80
90
100
110
Tube Number
VOID VOLUME
TOTAL VOLUME

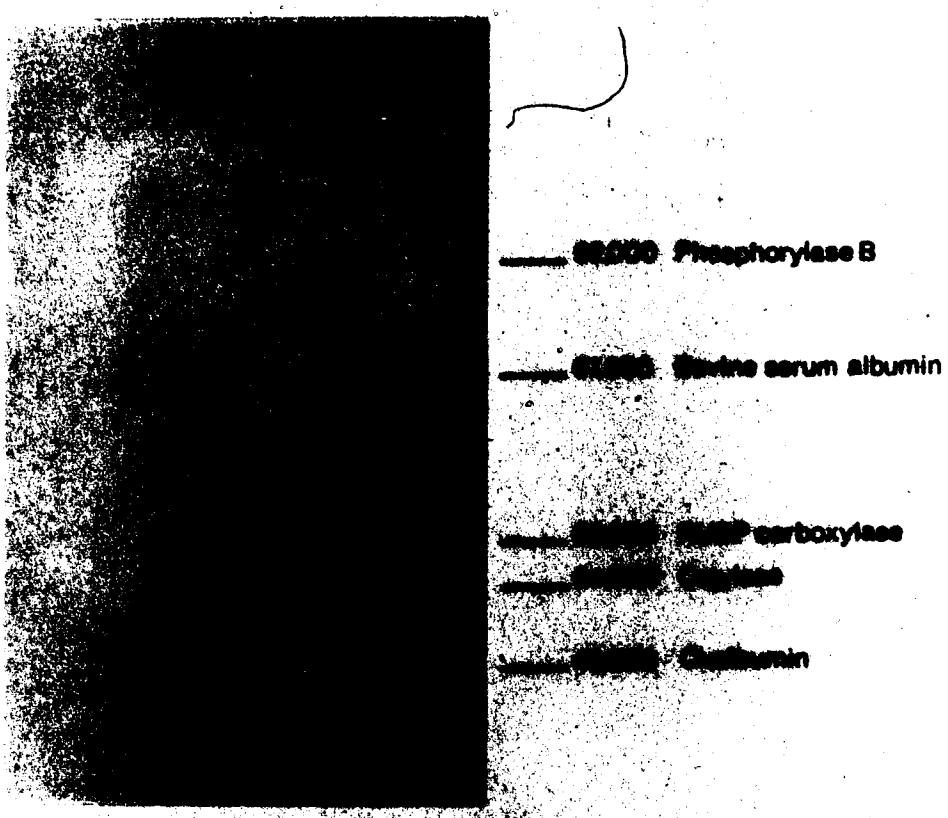


Plate 2.1 SDS-polyacrylamide [illegible]
of 20 ug of purified [illegible]

Plate 2 2 DNA agarose gel electrophoresis of *R. rubrum* DNA cut with restriction endonucleases (lanes 3–4) Lane 1 (1 0 ug) and lane 2 (0 1 ug) contain an Eco R1 digest of pRR1 16

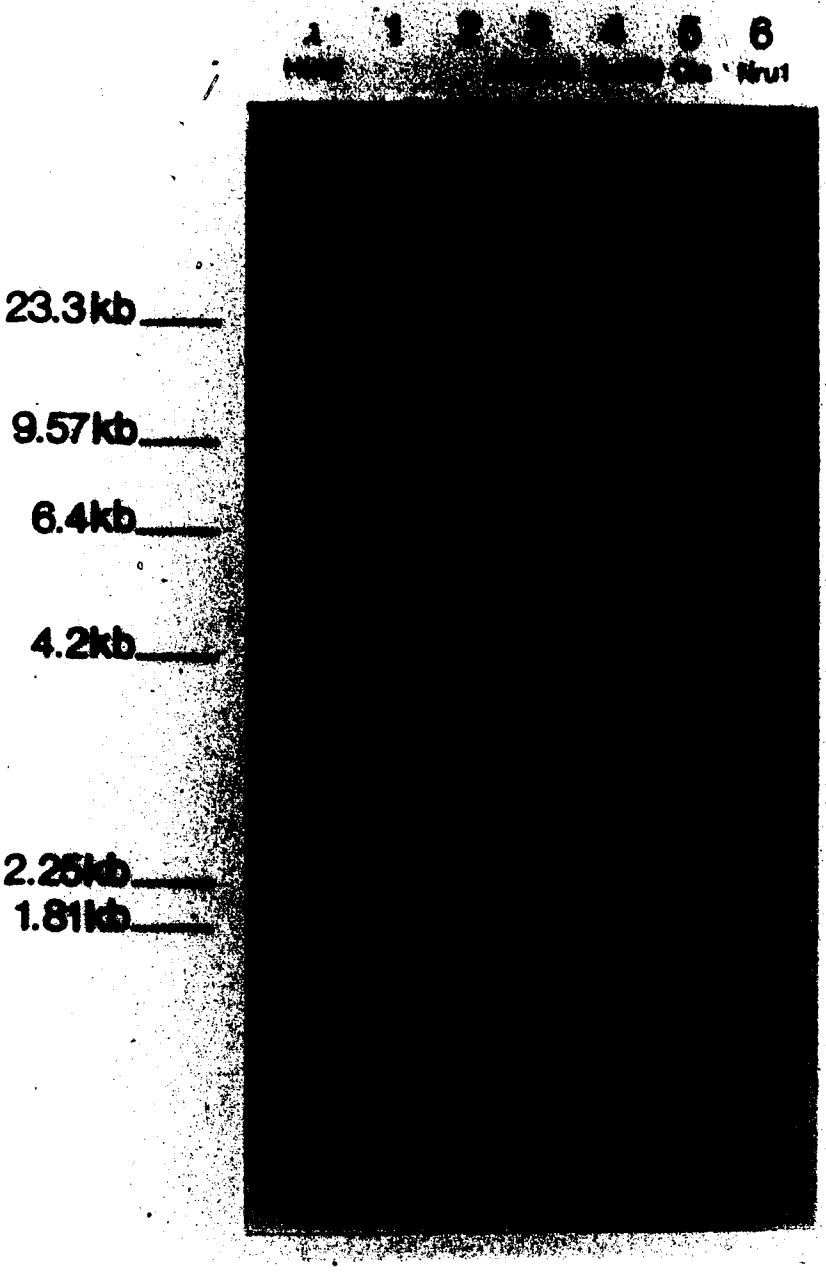

6
23.3kb
9.57kb
6.4kb
4.2kb
2.26kb
1.81kb

Plate 2 3 Southern analysis of agarose gel in Plate 2 2


2
3
4
5
6
BamH1
EcoR1
Cla
Nru1

Because there is only one Eco R1 band (Plate 2.3, lane 2) and one Bam H1 band (Plate 2.3 lane 3) homologous to the carboxylase probe, it suggests that *R. rubrum* has one unique gene for RUBP carboxylase. This has been verified by using the restriction enzymes Cla and Nru 1 which cleave the 6.6 Eco R1 fragment into two equal pieces (in press, Somerville 1983).) If there is only one gene for RUBP carboxylase then *R. rubrum* DNA digested with either of these enzymes should generate two bands complementary to the radioactive probe. Since each band has approximately half the amount of homologous DNA, the radioactive signal from each band would be half the intensity of the signal from the one Eco R1 band. (The size of the two radioactive bands is not predictable because the genomic DNA adjacent to the carboxylase gene is unique and thus the distances to the flanking restriction sites differ.) On the other hand if there is more than one carboxylase gene residing on the 6.6 kb Eco R1 fragment a Cla or Nru1 digest of *R. rubrum* genomic DNA probed with ^{32}P-pRR1 16 would generate not two but many DNA bands. From the Southern autoradiograph (Plate 2.3, lane 5 and 6) it was determined that the radioactive decay is equally distributed between two bands in each of the Cla and Nru1 digest of *R. rubrum* DNA. (The molecular weight of each band is proportional to the distance to the flanking restriction site.) Because the ^{32}P-probe is homologous to one Eco R1 band and two Cla and Nru1 bands of *R. rubrum* DNA it is concluded that there is one gene for RUBP carboxylase.

DISCUSSION

R. rubrum, like other photosynthetic bacteria, has the enzyme RUBP carboxylase. The molecular weight of the enzyme has been estimated from polyacrylamide gels and light scattering as 112,000 daltons (Tabita and McFadden 1974b). According to SDS gel electrophoresis there are two subunits of equal size (Plate 2.1). Based on partial amino acid sequence information the two subunits are identical (Schloss *et al*. 1979; Stringer *et al*. 1981). Unlike the higher plant enzyme no small subunit could be detected on the SDS gel, rather only one band of 56,000 M.W. appeared.

Jensen and Bahr (1977) have suggested that the absence of the small subunit may be an artifact of isolation. However, several lines of evidence argue against this possibility: the sedimentation constant of the purified enzyme is the same as that of the

crude extracts (Anderson and Fuller 1969), and enzymes purified under a number of different conditions also lack the small subunit (Anderson and Fuller 1969, Tabita and McFadden 1974a, Schloss and Hartman 1977). Akazawa *et al*. (1970) concluded that the physical and catalytic properties of RUBP carboxylase were identical when purified from cells grown under three different sets of growth conditions.

Under the photoheterotrophic growth conditions used in this paper, the 112,000 M.W. homodimer was the only molecular form of RUBP carboxylase detectable by gel filtration and ion-exchange chromatography. From gel filtration chromatography neither the 550,000 M.W. nor the 360,000 M.W. carboxylase were detectable in the void volume or the final eluate fractions (Figure 2.2). In addition, only one RUBP carboxylase can be located on the basis of charge. It is known from isoelectric focusing that the charge on the higher plant holoenzyme (Garret 1978) and on the intermediate form (Akazawa *et al*. 1970) are different from that of the *R. rubrum* enzyme (Cliff and Somerville unpublished data). Because the enzymic forms have different charges they would be expected to behave differently on an ion-exchange column. Since only one peak of RUBP carboxylase activity was observed on the DEAE column (Figure 2.1) it suggests that, on the basis of charge as well as molecular weight, *R. rubrum* has only one form of RUBP carboxylase.

Since *R. rubrum* has one molecular form of RUBP carboxylase (Tabita and McFadden 1974a) encoded by a single gene the enzyme from this source is useful as a genetic tool. Other members of the Rhodospirillaceae family such as *R. capsulate* would be unsuitable for *in vivo* genetic modification since this organism has two genes for the catalytic subunit of RUBP carboxylase (Marrs, personal communication to C.R. Somerville). Since *in vivo* mutagenesis will only inactivate one out of the two RUBP carboxylase genes, the alternate form of the enzyme will continue to fix CO_2 and thus the reduction in the rate of CO_2 assimilation may be phenotypically undetectable. In *R. rubrum*, however, it should be possible to completely eliminate CO_2 assimilation by altering the one gene for RUBP carboxylase. A genetically modified RUBP carboxylase would reveal information about enzyme structure and function. An enzyme defective in carboxylation while maintaining its oxygenation capability would allow the identification of active site residues unique to the carboxylation function. Chapter 3 describes a screening procedure, whereby, CO_2

assimilating mutants of *R. rubrum* can be obtained.

Chapter 3

Screening for RUBP Carboxylase Mutants in Rhodospirillum rubrum

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase is recognized as the cardinal enzyme catalysing CO_2 fixation. Besides catalysing the carboxylation of ribulose-1,5-bisphosphate (RUBP) to give two molecules of 3-phosphoglycerate, this enzyme also acts as an oxygenase, adding O_2 to RUBP to give phosphoglycerate and phosphoglycolate. Without exception, the oxygenase activity has been detected in all RUBP carboxylase preparations including those from higher plants with C_3 and C_4 photosynthesis (Kestler *et al*. 1975; Miziorko and Lorimer 1983), green algae (Akazawa and Osmond 1976) and photosynthetic bacteria (Takabe and Akazawa 1973; McFadden 1974; Ryan *et al*. 1974). Extensive analysis of the higher plant enzyme reveals that: (1) O_2 competitively inhibits the carboxylation reaction, and (2) CO_2 competitively inhibits oxygenation (Bowes and Ogren 1972; Badger and Andrews 1974). When the enzyme activity is affected by inhibitors and activators both carboxylase and oxygenase activities are modulated in parallel (Ryan and Tolbert 1975; Paech and Tolbert 1978). Andrews and Lorimer (1978) have proposed that the oxygenation and carboxylation reactions are a consequence of the enzyme's reaction mechanism where the enzyme bound RUBP is susceptible to attack by both O_2 and CO_2. This concept that the bifunctional nature of RUBP carboxylase is an inherent property of the reaction mechanism rather than a property of the enzyme's active site could be disproved by a genetically modified ribulose-1,5-bisphosphate carboxylase/oxygenase lacking either activity.

There have been sporadic reports of RUBP carboxylase mutants in plants, algae, and photosynthetic bacteria. Anderson *et al*. (1970) reported an electrophoretic variant of RUBP carboxylase, which was recovered by random screening of photosynthetically impaired mutants of tomato. Their analysis was preliminary and has not been pursued. Nelson and Surzucki (1967a,b) reported a mutant of *Chlamydomonas reinhardi* which was also impaired in photosynthesis. This was attributed to an alteration in the small subunit since the effect was biparentally inherited. In 1980, Spreitzer and Mets obtained mutants defective in the large subunit (LS) of RUBP carboxylase. The difference in

isoelectric focusing properties between the mutant and wild-type LS is the result of an amino acid substitution (glycine-->aspartate) at residue 171 (Dron *et al*. 1983). Although the mutant has a 10-fold reduction in CO_2 fixation, oxygenase assays were not performed. In 1978, Anderson, King, and Valentine isolated six RUBP carboxylase/oxygenase mutants in the plant-type enzyme from *Alcaligenes eutrophus* but found that the catalytically incompetent enzymes had lost their carboxylase and oxygenase activities to similar extents. Since it is of great interest to understand the bifunctional nature of this enzyme the photosynthetic bacterium *Rhodospirillum rubrum* was chosen for *in vivo* mutagenesis in the hope that a large number of genetically modified RUBP carboxylase/oxygenases could be obtained.

R. rubrum was chosen as the experimental organism for the following reasons:

1. It fixes CO_2 through the Calvin cycle (Anderson and Fuller 1967b) and possesses an RUBP carboxylase which has been extensively characterized (Tabita and McFadden 1974a,b; Schloss *et al*. 1979).
2. The one form of RUBP carboxylase present in *R. rubrum* has an oxygenase activity (McFadden 1974).
3. Because one gene encodes the enzyme (Chapter 2) elimination of enzyme activity is possible as well as phenotypically detectable.
4. Due to the simplicity of prokaryotic gene expression there are no problems with incorrect processing of mRNA.
5. The carboxylase gene from *R. rubrum* has been cloned (Somerville in press), and antibody has been raised against the enzyme so defective carboxylases can be examined at the DNA and immunological levels.

The strategy for screening for CO_2 fixation mutants is based on the assumption that during the screening process the bacterial cells are expressing RUBP carboxylase. The amount of carbon dioxide assimilated after 1 1/2 hr of $^{14}CO_2$ feeding was quantified by the intensity of the radioactive signal eminating from the bacterial colony. Bacterial colonies failing to fix $^{14}CO_2$ may be defective in either the carboxylation or regeneration of RUBP, the substrate for CO_2 fixation. CO_2 fixing variants would then be assayed *in vitro* for RUBP carboxylase activity. Bacteria defective in the regeneration of RUBP should have a normal specific activity for RUBP carboxylase whereas those with enzymes that are

genetically altered would be expected to have a reduced enzyme activity.

When 66 CO_2 assimilating variants were assayed for RUBP carboxylase none had a genetically altered enzyme. In all cases the enzyme's activity was close to that of the wild-type enzyme. The failure to obtain an RUBP carboxylase mutant suggests that the Calvin cycle is not the sole route for CO_2 assimilation in photoheterotrophically grown *R. rubrum*. Other CO_2 assimilating enzymes may be actively contributing to cellular metabolism of *R. rubrum* (Chapter 4).

The ten light-sensitive mutants obtained from the screen, were not assayed for RUBP carboxylase because they failed to grow photoheterotrophically. These mutants could not be examined as a source RUBP carboxylase mutations because dark-grown cultures of *R. rubrum* do not express RUBP carboxylase (see Results below).

METHODS

Stocks

R. rubrum strains were maintained on solid malate medium in the dark at 22°C.

Mutagenesis

R. rubrum was cultured photoheterotrophically on malate and CO_2 and treated with N-methyl-N'-nitro-N-nitrosoguanidine (NG) to induce base substitutions at the replicating fork (Cerda-Olmedo *et al*. 1968). 4 ml of mid-logarithmic cells were washed and resuspended in an equal volume of 0.1M sodium citrate (pH=5.5) and incubated for 30 min at 30°C with one tenth the volume of saturated solution of NG. After mutagenesis the culture was plated on solid malate medium at a density of 100-300 colonies per plate. The bacteria were grown in the dark at 25°C. Single colonies were picked and patched and replica-plated for screening.

Calvin Cycle Screen

Mutagenised *R. rubrum* cells patched on malate plates were grown in the dark and transferred to anaerobic light conditions for 4 hr. The plexiglass chamber (Tyler Research Inc.) was continuously flushed with nitrogen at a flow rate of 300 ml/min while being

illuminated from the bottom with a panel of 100 W tungsten bulbs (3500 lux). The sealed chamber was then filled with $^{14}CO_2$ by injecting 50 ul $NaH^{14}CO_3$ into a solution of 3% phosphoric acid. The nitrogen and $^{14}CO_2$ in the chamber were continuously circulated with a Cole-Parmer pump. After allowing the cells to fix $^{14}CO_2$ for 1 1/2 hours, unbound $^{14}CO_2$ was flushed from the chamber into a limestone column. The bacteria were transferred to a solid support by overlaying the petri dish with circular filter paper (Whatman #4). Filter paper discs were then air dried and CO_2 fixation rates of each colony quantified by the intensity of the radioactive decay on X-OMAT film.

Growth of R. rubrum

R. rubrum cells defective in CO_2 fixation were grown in 10 mm X 75 mm glass screwcap tubes filled to the top with Ormerod medium in which 0.6% butyrate replaced malate as the carbon source. The final concentration of $NaHCO_3$ was 10 mM. Cells were illuminated with a panel of 100 Watt tungsten bulbs at a light intensity of 20,000 lux.

Specific Activity of RUBP Carboxylase

One milliliter of mid-logarithmic cells were sonicated for two 15 sec medium frequency bursts with a Fisher dismembrator (4°C). Lysed cells were microfuged for 5 min and RUBP carboxylase assays performed as described previously (Chapter 2) with 72 ul of the supernatant. Protein determinations were done according to Spector (1978) and CO_2 fixation rates were expressed as umoles of CO_2 fixed per minute per milligram of crude soluble protein.

Immunoelectrophoresis

A 2 ml 0.7% agarose gel containing 60 ul of antibody directed against the *R. rubrum* carboxylase was cast in a 0.7% agarose mold. Soluble cytoplasmic protein (8.5 ug) from 4 bacterial lines grown photoheterotrophically on butyrate were electrophoresised for 16 hr at 30 V into the antibody containing gel. The electrode buffer was Biorad barbitol (pH=8.6) made to 1/2 strength (6.8 g/200 ml). After electrophoresis the gel was soaked in 0.15M NaCl to remove the antibody (Laurell 1966) and immunoprecipitan arcs were visualized by staining with Coomassie Blue R250

(Chapter 2)

Preparation of Antibodies

Antibodies against the holoenzyme of ribulose-1,5-bisphosphate from *R. rubrum* were developed in rabbits by C.R. Somerville using the standard procedures of Hames and Rickwood (1981). Prior to the injection series, rabbits were bled to obtain preimmune sera. The immunoglobulins were precipitated with 18% (w/v) ammonium sulfate.

Extraction of Bacterial mRNA

Total cellular RNA was extracted using a procedure similar to that of Miller (1972) in which 10 ml of mid-logarithmic butyrate-grown cells were pelleted and resuspended in an equal volume of SAE (0.02 M sodium acetate, 0.001 M EDTA, and 15% SDS (pH=5.5)). Two volumes of 60°C SAE saturated phenol was added to the solution and the proteins were extracted for 15 min in a 60°C water bath. The phenol and SDS, at this pH and temperature, partition the denatured proteins and DNA from the RNA. After phenol extraction, the phases were separated by spinning in a clinical centrifuge at top speed for 15 min. The aqueous phase was extracted at room temperature and adjusted to 0.1 M KCl. The nucleic acids were precipitated from the solution by adding 2 volumes of precooled 95% ethanol and chilling to -20°C for 8 hr. RNA was pelleted at 18,000 xg for 30 min and resuspended in 0.3 M sodium chloride and 0.03 M sodium citrate.

RNA Gel Electrophoresis

mRNA was ethanol-precipitated and resuspended at 0.5 mg/ml in 50% formamide, 6% formaldehyde, 0.02 M borate (pH=8.3), 10% glycerol, and 0.2 mM EDTA. Samples were heated at 65°C for 2 min to fully denature the RNA (Rave *et al.* 1979) and loaded onto 1.25% agarose gel buffered with BEF (0.02 M borate (pH=8.3), 0.2 mM EDTA, 3% formaldehyde). The gel was electrophoresed for 5 hr at 96 V using BEF as the electrode buffer.

Preparation of the Plasmid Probe

Plasmid pRR116 was extracted from HB101 (hsdR hsdM recA13 supE44 lacZ44 leuB6 proA2 thi-1 rpsL) and nick translated according to the procedure described previously (Chapter 2).

Northern Transfer and Hybridization

Size fractionated RNA was blotted from the agarose formaldehyde gel to a nitrocellulose sheet in the same manner as DNA (Chapter 2), except the RNA required no alkali pretreatment or salt equilibration before transfer to the solid support with 10X SCC (1.5 M NaCl and 0.15 M Nacitrate). The blots were baked *in vacuo* for 2 hr at 80°C and prehybridized at 42°C for 12 hr with 5X SCC, 50 mM Na_2HPO_4, (pH=6.5), 2.5 mg SCTD, and 1X Denharts (Chapter 2). Hybridization was performed at 42°C in the prehybridization buffer plus 10^6 cpm/ml of denatured nick translated specific plasmid. After a 24 hr hybridization, the blots were washed 4 times with 0.2% SDS in 2X SCC for 5 min at room temperature, followed by two 15 min washes of 0.1% SDS in 0.2X SCC at 50°C. Damp filters were wrapped in plastic wrap and autoradiographed using intensifying screens.

RESULTS

Mutagenesis

R. rubrum was mutagenised with NG for 30 min at 30°C, introducing base substitutions at the growing fork (Cerda-Olmendo *et al.* 1968). The success of mutagenesis was monitored by the presence of abnormally pigmented colonies. Approximately 5-7 green colonies were observed per 100 red pigmented wild-type colonies.

Screening for Calvin Cycle Mutations

Dark-grown replica-plated mutagenised colonies (1100) were transferred to the light for the induction of RUBP carboxylase (Lascelles 1960) .The bacteria were allowed to photosynthesize for 4 hr before 50 uCi of $^{14}CO_2$ was injected into the plexiglass chamber. After 1 1/2 hr of $^{14}CO_2$ fixation the colonies were transferred to a solid support and

overlayed with X-ray film. The intensity of the radioactive decay on the X-ray film was used as a crude measure of relative Calvin cycle activity. Colonies with reduced CO_2 fixation were assumed to be defective in the carboxylation of RUBP or its regeneration (Plate 3.1, 19-1, 19-2, 19-3, and 10-1). Eighty putative Calvin cycle mutants were streaked for single colonies, rescreened, and assayed for RUBP carboxylase activity.

No Genetically Modified RUBP Carboxylase

RUBP carboxylase assays were performed on sonicated mid-logarithmic cells that had been photoheterotrophically grown on butyrate and CO_2. The quantity of $^{14}CO_2$ fixed in 1 min was expressed as the umoles CO_2 fixed / min / mg of crude soluble protein. The assay was specific for RUBP carboxylase because controls without RUBP gave <0.1% of the RUBP carboxylase activity. Of the 66 RUBP carboxylase assays performed (one determination per variant) on the CO_2 fixing variants, none showed a ten fold reduction in enzyme activity (Table 3.1). Although the enzyme activity showed a great deal of heterogeneity, no bacteria appeared to be genetically modified for RUBP carboxylase.

Light-sensitive Bacteria

Of the CO_2 fixing variants identified in the screen, approximately 10% were unable to grow in the light. These light-sensitive bacteria (LS^-) fell into three categories: (1) those incapable of photometabolizing malate (LSM^-); (2) or butyrate (LSB^-); and (3) those unable to use either substrate (LSM^-B^-). The cells incapable of growing on butyrate (LSB^-) in the light did grow on malate in the light, suggesting that the cause of death is the inability to use butyrate as a source of carbon.

The light-sensitive malate cells (LSM^-), in contrast to the LSB^-, are able to utilize malate as a carbon source because they are maintained on malate in the dark. If the metabolism of malate is the same in the light as in the dark, the cause of death is the result of a light induced phenomenon. Similarly, cells incapable of using either malate or butyrate in the light (LSM,B^-) are undoubtedly blocked in light-regulated metabolism.

Plate 3 1 An autoradiograph showing the $^{14}CO_2$ assimilated by mutagenised *R. rubrum* cells Bacterial colonies 19-1, 19-2, 19-3, and 10-1 have reduced CO_2 fixation

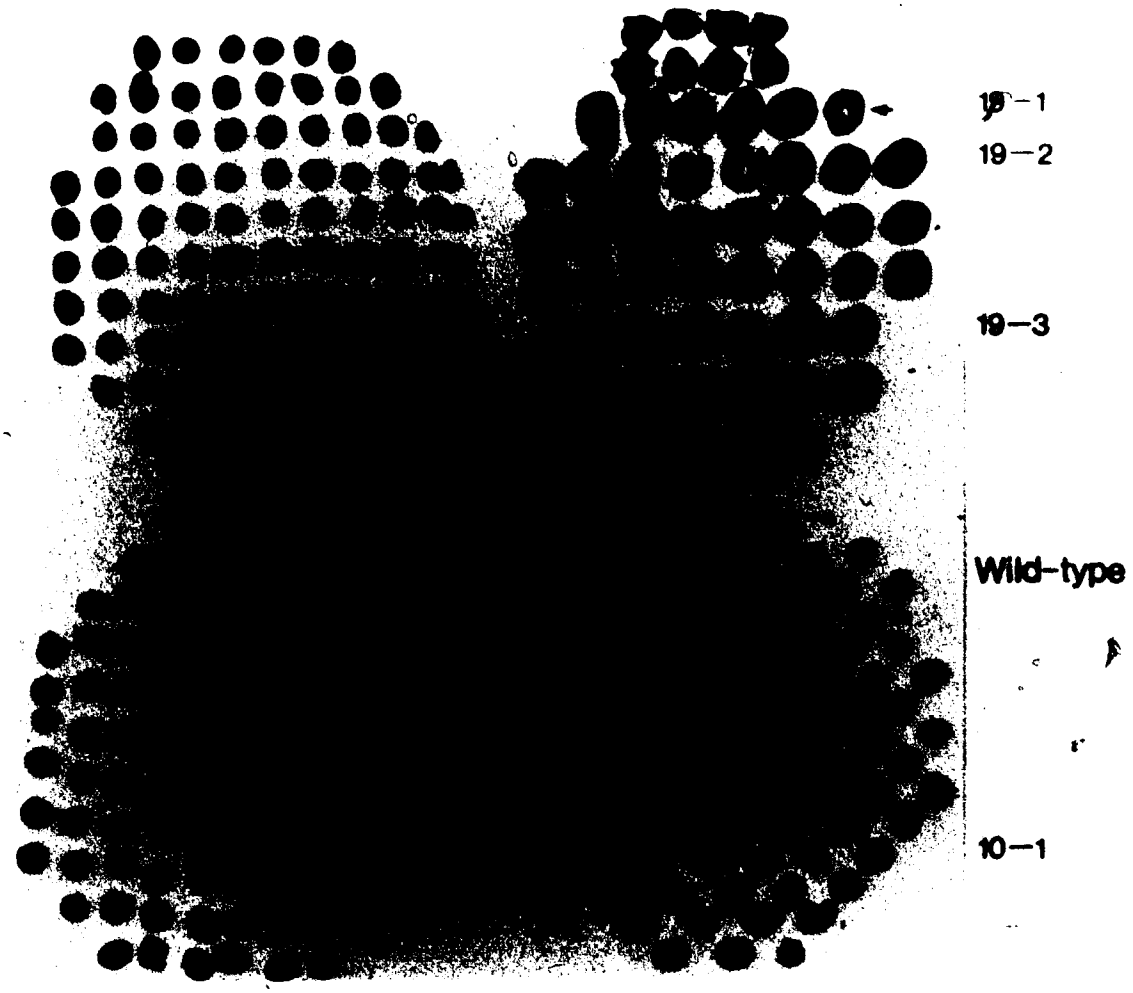

19—1
19—2
19—3
Wild-type
10—1

Table 3.1

Specific Activity of RUBP carboxylase from CO_2 Assimilating Variants of R. rubrum

Str.[1]	S.A.[2]	Str.	S.A.	Str.	S.A.	Str.	S.A.
1-1C	.39	2-6	LSB	10-1	.212	14-5	.225
1-3	.175	3-1	.15	10-3	.292	15-1	.17
1-3F	---[3]	3-4	.258	10-4	.408	15-3	.23
1-4	.30	4-1	.218	10-5	.253	15-4	.24
1-4F	.109	4-2	.327	10-6	.335	15-7	.387
1-4C	.236	4-3	.460	11-4	.135	17-1	.206
1-5	.15	4-20	.19	11-5	.228	17-2	---
1-5C	.204	5-1	.154	11-6	LSB	17-3	.42
1-6	.335	6-1	.265	11-7	.183	17-5	.259
1-6C	---	6-3	.200	11-12	LSB	17-7	.24
1-6F	.154	6-4	.201	12-1	.249	18-2	.159
1-7	LSM	6-5	.193	12-2	LSM	19-1	.275
1-9	LSB	7-2	.362	12-7	.249	19-2	.191
1-10	.202	7-6	.225	12-9	.460	19-3	.219
1-11	.424	7-7	LSM	12-14	.309	19-4	.287
1-13	LSM	8-1	.187	13-1	----	19-5	.26
1-14	.284	9-3	LSB	13-5	.268	22-1	.196
2-1	.15	9-9	.365	13-9	.312	22-2	.196
2-3	.25	9-10	.46	14-1	.17	24-2	.36
2-4	LSM,B	9-11	.212	14-3	.13	24-3	.35

[1]strain

[2]specific activity of RUBP carboxylase (umoles CO_2 fixed / min / mg of soluble protein)

[3]specific activity not determined

specific activity of wild-type = 0.25±0.05 umoles CO_2 / min / mg of soluble protein

LSM---will not grow in the light using malate as the carbon source

LSB---will not grow in the light using butyrate as the carbon source

LSM,B---will not grow in the light using malate or butyrate as the carbon source

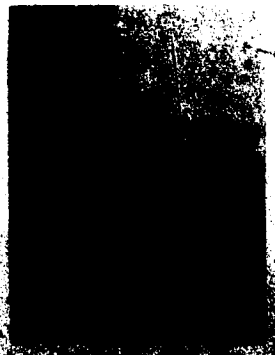
Varying Amounts of RUBP carboxylase

The quantity of RUBP carboxylase in 8.5 ug of cytoplasmic protein was examined in four bacterial lines. 1-10, 11-7, 3-1, and wild-type. Each strain of *R. rubrum* when harvested at the same optical density (A_{650}=4.5) expressed a different amount of RUBP carboxylase as represented by the varying heights of the immunoprecipitan arcs (Plate 3.2).

RUBP Carboxylase is Not Expressed in the Dark

R. rubrum RNA has been fractionated on the basis of molecular weight in a denaturing formaldehyde-agarose gel. After transferring the RNA to a solid support and probing with the cloned RUBP carboxylase gene (^{32}P-pRR116) a small molecular weight mRNA from the light grown cultures hybridized with the carboxylase probe (L_1, L_2, and L_3 in Plate 3.3). In lane D_1, however, there is no RNA species co-migrating with the messenger-RNA of RUBP carboxylase (1-2kb). This finding suggests that the RUBP carboxylase gene is either not transcribed in the dark or its transcript is present in undetectably low quantities. Thus, in 1 ug of messenger-RNA extracted from dark-grown cells, an RUBP carboxylase mRNA is not present. The large RNA species (~4 kb) present in Plate 3.3, lane D_1 is believed to be a transcript homologous to another gene carried on the 6.6 Eco R1 *R. rubrum* probe. Since RNA splicing has not been reported in prokaryotic organisms, it is considered unlikely that this 4kb mRNA represents a precursor to the RUBP carboxylase transcript. To rigorously demonstrate that this 4 kb polycistronic mRNA has no homology with the RUBP carboxylase gene a smaller probe internal to the gene would have to be used in the Northern hybridization. (This 4kb transcript, unique to RNA from dark-grown cultures of *R. rubrum*, is thought to encode protein(s) for dark respiration.)

The high molecular weight band at the origin was shown to be contaminating DNA in the RNA preparation. Treatment of the sample with DNAse specifically eliminated this high molecular weight band, whereas it remained unchanged in an RNAse digest (data not shown).



[illegible]

[illegible] from bacterial lines [illegible]

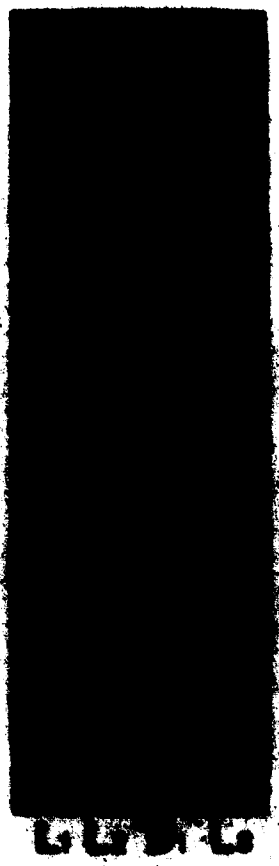


Plate 3.3 *R. rubrum* mRNA was separated on the basis of molecular weight and probed with ^{32}P-pRR116. Lanes L_1, L_2 and L_3 have 1 ug of mRNA from three light-grown cultures. Lane D_1 has 1 ug of mRNA from a dark-grown *R. rubrum* culture.

DISCUSSION

R. rubrum is able to grow photoheterotrophically on CO_2 and a variety of organic carbon compounds. In an atmosphere of N_2 and CO_2, *R. rubrum* will grow on malate (Porter and Merrett 1972), lactate (Ormerod 1956), acetate (Glover *et al*. 1952; Hoare 1963), or butyrate (Takabe *et al*. 1979) and assimilate CO_2. In 1967(d), Anderson and Fuller assayed the enzymes of the Calvin cycle from extracts of *R. rubrum* grown on acetate or malate in an atmosphere of CO_2 and hydrogen and reported that in malate photoheterotrophs the Calvin cycle is considerably less active than in autotrophs. However, more recent investigations by Slater and Morris (1973a,b) with turbidostat continuous-flow cultures have shown that the the changing rates of carbon dioxide assimilation are paralleled by changing activities of RUBP carboxylase. In addition the maximum rate of carbon dioxide fixation of photoheterotrophs was found to approach that shown by autotrophically grown bacteria.

Since the Calvin cycle is expressed during photoheterotrophic growth, the strategy for obtaining Calvin cycle mutants (RUBPcarb$^-$) is to screen bacteria for the inability to assimilate CO_2. Bacteria defective in the carboxylation or regeneration of RUBP will fail to fix $^{14}CO_2$ and be identified in the screen (Plate 3.1). As there is no direct selection for Calvin cycle mutations, this screen will only identify bacteria which failed to assimilate CO_2. This may have occurred for a number of reasons:

1. Bacteria unable to survive in the light (LS$^-$) because of a defect in photophosphorylation (Gimenez-Gallego and Del Valle-Tascon 1978) or electron transport (Del Valle-Tascon and Malkin 1981) would be unable to assimilate CO_2.
2. Phototsynthetic pigments altered in quality or type (pig$^-$) would effect $^{14}CO_2$ incorporation (Schick and Drews 1969; Rivas *et al*. 1970).
3. Slow growers, resulting from transport or metabolic deficiencies (aux$^-$), would have a lower cell density and fix $^{14}CO_2$ at a reduced rate (Beauchemin-Newhouse and Cedergren 1978).

Bacteria from the first two categories were eliminated from the analysis procedure because it was felt that the secondary mutations would indirectly affect CO_2 fixation. The light-sensitive bacteria (LS$^-$) (Table 3.1) were detected when they failed to grow phototrophically and the pink, blue, green, and white bacteria (pig$^-$) were easily

spotted against the red pigmented wild-type. The auxotrophic bacteria (aux) on the other hand, could not be eliminated from the screen because the rich medium would supplement their genetic defect. If bacterial growth was retarded (Appendix 1) a lower cell density would result in less reduced $^{14}CO_2$ (Takabe *et al*. 1979). These aux bacteria would have altered CO_2 fixation and thus be included amongst those bacteria assayed *in vitro* for RUBP carboxylase activity.

Of the 66 RUBP carboxylase assays performed on mid-logarithmic, normally pigmented bacteria none had a drastically reduced enzyme activity (Table 3.1). A lowered specific activity may be the result of a differential expression of the gene or a "leaky" mutation in the RUBP carboxylase gene. In order to distinguish between these two possibilities, the umoles of CO_2 assimilated / min would have had to be expressed as a proportion of the cellular RUBP carboxylase rather than crude soluble protein. A genetically modified enzyme with a slightly reduced specific activity would be unidentifiable against the background variation.

The variability in enzyme activity of the CO_2 fixing variants can be explained by the fact that the CO_2 fixing variants have different dry weights when harvested at the same optical density (Appendix 2). CO_2 assimilating variants with low RUBP carboxylase specific activities had high dry weights when compared to wild-type, whereas variants 1-1C and 17-3, with high specific activities, had low dry weights. Because the CO_2 assimilating variants have different dry weights the amount of soluble protein would be affected and thus specific activity of RUBP carboxylase would fluctuate. The variation in the specific activity of RUBP carboxylase can therefore be explained by the fact the CO_2 assimilating variants have different quantities of soluble protein when harvested at the same optical density.

The expression of RUBP carboxylase is also altered in the CO_2 fixing variants. In Plate 3.2 cytoplasmic protein (8.5 ug) from three CO_2 fixing variants (harvested at the same optical density) has been quantified for RUBP carboxylase by immunoelectrophoresis. Of the variants examined (1-10, 11-7, 3-1) the heights of the immunoprecipitan arcs were directly proportional to the specific activities of RUBP carboxylase suggesting that the variation in enzyme activity is the result of differential gene expression. Although this observation may be explained by a missense mutation

affecting both the cross reactivity and specific activity it can more easily be explained by an alteration in the expression of the RUBP carboxylase gene. For each bacterial variant tested the reduced specific activity of RUBP carboxylase is correlated with reduced enzyme levels.

The failure to obtain a mutation in the RUBP carboxylase gene can be explained in two ways. First, the Calvin cycle may make a smaller contribution to the metabolism of *R. rubrum* under photoheterotrophic conditions than it does in photoautotrophically grown cells (Chapter 4). Second, bacteria having mutations in the Calvin cycle may be unable to grow in the light (Spreitzer and Mets 1980) so that the RUBPcarb$^-$ mutants would be found amongst the light-sensitive bacteria. Spreitzer and Mets reported that the RUBPcarb$^-$ mutant of *C. reinhardi* is a stringent acetate requirer that must be maintained below 2,000 lux. In the experimental procedure used in this paper only those CO_2 fixing variants that grew at high light intensity (20,000 lux) were assayed for RUBP carboxylase. The failure to detect RUBPcarb$^-$ mutants in *R. rubrum* could therefore be explained by light-sensitivity of RUBPcarb$^-$ mutants.

Since *R. rubrum* does not express RUBP carboxylase in the dark on either malate (Anderson and Fuller 1967d) or butyrate (Plate 3.3) medium it is impossible to grow the light-sensitive bacteria in the dark and analyze them for RUBPcarb$^-$ mutations.

Knowing that malate may limit the expression of the Calvin cycle (Chapter 4) and that Calvin cycle mutants may be sensitive to the light, it should be possible to modify the selection protocol to enhance the recovery of RUBP carboxylase$^-$ cells. Mutagenised *R. rubrum* cells growing well on a carbon source in the dark would be replica-plated to minimal medium. The bacterial colonies unable to grow on minimal medium under autotrophic conditions would require carbon for growth and be identifiable on the dark-grown plate. The carbon-requiring bacteria (Calvin cycle mutants) could then be grown under low light intensity (2,000 lux) on butyrate medium for analysis of Calvin cycle mutations. Since 6 to 8% of the soluble protein on butyrate grown cells (Tabita and McFadden 1974a) is RUBP carboxylase, protein extracts from carbon-requiring bacteria could be screened by isoelectric focusing (IEF) for an altered RUBP carboxylase. IEF of cytoplasmic proteins would reveal two classes of RUBPcarb$^-$ mutants - those lacking detectable RUBP carboxylase protein and those enzymes having an altered charge. The

defective RUBP carboxylases could then be examined at the biochemical and immunological levels.

Chapter 4

Carbon Dioxide Assimilation in Photoheterotrophically Grown *Rhodospirillum rubrum*

INTRODUCTION

Rhodospirillum rubrum is a facultative photoautotroph capable of utilizing organic compounds as well as CO_2 as a source of carbon. (Ormerod *et al.* 1961). Under photoautotrophic conditions, where carbon dioxide is the sole source of carbon, CO_2 is assimilated through RUBP carboxylase and the primary product of photosynthesis after a short-term $^{14}CO_2$ labelling is 3-phosphoglycerate (3-PGA). Also representative of Calvin cycle activity is the negative slope of isotope incorporation into the phosphorylated compounds and a high specific activity of all the Calvin cycle enzymes (Anderson and Fuller 1967d).

When photosynthetic bacteria (Hurlbert and Lacelles 1963; Anderson and Fuller 1967d; Cherniadiev *et al.* 1974) are grown on CO_2 in the presence of organic compounds the specific activity of RUBP carboxylase is reduced. Anderson and Fuller (1967d) have found that when *R. rubrum* is grown anaerobically in the light on CO_2 and malate, the specific activity of the Calvin cycle enzymes are low but significant. The reduction in specific acitivity of RUBP carboxylase, however, is not paralleled by a decrease in the rate of CO_2 fixation as measured by $^{14}CO_2$ incorporation (Anderson and Fuller 1967c; Slater and Morris 1973b). How malate regulates the Calvin cycle enzymes either at the DNA or protein level is not understood. In fact, the contribution of the Calvin cycle to the metabolism of photoheterotrophically grown *R. rubrum* has not been quantitatively assessed.

In order to estimate the flow of carbon in the Calvin cycle short-term $^{14}CO_2$ fixation experiments have been performed. Since CO_2 fixation varies with the cell cycle (Slater and Morris 1973b; see Results below) the short-term labellings were done with early logarithmic cells to maximize the rate of $^{14}CO_2$ incorporation. The radioactive amino acids in the photosynthate were removed from the organic acids and phosphorylated compounds by ion-exchange chromatography and identified by automatic amino acid analysis. The organic acids and phosphorylated compounds were separated and identified

by thin-layer, paper, and high performance liquid chromatography. When the percent incorporation of isotope into various metabolic intermediates was examined with respect to time the conclusion was reached that other CO_2 assimilating enzymes besides RUBP carboxylase must be actively contributing to the fixation of carbon dioxide. When malate is added to the growth medium it alters the flow of carbon in the metabolism of *R. rubrum*.

METHODS

Growth Curve

Three cultures of *R. rubrum* were grown, each in a 150 ml square pyrex bottle containing 100 ml of malate medium (Ormerod *et al*. 1961) with the concentration of malate being 0, 2, and 6 grams per liter. Cultures were grown under low light intensity (4000 lux) at 30°C. Beginning 12 hr after the initial inoculation (1 ml / culture), the cultures were sampled using sterile technique (300 ul) every two hours and the cell density measured at 650 nm. (Appendix 3 indicates that bacteriochlorophyll does not absorb at this wavelength (650 nm).)

CO_2 Fixation Rates During the Cell Cycle

R. rubrum was grown at low light intensity in pyrex tubes (Chapter 3) filled to the top with malate medium (6 g malate / liter). Cell cultures at various optical densities were harvested (12,000 xg for 6 min) and resuspended in $NaHCO_3$ free media. Cells (0.3 ml) were then transferred to a serum-stoppered minivial (Kimble 7 ml scintillation vial) and gassed for 5 min with N_2. The anaerobic cultures were preincubated for 30 min on an LKB polylite of 12,000 lux and the CO_2 fixation rates were determined by injecting 5 uCi of $NaH^{14}CO_3$ (1 uCi / ul) into the vial and allowing the bacteria to assimilate $^{14}CO_2$ for 15 min. CO_2 incorporation was terminated by injecting 0.5 ml of 4 N formic acid in 50% ethanol. The lysates were dried under nitrogen, resuspended in 350 ul of 100 mM HCl and counted for ^{14}C-carbon. CO_2 incorporation was expressed as a proportion of the cellular dry weight which was determined by weighing 0.3 ml of cells which had been vacuum dried in a preweighed minivial.

Short-term Labelling

R. rubrum was cultured photoheterotrophically on malate and CO_2 as previously described in Chapter 3. At early log-phase (OD=2.98) cells were harvested, washed, and resuspended in an equal volume of $NaHCO_3$ free medium. Bacterial cells (0.3 ml) were then transferred to a serum-stoppered glass minivial (Kimble 7 ml scintillation vial) and gassed with N_2 for 5 min. After a 30 min preincubation period on an LKB polylite (12,000 lux) at 28°C, 75 uCi of $NaH^{14}CO_3$ (1 uCi/ul) was injected into the vial.

Experiments were terminated by the injection of 0.5 ml 4 N formic acid in 50% ethanol which simultaneously drives off unbound CO_2 and lyses the bacterial cells. The extracts were dried with nitrogen, resuspended in 900 ul of H_2O, and a 10 ul sample counted for ^{14}C-carbon. Cellular debris (insoluble fraction) was pelleted in a microfuge for 5 min and the soluble supernatant was counted and subjected to Dowex-1 and -50 ion-exchange chromatography.

Ion-exchange Chromatography

Analysis of the $^{14}CO_2$ photosynthate was achieved by ion-exchange chromatography. The soluble fraction aliquots were applied to a Dowex-50 (H^+) column (0.5 X 3.5 cm) and washed with 2.5 ml of H_2O. The basic compounds (predominantly amino acids) remaining bound to the column were eluted with 2 N NH_4OH (8 ml).

The Dowex-50 (H^+) wash containing neutral and acidic compounds was applied to a Dowex-1 (formate) column (0.5 X 2.5 cm). The column was washed with 2.5 ml of H_2O and the eluate labelled as the neutral fraction (7 ml). The Dowex-1 column was washed with 8 ml of 1.6 N formic acid, then 8 ml of 8 N formic acid, followed by 6 ml of 6 N HCl. The first acid-1 fraction (A1) contains primarily organic acids, the second acid-2 fraction (A2) consists of monophosphorylated compounds, and the third acid-3 fraction (A3) is largely diphosphorylated compounds. (The quantity of ^{14}C-radioactivity was determined by counting a 100 ul aliquot from the amino acid, neutral, and acid fractions.) At each time point (10,20 and 45 sec), three separate labellings were preformed and the distribution of radioactivity in the AA, N, A1 and A2-3 fractions averaged. In all cases the recovery of radioactivity exceeded 90%.

High Performance Liquid Chromatography

^{14}C-organic acids (A1, A2 and A3) were eluted from a Dowex-1 column with 6 N HCl. They were lyophilized at 60°C, resuspended in 20 ul of deionized millipore filtered H_2O, and analysed by high performance liquid chromatography (HPLC) (Appendix 4). The amount of radioactivity in each peak was expressed as a proportion of the total radioactivity in the soluble fraction. (The distribution of radioactivity at each time point resulted from one run on the HPLC column.)

Amino Acid Analysis

The radioactive amino acids eluting from the Dowex-50 (H^+) column were dried in a Labconco Lyophilizer (-60°C), resuspended in Nacitrate buffer (pH=2.2), and identified on a Beckman (Model 121) automatic amino acid analyzer (Appendix 5).

TLC of Organic Acids

A1 fractions from ion-exchange chromatography were identified by thin-layer chromatography (TLC). Samples were dried under vacuum, resuspended in 10 ul of H_2O and spotted on cellulose MN300 plates (Analtech 250 microns). The plates were equilibrated in chromatography tanks for 30 min before separation of the organic acids using diethyl ether:acetic acid:H_2O (13:5:1). The standards were detected with a 0.04% bromphenol blue reagent in 95% ethanol pH=7.0 (Buch 1965).

Paper Chromatography

After a preparative run on the HPLC of the acid photosynthate from a 10 sec labelling, the first peak of sugar phosphates was subjected to paper chromatography. After separation using ethyl acetate:acetic acid:H_2O (3:3:1 v/v), the paper was autoradiographed and the radioactive spots quantified using a scanning densitometer. Unknown radioactive compounds were identified by comparing their migration distance to published Rf values (Mortimer 1952). Some unknown radioactive phosphates remained at the origin, but radioactive 3-phosphoglyceric acid co-migrated with the standard.

RESULTS

A Diauxic Growth Curve for Heterotrophically Grown Cells

R. rubrum cells exhibited diauxie when grown photoheterotrophically on malate. In Figure 4.1 it can be seen that a biphasic growth curve occurred only when malic acid was used as the carbon source. At the two concentrations of malic acid tested, the initial exponential growth (phase I) was followed by the longer doubling time of phase II (as indicated by the reduced slope of the curve).

The final cell density at stationary phase was proportional to the availability of the carbon source (malate); bacterial cells growing on 6 grams of malate / liter had a greater cell density than cultures containing 0 or 2 g of malate / liter.

CO_2 Fixation Peaks with Early Log-Phase Cells

In photoheterotrophically grown cells the highest rate of $^{14}CO_2$ fixation occurred with early log-phase cells (Figure 4.2). At an A_{650} between 1.7 and 3.6 the maximum rate of CO_2 fixation occurred. As the cells reached mid-log phase there was a sharp decline in the CO_2 fixation rate and at stationary phase the rate of CO_2 fixation was only 45.9% that of early log-phase cells. The drop in the rate of CO_2 assimilation is not the result of $^{14}CO_2$ depletion because carbon dioxide fixation is in equilibrium (Appendix 6).

Partial Contribution of the Calvin Cycle to the Metabolism of Malate-grown R. rubrum

The radioactive photosynthate from $^{14}CO_2$ labelling of *R. rubrum* for various time intervals was separated by ion-exchange chromatography into four categories: amino acid (AA), neutral (N), acid-1 (A1), and acid 2-3 (A2-3). When the percent incorporation of isotope into the phosphorylated compounds (A2-3) was examined with respect to time, a greater percentage of the assimilated $^{14}CO_2$ was found in the phosphorylated compounds at short labelling intervals (3 sec) than at longer ones (45 sec) (Figure 4.3). At 3 sec of $^{14}CO_2$ labelling, 74.6% of the assimilated $^{14}CO_2$ appeared in the phosphorylated compounds (Table 4.1), whereas at 45 sec only 28.5% of the radioactivity was present in this fraction. As the $^{14}CO_2$ fixation period increases, intermediates of the Calvin cycle are withdrawn from the cycle for biosynthetic purposes, resulting in a decrease in the percentage of

Figure 4.1 *R. rubrum* cells growing on three different concentrations of malate were monitored for cell density by withdrawing a 300 ul sample (using sterile technique) and reading the absorbance at 650 nm. (Each point on the curve represents the average of three A_{650} readings.)

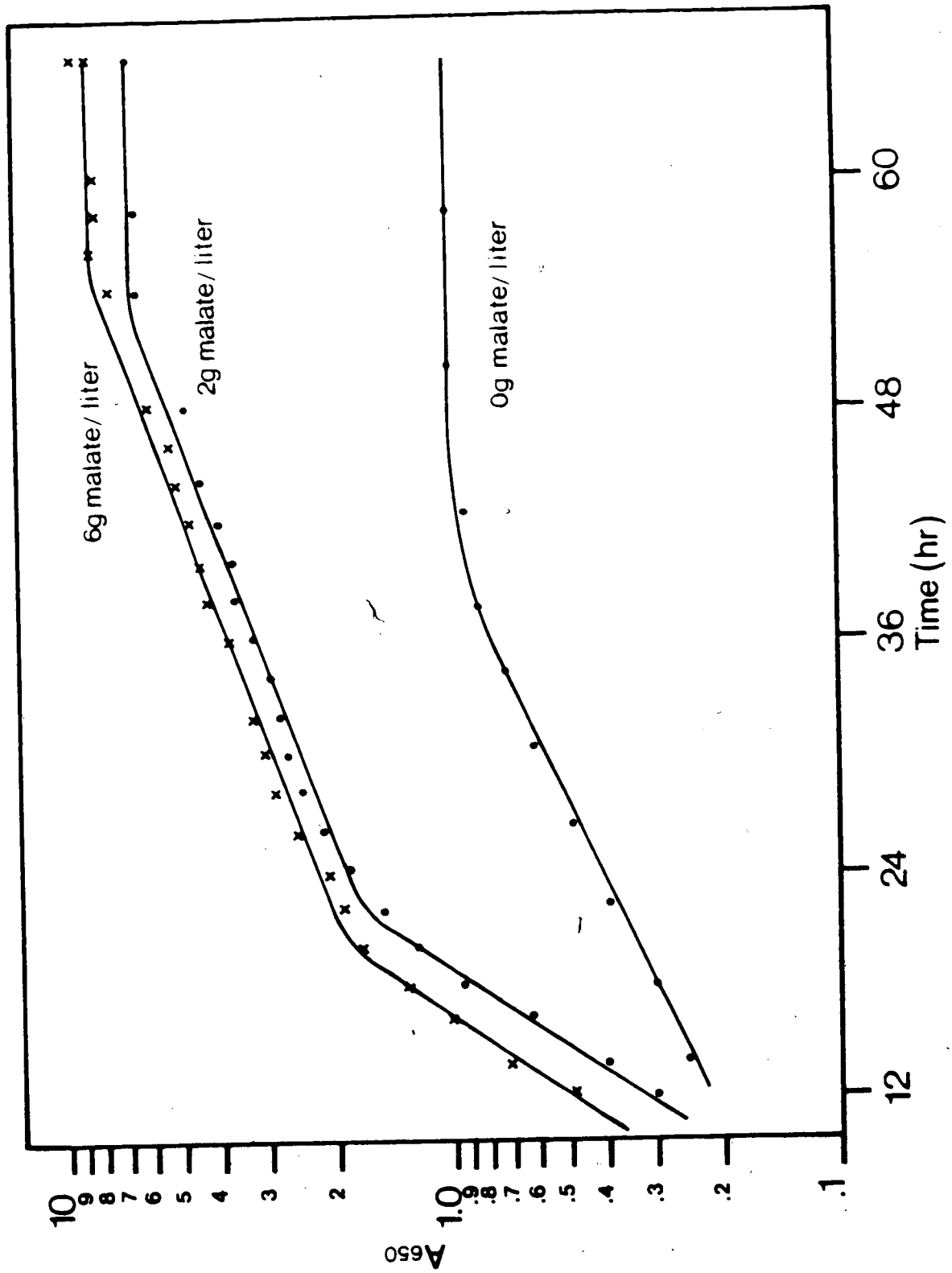

6g malate/ liter
2g malate/ liter
0g malate/ liter
A650
10
9
8
7
6
5
4
3
2
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
12
24
36
48
60
Time (hr)

Figure 4.2 Bacterial cells at different stages in the cell cycle have been quantified for the amount of carbon dioxide ($^{14}CO_2$) they assimilated in 15 min. The amounts of radioactivity incorporated into the soluble and insoluble fractions (total incorporation) were plotted against the culture's optical density (A_{650}).

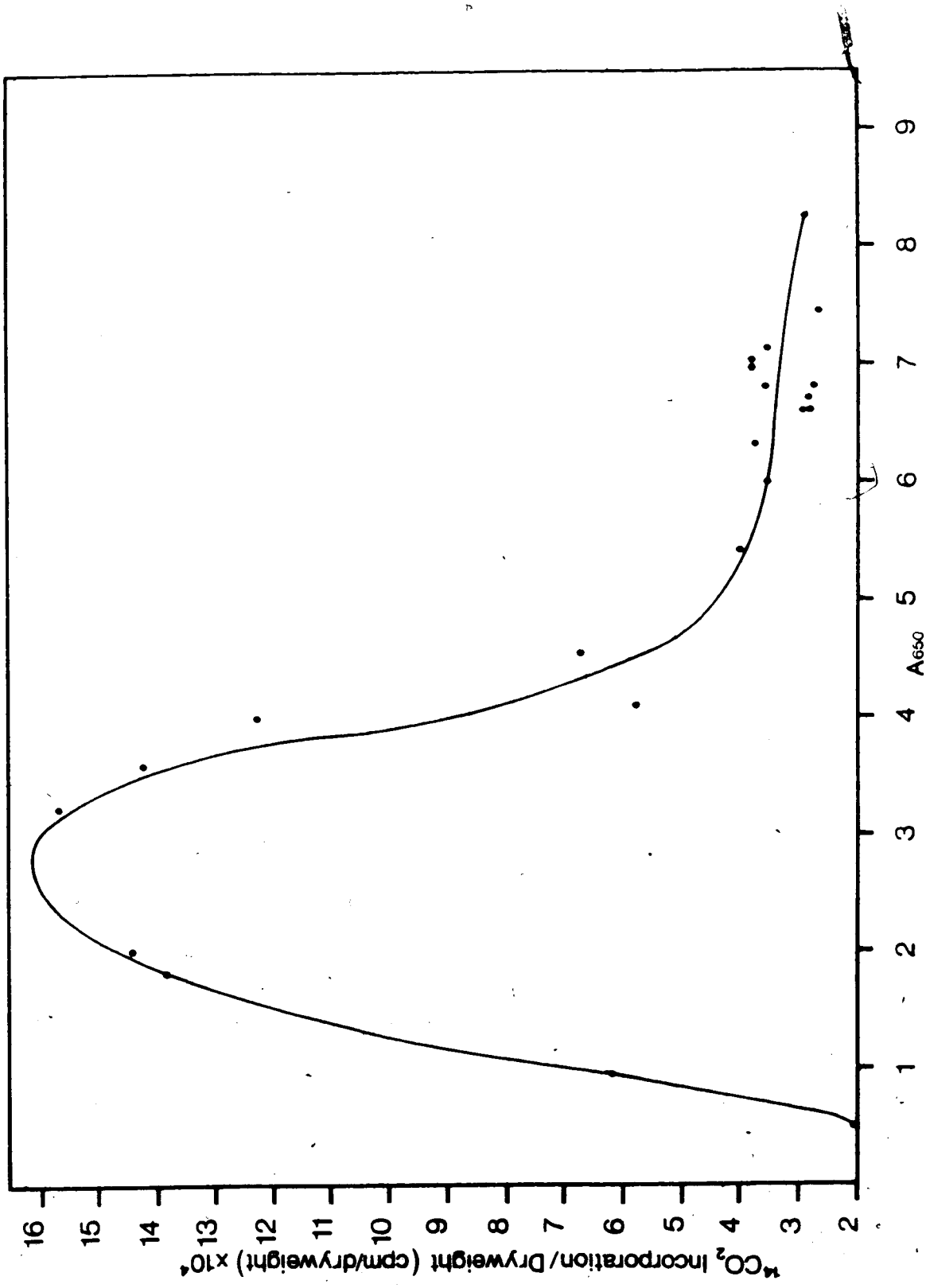

$^{14}CO_2$ Incorporation/ Dryweight (cpm/dryweight) x10^4
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
2
3
4
5
6
7
8
9
A_{650}

Figure 4.3 Photosynthates at 10 [redacted] and 45 sec of $^{14}CO_2$ fixation have been subjected to ion-exchange chromatography (see Methods) and the percent of radioactivity incorporated into each fraction (AA, N, A1, A2-3) have been plotted against time.

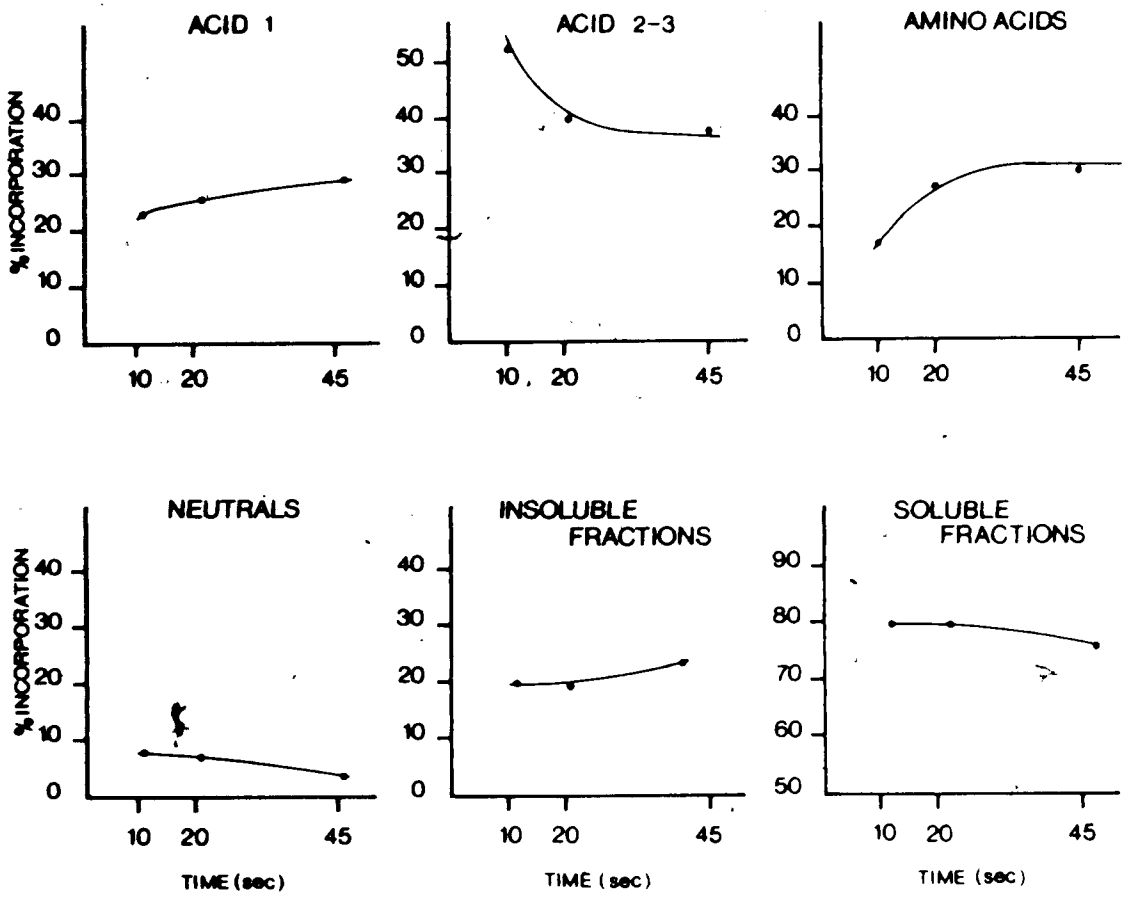

ACID 1
ACID 2-3
AMINO ACIDS
NEUTRALS
INSOLUBLE FRACTIONS
SOLUBLE FRACTIONS
% INCORPORATION
TIME (sec)
10
20
45

Table 4.1

HPLC Separation of Radioactive Photosynthates

(% Incorporation)

sec[1]	AA	N	A2-3[2]	A1[3]				
				Isocit/α-Kg	Mal/Pyr	Glyco/Succ	Acetate	Fumarate
3	13.2	3.0	74.6	6.1	4.3	—	—	—
10	17.4	7.9	51.8	4.1	13.4	1.9	1.7	2.1
15	22.4	7.8	40.0	6.6	18.2	3.1	3.0	3.0

[1]The short-term $^{14}CO_2$ labelling interval (in sec).

[2]Radioactivity in the A2-3 fraction (10 sec) (elution time=6-7.5 min) was identified by paper chromatography (see Results).

[3]Short-term photosynthate was subjected to ion-exchange chromatography to remove the neutral (N) and basic (AA) fractions. The acid fraction (A1, A2, and A3) was fractionated by HPLC and radioactive organic acids identified according to their elution time (Appendix 4).

radioactivity remaining in the A2-3 fractions. The strongly negative curve of these mono and diphosphorylated compounds indicates a functional Calvin cycle. The phosphorylated compounds at 10 sec have been identified by paper chromatography as: 3-phosphoglycerate (12.7%), 2-phosphosphoglycerate(11.5%), fructose-1,6-bisphosphate (14.8%); 3-phosphoglyceraldehyde (18.5%); and origin (57.5%). Phosphoenol pyruvate (PEP), not listed in the published table of Rf values (Mortimer 1952), is believed to remain at the origin or have an Rf value similar to 3-phosphoglyceraldehyde.

Carbon Dioxide is Rapidly Being Incorporated into Organic Acids

During short-term $^{14}CO_2$ labelling, the phosphorylated compounds are not the only radioactive products of photosynthesis for at 10 sec, 23.2% of the radioactivity is found in organic acids.

Table 4.1 lists the organic acids that have been identified by high performance liquid chromatography after 3, 10, and 15 seconds of $^{14}CO_2$ labelling. At 3 sec of $^{14}CO_2$ labelling α-ketoglutarate (α-kg) and malate became radioactively labelled (this was confirmed by TLC), whereas fumarate and acetate, which appear as distinct peaks from the HPLC column, did not appear until 10 sec of $^{14}CO_2$ labelling (Table 4.1). Because organic acids such as citrate, isocitrate, and α-ketoglutarate elute from the column under the same radioactive peak (Appendix 4) it is not possible to state whether *all* the TCA cycle intermediates have become radioactively labelled in 10 sec. (From Table 4.1 it is evident that malate and pyruvate, as well as glycolate and succinate, also co-elute). The failure to observe radioactivity in some compounds may be due to their unstable nature. For instance, oxalacetic acid (OAA) is lost during the first drying down procedure before HPLC analysis (data not shown).

Consistent with the labelling of organic acids is the presence of radioactivity in the amino acid fraction. At 10 sec of $^{14}CO_2$ labelling radioactivity is predominantly distributed between the serine (62.2%) and pyruvate (22.5%) families with small amounts of radioactivity in aspartate (1.6%), glutamic acid (1.5%), and proline (1.3%) (Appendix 5).

DISCUSSION

The Calvin cycle is implicated in the pattern of CO_2 fixation because there is a negative slope of $^{14}CO_2$ incorporation into the phosphorylated compounds when percent incorporation of isotope is plotted against time (Figure 4.3). Between 3 and 45 seconds of $^{14}CO_2$ labelling, the percentage of radioactivity in the phosphorylated compounds dropped from 74.6% to 28.5%. Although the presence of radioactivity in the Calvin cycle intermediates is not sufficient to conclude the functioning of the Calvin cycle, Anderson and Fuller (1967d) have demonstrated that all Calvin cycle enzymes are present in extracts prepared from malate-grown *R. rubrum*.

At 10 sec of $^{14}CO_2$ labelling the phosphorylated compounds have been identified by paper chromatography, and 3-PGA represents 6.6% of the total assimilated radioactivity. In photoautotrophic cells, however, 3-PGA represents 52.0% of of the total assimilated $^{14}CO_2$ (10 sec) (Anderson and Fuller 1967c). Because the rate of 3-PGA formation and the specific activity of RUBP carboxylase are low (Anderson and Fuller 1967d; Takabe *et al.* 1979) it appears that the Calvin cycle is less functional in malate-grown bacteria. Since the drop in RUBP carboxylase activity is not paralleled by a reduced rate of CO_2 fixation, as measured by $^{14}CO_2$ incorporation, (Takabe *et al.* 1979) it appears that other CO_2 assimilating enzymes besides RUBP carboxylase contribute to the metabolism of *R. rubrum*. Photoheterotrophic cells fix 1.5×10^4 dpm/min/mg of dry weight whereas photoautotrophic cells fix 1.8×10^4 dpm/min/mg of dry weight (Anderson and Fuller 1967d).

The difference in CO_2 fixation pathways between photoheterotrophic and photoautotrophic cells is also reflected in the rate of labelling of organic acids. Radioactive α-kg and malate appear within 3 sec of $^{14}CO_2$ labelling with photoheterotrophic cells (Table 4.1), whereas under photoautotrophic labelling conditions they do not appear until 1 and 3 minutes respectively (Anderson and Fuller 1967c). At 10 sec of $^{14}CO_2$ labelling with malate and CO_2 grown cells, 17.4% of the total radioactivity is found in the amino acids. Although most of the radioactivity in the amino acids is derived from 3-PGA, 27% of the radioactive amino acids are synthesized from organic acids. In order for alanine, aspartate, glutamic acid and proline to become labelled in 10 sec (Appendix 5) their precursors pyruvate, OAA, and α-kg must contain radioactivity. Although ^{14}C-3-PGA

could be interconverted to ^{14}C-pyruvate and ^{14}C-OAA (Figure 4.4) it appears that the Calvin cycle is operating at an insufficient rate to explain 23.2% of the total assimilated radioactivity in organic acids after 10 sec of $^{14}CO_2$ labelling.

One explanation for the rapid appearance of ^{14}C-organic acids is the action of pyruvate and α-kg synthase. These two ferredoxin-dependent carboxylases were discovered in 1965 (Evans and Buchanan) and are present in numerous photosynthetic anaerobes including malate-grown *R. rubrum* (Buchanan *et al*. 1967). According to Buchanan these two enzymes would allow the reversal of the TCA cycle (Buchanan *et al*. 1967). For each turn of the cycle, four molecules of CO_2 would be assimilated for the net synthesis of oxalacetate (Figure 4.4). The carboxylation of the reductive carboxylic acid cycle include, apart from the pyruvate and α-ketoglutarate synthase reaction, isocitrate dehydrogenase which catalyses reversibly the carboxylation of α-kg to isocitrate (Figure 4.4, reaction 10) and PEP carboxylase which catalyses the carboxylation of PEP to OAA. (Figure 4.4 reaction 4). A variant of the complete reductive carboxylic acid cycle is the "short" reductive carboxylic acid cycle which, in one turn, incorporates two molecules of CO_2 and yields one molecule of acetate. Although the enzymes for the operation of the reductive TCA are present in *R. rubrum* (Buchanan *et al*. 1967), their activity *in vivo* is not known.

In order to establish that a CO_2 assimilating pathway is functional, it is necessary not only to show enzyme activities but also that the first compound in the pathway has the highest specific activity when the organism is fed with $^{14}CO_2$. The data in Table 4.1 show the percent isotope incorporation into the various TCA cycle intermediates and therefore can be misleading in ascertaining a metabolic pathway. The relative size of the metabolic pools of various compounds can have a more drastic effect on the amount of label in any one compound than can the sequence in which the compounds are labelled. For instance, the high percentage of radioactivity observed in malate (3 sec) may be the result of a large malate pool rather than malate being the first product of CO_2 fixation. Since malate was used as the growth substance, it is likely that there is a large pool of it within the cell serving as a "sink" for radioactivity that must be filled before radioactivity could pass to the next metabolic intermediate. For this reason the amount of radioactivity in the CO_2 fixation products must be expressed as a proportion of their total pool size. The data in

Figure 4 4

1 acetyl-CoA synthase

2 pyruvate synthase

3 phosphoenolpyruvate synthase

4 phosphoenolpyruvate carboxylase

5 malate dehydrogenase

6 fumerate dehydrogenase

7 fumerate reductase

8 succinyl-CoA synthetase

9 α-ketoglutarate synthase

10 isocitrate dehydrogenase

11 aconitase hydratase

12 citrate lyase

13 α-ketoglutarate dehydrogenase

14 succinate dehydrogenase

15 citrate synthase

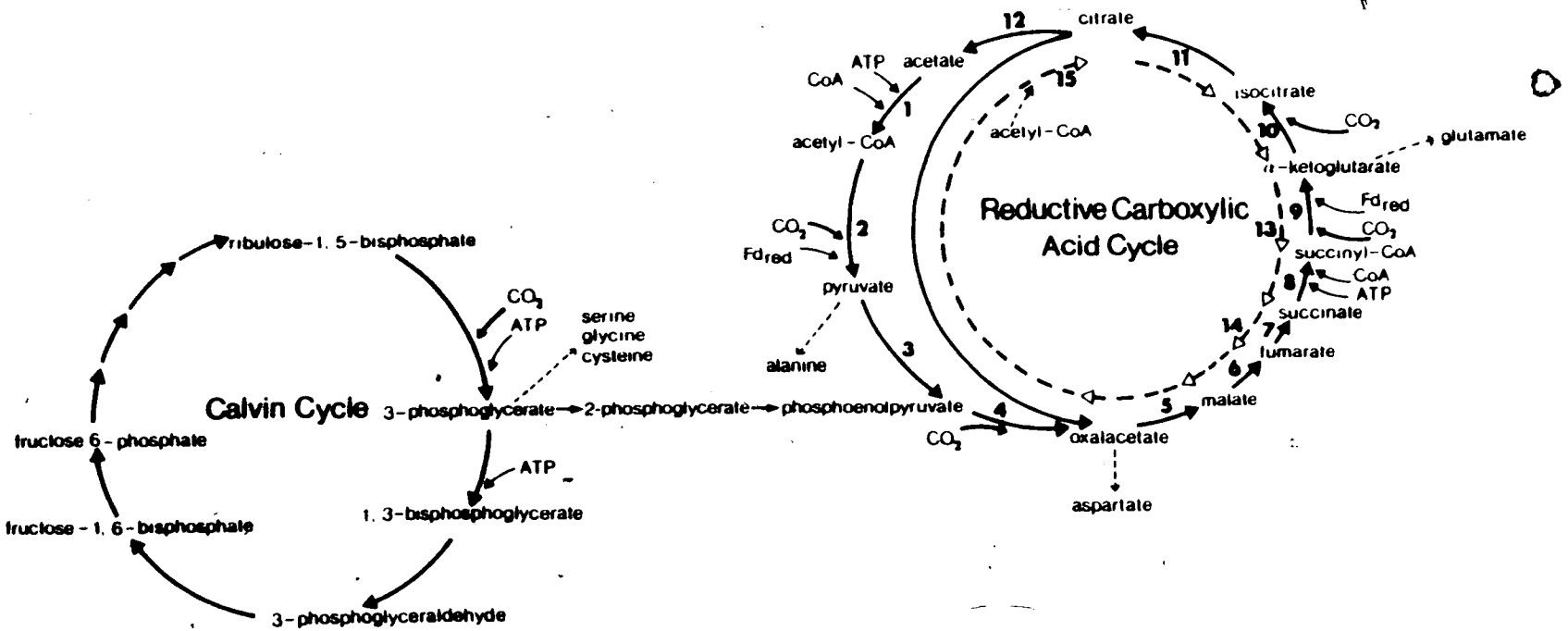

Reductive Carboxylic Acid Cycle
Calvin Cycle
citrate
isocitrate
α-ketoglutarate
succinyl-CoA
succinate
fumarate
malate
oxalacetate
aspartate
acetyl-CoA
pyruvate
alanine
phosphoenolpyruvate
2-phosphoglycerate
3-phosphoglycerate
1, 3-bisphosphoglycerate
3-phosphoglyceraldehyde
fructose - 1, 6 - bisphosphate
fructose 6 - phosphate
ribulose-1, 5-bisphosphate
acetate
glutamate
serine
glycine
cysteine
CoA
ATP
CO_2
Fd_{red}
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Table 4.1 were not expressed in this fashion because many of the TCA cycle organic acids in 0.3 ml of *R. rubrum* were present in undetectable quantities (<<nmoles). The radioactive organic acids were therefore the only compounds identifiable when fractionated on the HPLC column (Appendix 4).

Anderson and Fuller (1967c) have performed a $^{14}CO_2$ fixation time-course with malate grown *R. rubrum*, however, their time-course data do not resemble that illustrated in Figure 4.3. Anderson and Fuller observed a positive slope in the phosphorylated compounds. At 3 sec of $^{14}CO_2$ labelling the phosphorylated compound that was radioactively labelled was not a Calvin cycle intermediate but phosphoenolpyruvate. Radioactive glycolate at 1 sec represented 85% of the total assimilation CO_2 with a strongly negative slope of percent incorporation with respect to time. The plots of percent incorporation into alanine, malate, succinate, and fumarate acids were initially positive but became negative after 5 sec of $^{14}CO_2$ fixation. The difference in CO_2 fixation data may be due to the fact that these investigators used stationary-phase cells, whereas the data in this paper were generated with early log-phase cells in which the rate of CO_2 fixation is maximal. Figure 4.2 illustrates that the age of the bacterial culture plays a critical role in optimizing and reproducing $^{14}CO_2$ fixation data. Differences in the CO_2 fixation data (Sarles and Tabita 1983) could be explained by the fact that the cells used by Anderson and Fuller were grown on a higher concentration of $NaHCO_3$. The appearance of radioactive glycolate was not duplicated in the experiment reported in this paper nor in the unpublished data of Akazawa *et al.* (1978). Although Anderson and Fuller do not explain the synthesis of radioactive glycolate; the failure to maintain anaerobic conditions would allow the oxygenation of RUBP and the generation of radioactive glycolate.

Figure 4.2 illustrates that the rate of CO_2 assimilation varies with the bacterial growth cycle. Although the reason for the decline in CO_2 fixation at mid-log phase is not understood it is correlated with a slower generation time (Figure 4.1, phase II). A slower generation time is often the result of a switch to an alternate carbon source (Loomis and Magasanik 1967). Since the shift from growth phase I to phase II occurs at the same optical density for cells grown on either 2 or 6 g of malate/liter, the slower division time cannot be due to a depletion of malate. Rather, the carbon source is not exhausted until stationary phase when bacterial cells growing on 6 g of malate/liter reach a higher density

than cells growing on 2 g of malate / liter. The primary event that would have triggered the slower generation time and the decline of CO_2 fixation is unknown.

In conclusion, CO_2 fixation varies with the cell cycle when *R. rubrum* is grown photoheterotrophically on malate and CO_2. At an A_{650} between 1.7 and 3.6 the maximum rate of CO_2 fixation occurs. Cells labelled with $^{14}CO_2$ at this stage of the growth cycle assimilate CO_2 via the Calvin cycle but also utilize secondary carboxylases (α-kg and pyruvate carboxylase) to rapidly incorporate CO_2 into the TCA cycle organic acids. Since the specific activity of RUBP carboxylase (Anderson and Fuller 1967d) and the rate of isotope incorporation into 3-PGA under photoheterotrophic conditions is only one tenth that observed under photoautotrophic conditions, it appears that the Calvin cycle is less functional when *R. rubrum* is grown in the light on malate and CO_2.

Chapter 5

Rhodospirillum rubrum Variants Defective in Carbon Dioxide Assimilation

INTRODUCTION

Rhodospirillum rubrum, when grown photoheterotrophically on malate and CO_2, incorporates carbon dioxide into phosphorylated compounds, organic acids, and amino acids after 3 seconds of $^{14}CO_2$ fixation. The rapid incorporation of radioactivity into these compounds suggests that other carboxylases besides RUBP carboxylase contribute to CO_2 fixation in *R. rubrum* (Chapter 4). This concept is supported by the analysis of CO_2 assimilating variants which, when grown on malate, are defective in CO_2 fixation yet have normal levels of RUBP carboxylase. The CO_2 assimilating variants incorporated $^{14}CO_2$ at rates ranging from 13.1% to 58.2% of that of the wild-type control.

In the CO_2 assimilating variant 12-1, the quantity of isotope in the phosphorylated compounds and the specific activity of RUBP carboxylase are equivalent to wild-type yet the CO_2 fixation rate is reduced by 85.4%. Since the reduction in CO_2 fixation, as measured by $^{14}CO_2$ incorporation, cannot be accounted for by the elimination of RUBP carboxylase this variant provides evidence for the role of other CO_2 assimilating pathways besides the Calvin cycle in the metabolism of *R. rubrum*. The residual CO_2 fixation of 14.6% in variant 12-1, may represent the maximum contribution that the Calvin cycle makes to CO_2 fixation in malate grown *R. rubrum*.

METHODS

Growth of R. rubrum

R. rubrum strains defective in CO_2 fixation were grown in 10 mm X 75 mm glass screwcap tubes filled to the top with Ormerod medium (Ormerod *et al.* 1961). The organic carbon sources were malate and casamino acids, present at 6.0 and 4.0 grams per liter respectively. The final concentration of $NaHCO_3$ was 10 mM and the light intensity was 20,000 lux.

Quantifying CO_2 Assimilation

R. rubrum variants in CO_2 fixation were quantified for CO_2 fixation rates using early logarithmic cells resuspended in $NaHCO_3$ free media. The cells were gassed with N_2 (5 min) and preincubated in the light for 30 min at 12,000 lux before injection of 10 uCi of $NaH^{14}CO_3$ (1 uCi / ul) as described in Chapter 4. The fixation reaction was stopped after 15 minutes with 4N formic acid in 50% ethanol. The dried lysate was resuspended in 350 ul of 100 mM HCl and counted for ^{14}C-carbon. The CO_2 fixation rates were expressed as the ^{14}C-cpm incorporated for the variant as proportion of the ^{14}C-cpm incorporated by wild-type *R. rubrum* which had been harvested at the same optical density.

Ion-exchange Chromatography

The photosynthate from a 15 min $^{14}CO_2$ labelling was subjected to ion-exchange chromatography according to the procedure described in Chapter 4. The percent radioactivity in the amino acid, neutral, and acid 1-3 fractions were determined by counting 700 aliquot samples. In all cases the recovery of ^{14}C-carbon from the column exceeded 90%.

TLC of Amino Acids

The radioactive amino acids from a 15 min $^{14}CO_2$ labelling were separated by thin-layer chromatography using MN300 plates (Analtech). The plates were equilibrated for 30 min before butanol:acetone:diethylamine:H_2O (10:10:2:8) was allowed to ascend 15.5 cm up the plate. The solvent was evaporated from the plate before autoradiographing for 48 hr. The radioactive amino acids were identified by co-migrating standards (20 ug) which were detected with a ninhydrin reagent (0.1% ninhydrin in aqueous butyl alcohol).

RESULTS

The CO_2 Fixing Variants Quantitatively Fix Less Carbon Dioxide

Since CO_2 fixation varies with the bacterial growth cycle, *R. rubrum* variants have been quantified for CO_2 assimilation in early log-phase at an optical density (A_{650}) between 2.1-3.9. At this optical density CO_2 fixation rates peak (Chapter 4) so that the incorporation of ^{14}C-carbon into the bacterial cell is a sensitive measure of the cell's total CO_2 assimilating capability. The CO_2 fixation rates for the variants range from 13.1% to 58.2% of that of wild-type *R. rubrum* (Table 5.1). When the specific activity of RUBP carboxylase was assayed in variants 1-3, 1-4F, and 12-1 it was never reduced by more than 50% (Chapter 3), yet the CO_2 fixation rate dropped by 86.9%, 81.1%, and 85.4% respectively. The reduction in RUBP carboxylase activities do not explain the drastic drops in CO_2 fixation.

The Distribution of Radioactivity in the Photosynthate from CO_2 Fixing Variants is Different from that of Wild-type R. rubrum

The radioactive photosynthate from *R. rubrum* variants was separated by ion-exchange chromatography into five fractions: amino acid (AA), neutral (N), and acids (A1, A2, A3). The A1 fraction contains primarily organic acids, the A2 fraction consists of monophosphorylated compounds and the A3 fraction is largely diphosphorylated compounds. The $^{14}CO_2$ labelling was done for 15 min with cells capable of maximum CO_2 fixation rates (A_{650}=1.9-3.1). Since CO_2 fixation is in equilibrium during the 15 min labelling period (Appendix 6), the distribution of radioactivity in the photosynthate reflects the flow of carbon in the cell's metabolism. The distribution of radioactivity in the wild-type photosynthate was: AA=24.0%, N=8.2%, A1=26.2%, A2=19.1% and A3=22.7%. From Table 5.2 it is apparent that the distribution of radioactivity in the variants was found to be significantly different than that of wild-type.

Table 5.1

Quantification of CO_2 Assimilation in R. rubrum Variants

Strain	%[1]	A_{650}
W-T[2]	100.0	3.50
1-3	13.1	2.80
1-3F	17.5	2.14
1-4F	18.9	2.60
1-6C	18.5	2.86
1-6C2	44.8	2.04
6-1	34.8	3.62
7-2	58.2	3.93
11-8	50.8	3.77
12-1	14.6	3.21
13-1	35.0	3.21
17-2	33.5	4.30
19-1	51.0	3.36
19-5	42.0	2.39

[1]incorporation of CO_2 as compared to W-T at the same optical density

[2]wild-type

Table 5.2*

The Distribution of Radioactivity in the Photosynthate From R. rubrum CO_2 Assimilating Variants

Strain	%[1]	A_{650}	AA	N	A1	A2	A3
W-T_2	66.3	3.50	24.0	8.2	26.2	19.1	22.7
1-3	63.7	3.06	23.9	7.6	20.8	21.4	26.3
1-3F	56.1	2.10	17.7	7.8	31.7	24.6	18.1
1-4F	60.0	2.19	7.8	2.8	65.8	10.9	12.6
1-6C	33.4	2.86	78.8	2.4	6.6	6.6	5.5
12-1	66.6	3.20	39.3	5.4	13.7	14.9	26.6
13-1	45.2	2.35	27.2	13.3	14.1	19.8	25.5
17-2	57.6	1.93	20.5	15.2	16.6	20.9	26.8

[1]% incorporation into soluble fraction

[2]wild-type

DISCUSSION

Of the CO_2 assimilating variants listed in Table 5.1, seven were chosen for further analysis because they had the lowest CO_2 fixation rates and were thought to be defective in a CO_2 assimilating pathway. The radioactive photosynthates from a 15 min labelling of these CO_2 assimilating variants have been analysed by ion-exchange chromatography and TLC. Variants 12-1, 1-4F, and 1-3 are discussed first since they were assayed for RUBP carboxylase (Chapter 3).

In variant 12-1, the specific activity of RUBP carboxylase was identical to that of wild-type (0.25 umoles CO_2 fixed/min/mg) indicating that the enzyme had not been genetically modified. Because the proportion of radioactivity in the phosphorylated compounds (A2 + A3) was unaltered (41.5%) when compared to wild-type (41.8%) it appears that the Calvin cycle was operating at wild-type levels in this variant. The 85.4% reduction in CO_2 fixation can therefore only be explained by the elimination of another CO_2 assimilating enzyme or pathway. Upon examination of the radioactive photosynthate, the organic acids (A1) were found to contain less radioactivity while the amino acids (AA) accumulated. Perhaps the enzymic block that results in the reduction in CO_2 fixation prevents the synthesis of the substrate for CO_2 fixation (A1) and causes the diversion of carbon into amino acid biosynthesis. Because the Calvin cycle is operating at wild-type levels it appears that the residual CO_2 fixation of 14.6% represents the maximum contribution that the Calvin cycle makes to the metabolism of *R. rubrum*.

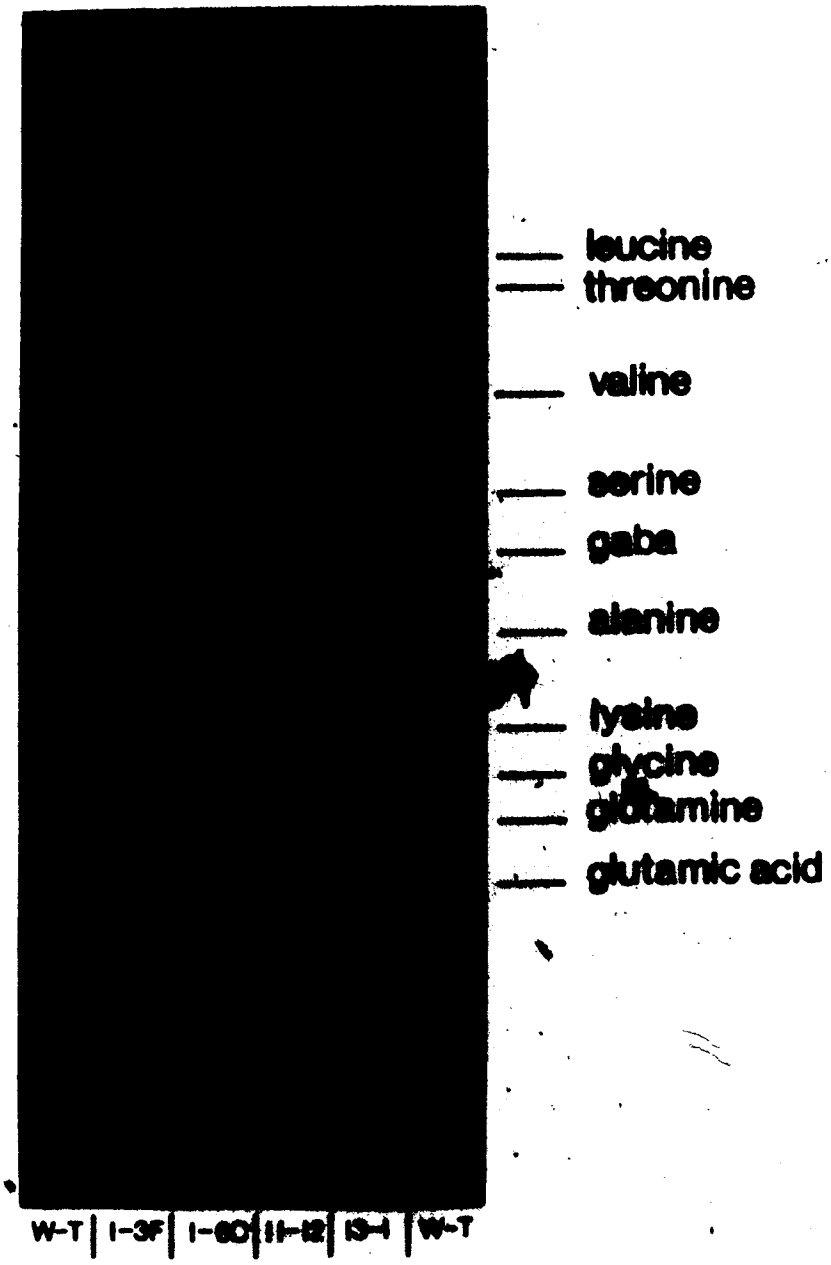
In the variant 1-4F CO_2 fixation was reduced 81.1% while the specific activity of RUBP carboxylase and the radioactivity in the phosphorylated compounds was only reduced by 50%. The reduction in Calvin cycle activity could be the result of a "leaky" mutation within the pathway, or a pleiotrophic effect from another genetic lesion. However, the partial loss of Calvin cycle activity cannot explain the drastic drop in the CO_2 fixation. Since the reduction of CO_2 fixation is accompanied by an accumulation of radioactivity in the A1 fraction (85.8% of the total assimilated $^{14}CO_2$) and a reduction of radioactivity in the AA fraction (7.8% of the total assimilated $^{14}CO_2$) this suggests that the enzymic block is either in amino acid biosynthesis or the synthesis of organic acids that serve as their precursors. Since CO_2 is not directly incorporated into amino acids during their biosynthesis, with the exception of phenylalanine (Allison and Robinson 1967), the

failure to fix CO_2 must result from the cells' inability to synthesize organic acids that serve as amino acid precursors. The increase in the organic acid fraction therefore represents a genetic defect in a biochemical pathway that serves in the synthesis of organic acids.

The 25% drop in specific activity of RUBP carboxylase in variant 1-3 also can not account for the 86.9% reduction in the rate of CO_2 fixation. The distribution of radioactivity in the photosynthate of this variant is the same as that of wild-type (Table 5.2), so further investigation would be needed to understand the metabolic changes that could reduce CO_2 fixation without altering the carbon flow. Because these CO_2 assimilating variants (12-1, 1-4F, and 1-3) were not defective in RUBP carboxylase, the intensity of the radioactive decay emanating from the bacterial colonies in the CO_2 assimilating screen (Chapter 3) was most likely indicative of other carboxylations besides RUBP carboxylase.

The CO_2 assimilating variants 1-6C, 13-1, 17-2, and 1-3F were obtained from the CO_2 screen but were not amongst those assayed for RUBP carboxylase. It is unlikely, however, that they are defective in the Calvin cycle enzyme because the screen (Chapter 3) favored the recovery of mutants in other CO_2 assimilating pathways. In variants 1-6C and 13-1 the proportion of radioactivity in the amino acid fraction increased, with a simultaneous decrease in the organic acid fraction. In variant 1-6C CO_2 assimilation is down 81.5% and the amino acid fraction contains 78.8% of the assimilated $^{14}CO_2$. The majority of the radioactivity in the AA fraction accumulated in leucine and γ-amino-butyric acid (gaba), which is formed from the decarboxylation of glutamic acid (Plate 5.1). Although the amount of radioactivity in the amino acid fraction increased, valine, alanine, lysine, glutamine, and glutamic acid appeared less intensely labelled. The reason leucine accumulates while the other pyruvate family amino acids do not is not understood, but perhaps the shortage of glutamic acid reduces the rate of transamination of oxo-methylbutyrate and pyruvate so that the valine and alanine precursors are quickly consumed in the biosynthesis of leucine. With the radioactivity drawn to the amino acid fraction the organic acids (A1) were reduced by 74.8% with a 65.4% and 75.8% reduction in the A2 and A3 fractions. Not only is the quantity and distribution of radioactivity in the soluble fraction highly altered (Table 5.2), but the doubling time of 1-6C is approximately half that of wild-type (Appendix 1). Although the precise genetic lesion is not known, a

Plate 5.1 The amino acid fraction from the CO_2 assimilating variants 1-3F, 1-6C, 11-12 and 13-1 were separated on thin-layer cellulose plates using the solvent butanol:acetone: diethylamine: H_2O (10:10:2:8). The 100,000 cpm that are present in each lane were detected by autoradiographing each plate for 48 hours. The radioactive amino acids were identified by co-migrating standards which were detected by 0.1% ninhydrin reagent in aqueous butyl alcohol. (Variant 11-12 is light-sensitive and not discussed in the text.)


leucine
threonine
valine
serine
gaba
alanine
lysine
glycine
glutamine
glutamic acid
W-T
I-3F
I-60
II-12
I3-I
W-T

mutation in PEP carboxylase would explain the reduction in CO_2 fixation and the accumulation of the pyruvate family amino acid leucine.

In variant 13-1 there is only an 11.3% increase in the quantity of amino acids that have become labelled after 15 min of $^{14}CO_2$ fixation. Radioactive gaba accumulates with a slight decrease in radioactive glutamine, glutamic acid, and lysine. The proportion of radioactivity in the A2 and A3 fractions is identical to that of wild-type although there is a 46.1% reduction in the organic acids which ultimately results in a 65% reduction in CO_2 fixation. The most striking feature of variants 1-6C and 13-1 is that glycine and gaba are more intensely labelled than the corresponding basic compounds of wild-type. Although the precise genetic lesion in these variants is not known, assimilated CO_2 is clearly being directed from the organic acids to the amino acids. In the case of variant 1-6C, leucine and glutamic acid biosynthetic pathways have an increased flow of carbon through them, whereas in variant 13-1 ^{14}C-carbon is diverted only into the glutamic acid pathway. The increased flow of carbon through pyruvate and/or α-ketoglutarate in variants 1-6C and 13-1 respectively, may reflect enzymic blocks in the TCA cycle so that these amino acids serve as a sink for radioactivity. Mutants of *E. coli* in α-ketoglutarate dehydrogenase (Herbert and Guest 1968; Creaghan and Guest 1972), and fumarate reductase (Spencer and Guest 1973) have incomplete TCA cycles and are unable to grow on intermediates of the TCA cycle when they are used as the *sole* source of carbon. Similarly, *R. rubrum* variants 1-6C and 13-1 could be tested for growth or no growth on succinate, fumarate, acetate, etc. to deduce their possible genetic lesion(s). (The rich medium used in the experiments described in this paper would allow the survival of TCA cycle mutants.)

In contrast to variants 1-6C and 13-1, which accumulate radioactive amino acids, the CO_2 assimilating variants 1-3F and 17-2 have 26.3% and 14.6% reductions respectively in their amino acid fractions. In variant 1-3F the following basic compounds are reduced in quantity: lysine and threonine (aspartate family); valine and serine (pyruvate family); and glutamic acid, glutamine, and gaba (α-kg family). Alanine is the only radioactive amino acid that appears in the mutant at the same intensity as in the wild-type. Although the nature of the genetic lesion that would cause a decline in CO_2 fixation is not known, this variant is dividing twice as slowly as wild-type (Appendix 1). In variant 17-2 the reduction in the amino acid fraction specifically affects alanine, glutamine, and gaba (data not shown).

Although glutamine and gaba contain less radioactivity than wild-type, glutamic acid increases in radioactive intensity. The failure to aminate both pyruvate and glutamic acid may reflect the cells inability to assimilate ammonia. Variants 1-3F and 17-2 clearly have drastically altered metabolisms with 82.5% and 66.5% reductions in CO_2 fixation.

In conclusion then, the presence of CO_2 assimilating variants with wild-type levels of RUBP carboxylase and normal levels of phosphorylated compounds argues for the presence of other CO_2 assimilating pathways in the photometabolism of malate. Although the precise enzymic defects have not been elucidated in these CO_2 assimilating variants, it has been demonstrated that they have grossly altered metabolisms.

The CO_2 assimilating variants reported in this chapter can be used to deduce the flow of carbon in photoheterotrophically grown *R. rubrum*. Because bacteria defective in an enzymic reaction accumulate its precursor, it is possible to deduce the sequence of metabolic intermediates in a biochemical pathway. The direction of carbon flow in the TCA cycle in *R. rubrum* is unclear, since all the enzymes for both the oxidative and reductive modes of the cycle are found in malate-grown cells. By examining enzyme activities and identifying accumulating organic acids (TLC or HPLC) the direction of carbon flow in the TCA cycle can be deduced. For instance, a bacterial strain defective in isocitrate dehydrogenase with an oxidative TCA cycle would accumulate isocitrate, whereas, if the TCA cycle was operating in the reductive mode, it would result in the accumulation of α-kg. However, before this analysis can be performed the genetic variants must be confined to this metabolic pathway and thus the bacteria need to be tested for the inability to grow on fumarate, succinate, and other TCA cycle organic acids as their *sole* carbon source (Creaghan and Guest 1972; Spencer and Guest 1973).

Chapter 6

Conclusions

The key enzyme of the Calvin cycle, RUBP carboxylase, was purified as a 112,000 M.W. dimer from the photosynthetic bacterium *Rhodospirillum rubrum*. Southern analysis has revealed that the enzyme is in fact a homodimer because it is encoded by a single gene (Chapter 2).

This gene was found to be regulated at the transcriptional level by light (Chapter 3). Messenger-RNA coding for RUBP carboxylase was found in light-grown cultures but undetectable in dark-grown cultures of *R. rubrum*. The level expression of RUBP carboxylase was also found to be influenced by the presence of organic compounds in the growth media. When malate as well as CO_2 are used as the carbon source, the specific activity of RUBP carboxylase is reduced when compared to the specific activity from photoautotrophically cultured cells. The rapid labelling of α-ketoglutarate, malate, and asparatate during $^{14}CO_2$ labelling experiments indicated that other carboxylases besides RUBP carboxylase contribute to the metabolism of *R. rubrum*.

In vivo mutagenesis of *R. rubrum* growing photoheterotrophically on $^{14}CO_2$ and malate has yielded CO_2 assimilating variants defective in CO_2 fixation (Chapter 3). When the rate of $^{14}CO_2$ fixation was quantified at the point of maximum CO_2 fixation, in early log-phase (Chapter 4), the variants incorporated $^{14}CO_2$ at rates ranging from 13.1-58.2% that of the wild-type control (Chapter 5).

When variant 12-1 was labelled with $^{14}CO_2$ for 15 min the quantity of ^{14}C-phosphorylated compounds and the specific activity of RUBP carboxylase were equivalent to that of wild-type yet the CO_2 fixation rate was reduced 85.4%. Since the reduction in CO_2 fixation as measured by $^{14}CO_2$ incorporation cannot be accounted for by the elimination of RUBP carboxylase, the residual CO_2 fixation of 14.6% may represent the maximum contribution that the Calvin cycle makes to CO_2 fixation in malate and CO_2 light-grown cultures of *R. rubrum*.

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Appendix 1

Growth Rates of CO_2 Assimilating Variants

Strain	Doubling Time (hr)
Wild-type	3.0
1-3F	6.1
1-6C	1.8
11-6	2.5
11-12	3.5
13-1	4.0
19-1	3.3

Bacterial cultures were grown photoheterotrophically on malate and CO_2 according to the description given in Chapter 3. After sterilizing the tip of a hamilton syringe, a 350 ul sample of culture was removed every 4 hours through the test tube's serum stopper. The culture's optical density at A_{650} was then plotted against time on semilogarithmic paper.

Appendix 2

Specific Activity of RUBP carboxylase is Affected by Dry Weight

Strain	Specific Activity[1,2]	mg[3]	A_{650}
Wild-type	25±.05	5.09±.05	1.90
1-4C	25±.05	4.93	1.87
14-5	25±.05	5.49	1.90
6-3	< 20	5.86	1.92
11-4	< 20	4.9	1.89
19-2	< 20	4.92	1.81
22-1	< 20	5.62	1.86
22-2	< 20	5.75	1.96
1-1C	> 30	4.95	1.92
17-3	>> 30	4.76	1.95

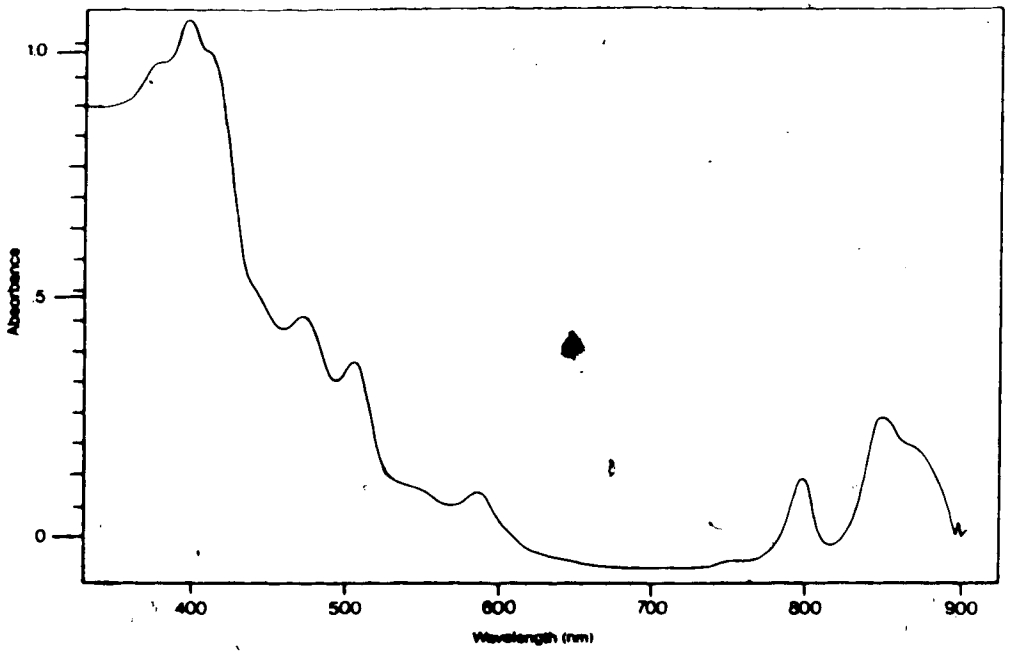
[1]umoles CO_2 fixed/min/mg soluble protein

[2]from mid-logarithmic cells (Chapter 3)

[3]dry weight of 0.3 ml of cells

Appendix 3

Pigment Absorption



One milliliter of late logarithmic cells were sonicated at top speed with a Fisher dismembraner for 1 min. The lysate was diluted ten fold and the absorbance read at a wavelength varying from 330-900 nm.

Appendix 4

Organic Acid Separation by HPLC

Compound	Retention Time[1]	Fraction[2]
Unretained fraction	6.08	15
3-PGA	6.3	15-17
Oxalacetate	7.90	19-21
Citrate	8.15	20-22
Hydroxypyruvate	8.26	20-22
α-Ketoglutarate	8.56	21-23
Pyruvate	9.30	23-25
^{14}C-Glyoxylate	9.37	23-25
^{14}C-Malate	9.77	25-27
Glycerate	10.82	24-26
^{14}C-Glycolate	12.36	31-33
Succinate	12.8	32-34
^{14}C-Formate	14.07	35-37
Acetic acid	15.41	38-40
Fumaric acid	16.78	41-43

[1]in minutes

[2]tube number

A Varian HPLC system with a Vista 401 microprocessor was used to analyze the ^{14}C-organic acids after short-term labelling with $^{14}CO_2$. The organic acids were applied to the Bio-Rad cation exchange column (Aminex HPX-8T) through a Rheodyne (model 7125) fixed volume (20 ul) loop injector. The column effluent was monitored by U.V. spectrophotometry (Varian 50) at 210 nm. When dilute sulphuric acid (0.1 N) was pumped through the column (76 atm) the organic acids eluted in the order of increasing pKa (Turkleson and Richards 1978) within 20 min. The above table states the retention time of organic acids (10-15 ug in 20 ul) used to standardize the column. With a flow rate of .6 ml/min, 4 drops (240 ul) of effluent were collected per tube. Four ^{14}C-radioactive organic acids were used to correlate retention time and fraction number.

Appendix 5

Radioactive Amino Acids After 10 Sec of $^{14}CO_2$ Labelling

RF[1]	%[2]	AA	Standard	TLC	AA Family
4-7	5.3	unbound		-	
9-10	1.8	?			
11-12	10.9	phosphoserine	11	origin	serine
20-21	1.6	aspartate	20-23	-	aspartate
26-27	1.6	serine	25-28	-	serine
32-33	1.5	glutamic acid	31-33	-	α-kg
35-36	1.3	proline	¬35-37	-	α-kg
41-42	3.8	?			
43-45	13.6	glycine	42-45	+	serine
49	22.5	alanine	45-49	+	pyruvate
55	36.1	cysteine	¬54-57	+	serine

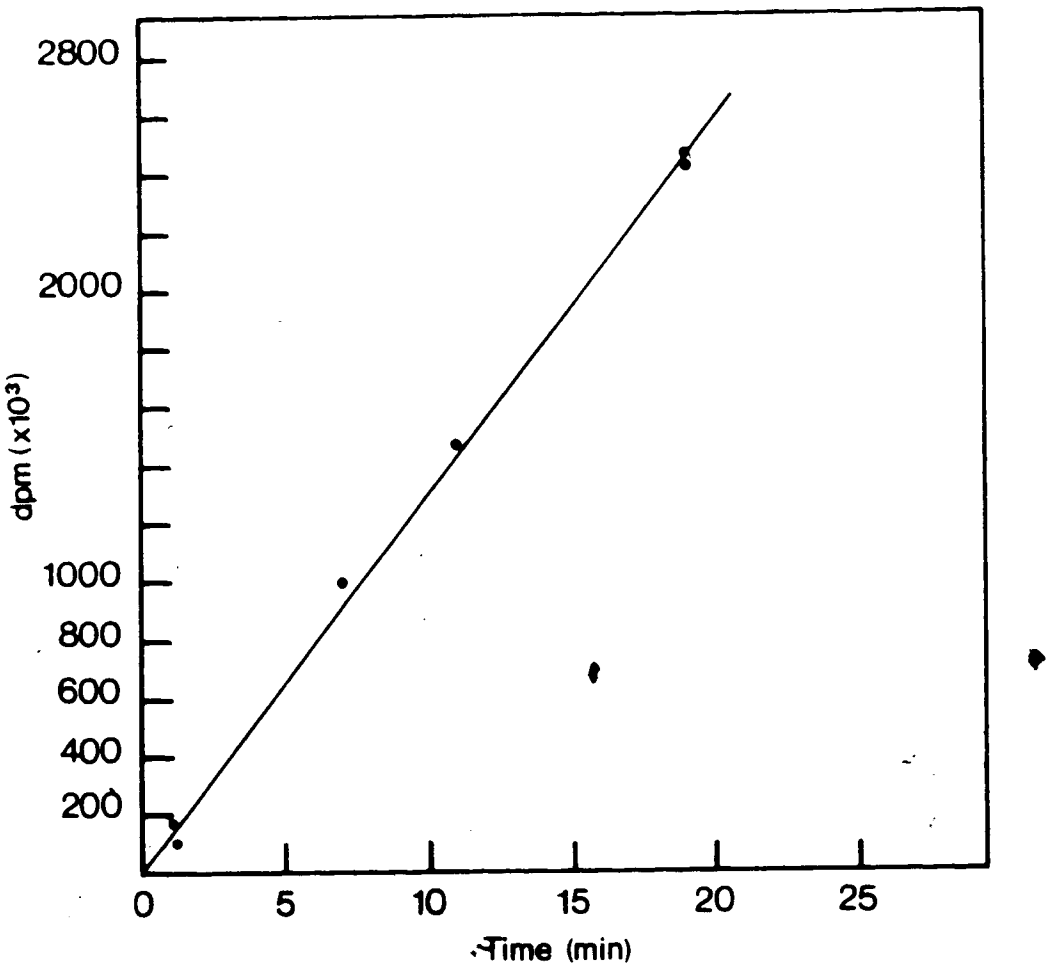
[1]radioactive fraction

[2]% of radioactivity

Amino acids eluting from the Beckman automatic amino acid analyzer were fractionated in a LKB Ultrarac. The amino acids containing radioactivity after 10 seconds of $^{14}CO_2$ feeding were identified by comparison to known standards. The identity of amino acids containing the highest proportion of radioactivity were verified by thin-layer chromatography (Chapter 5).

Appendix 6

Linear CO_2 Fixation



Rate of CO_2 fixation increases linearly with time. During the 15 min labelling experiments (Chapter 5) the atmospheric $^{14}CO_2$ had not been depleted, and CO_2 fixation was in equilibrium.