THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

NONAUTOTROPHIC FIXATION OF CO_2 BY SUSPENSION CULTURES OF PAUL'S SCARLET ROSE GROWN IN MINIMAL ORGANIC MEDIUM

A DISSERTATION

SUBMITTED TO THE GRADUATE COLLEGE

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

KNEELAND NESIUS

Norman, Oklahoma

1973

NONAUTOTROPHIC FIXATION OF CO_2 BY SUSPENSION CULTURES OF PAUL'S SCARLET ROSE GROWN IN MINIMAL ORGANIC MEDIUM

APPROVED BY

John S. Fletcher

Norman H. Boke

James R. Estes

Frank L. Seto

Simon H. Wender

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. John S. Fletcher for his guidance and helpful suggestions during the course of this study and in the preparation of this manuscript. Appreciation is also expressed to the members of my advisory committee, Dr. Norman Boke, Dr. James Estes, Dr. Howard Larsh, Dr. Elroy Rice, and Dr. Simon Wender for their advice and time.

A special note of thanks to my wife Carolyn for her assistance and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Suspension cultures of Paul's Scarlet rose were used to study the metabolic role of nonautotrophic CO_2 fixation. A minimal organic medium containing napthaleneacetic acid, 6-furfurylaminopurine, myo-inositol, and sucrose was developed and used in CO_2 privation studies. Myo-inositol was not essential, but sustained growth was reduced by 90% when it was omitted.

Nitrate could serve as the sole source of nitrogen, but maximum growth required nitrate plus a supplement of either ammonium or glutamine. The source of nitrogen influenced the morphology of the culture. Cells grown without ammonium or glutamine formed large cell-masses averaging approximately one cm in diameter. Ammonium at concentrations above 1 mM were inhibitory to growth.

The pH of culture media was maintained with a buffer (MES) which was nontoxic to growth and was not depleted. Maximum growth required a pH (5.2-5.4) during the division phase of growth (day 0-7) and a pH (5.8-6.0) during the expansion phase (day 7-14). Either a gradual shift of nonbuffered medium or an abrupt change of buffered medium led to maximum growth.

Carbon dioxide privation studies showed a partial requirement for exogenous CO_2. The fresh weight increase was reduced by approximately 22%, but the dry weight was not influenced when cells were grown for 14 days in a CO_2 deficient environment. The first five days of growth was the critical period of nonautotrophic CO_2 fixation when cells were grown in medium buffered at pH 5.4. The phosphoenolpyruvate carboxylase activity was highest during the period when nonautotrophic CO_2 fixation appeared to be critical for growth.

Nonautotrophically fixed $^{14}CO_2$ contributed carbon-14 to numerous cell constituents. Of primary importance carbon-14 was recovered in eleven different amino acids, several of which had never been associated previously with nonautotrophic CO_2 fixation. The labeled amino acids were readily available for protein synthesis.

Both exogenously provided bicarbonate-^{14}C and endogenously generated $^{14}CO_2$ were used for nonautotrophic CO_2 fixation. However, the distribution of ^{14}C among cell constituents differed depending upon the source of CO_2.

NONAUTOTROPHIC FIXATION OF CO_2 BY SUSPENSION CULTURES OF PAUL'S SCARLET ROSE GROWN IN MINIMAL ORGANIC MEDIUM

PART I

DEVELOPMENT OF A MINIMAL ORGANIC MEDIUM FOR SUSPENSION CULTURES OF PAUL'S SCARLET ROSE TISSUE

CHAPTER 1

INTRODUCTION

Tissue culture techniques have evolved to the point that cells isolated from many species of plants can be propagated for apparently indefinite periods of time on any one of several chemically defined media (35,44). Suspension cultures grown on defined media offer many desirable features for metabolic investigations (11,21,36,45). One strain of cells isolated by Tulecke from Paul's Scarlet rose has been demonstrated to be extremely well suited for such work (11,15). Over the past ten years at least eleven metabolic studies conducted in six different laboratories have been performed with rose cells (8,10-16,21,26,27,52). Despite the apparent popularity of these cells, no effort has been made to determine the minimal organic medium required for their growth. Such information would be of obvious value for

metabolic studies involving the requirement and utilization of organic compounds by this tissue. The work included in Part I was initiated to determine the minimal organic medium required for maximum sustained growth of suspension cultures of Paul's Scarlet rose.

CHAPTER 2

MATERIALS AND METHODS

Tissue Utilized

All of the work in this study was performed on suspension cultures of Paul's Scarlet rose. This tissue was isolated in 1957 from stem tissue by Walter Tulecke and has been propagated on defined media since 1960 (44). Since 1969 suspension cultures have been maintained in our laboratory on a modification of Tulecke's PN-25 medium (43,44). The medium was modified by omitting adenine, cytidylic acid, and guanylic acid from PN-25 medium (Table I-1). Suspension cultures propagated continuously over the past three years on this modified medium have consistently given yields comparable to those obtained previously with complete PN-25 medium. The control medium used in this investigation was the modified PN-25 medium (Table I-1) described above.

Growth and Measurement of Tissue

The cells were grown in 250 ml erlenmeyer flasks containing 75 ml of modified PN-25 medium with an initial pH of 5.5. To prepare a desired volume of medium, appropriate amounts of inorganic stock solution, hormones, vitamins,

TABLE I-1.--Tulecke's PN-25 and modified PN-25 medium.

	AMOUNT (mg/l) PN-25	mod. PN-25
INORGANIC SALTS		
Macronutrients		
Calcium nitrate (.4 H_2O)	280.00	280.00
Magnesium sulfate (.7 H_2O)	760.00	760.00
Potassium chloride	900.00	900.00
Potassium nitrate	80.00	80.00
Sodium nitrate	1800.00	1800.00
Sodium dihydrogen phosphate (.H_2O)	300.00	300.00
Sodium sulfate (.10 H_2O)	453.00	1.40
Micronutrients		
Boric acid	0.20	0.20
Cobalt chloride (.6 H_2O)	0.01	0.01
Copper sulfate (.5 H_2O)	0.02	0.02
Manganese sulfate (.H_2O)	0.80	0.80
Potassium iodide	0.50	0.50
Molybdic acid	0.01	0.01
Zinc sulfate (.7 H_2O)	0.50	0.50
ORGANIC COMPOUNDS		
Kinetin (6-furfurylaminopurine)	0.50	0.50
Naphthaleneacetic acid	1.00	1.00
myo-Inositol	100.00	100.00
Glutamine	200.00	200.00
Iron citrate	5.00	5.00
Sorbitol	100.00	100.00
Nicotinic acid	1.00	1.00
Thiamin	0.50	0.50
Pyridoxine	0.50	0.50
Calcium panthenate	1.00	1.00
Sucrose	20.00 g/l	20.00 g/l
Cytidylic acid	100.00	---
Guanylic acid	100.00	---
adenine	10.00	---

sugars, and iron citrate were added to column-distilled water. The pH was then adjusted to 5.5, using 0.1 N NaOH. Seventy-five ml aliquots were then placed into 250 ml erlenmeyer flasks. The flasks were tightly covered with aluminum foil and autoclaved at 15 psi for 17 min. After the medium cooled, 5 mls of a solution containing 16.6 mg of glutamine and 8.3 mg of myo-inositol were added to each flask aseptically by passing it through a 0.22 millipore filter held in a syringe adapter.

Transfer of 14 day old cells to fresh medium was made in a sterile chamber which had been swabbed with a germicide solution (O-syl) and exposed to U-V irradiation for at least 2 hr prior to transfer of the cells. During transfers a donor flask was mounted in a clamp and tilted so that its mouth was horizontal. The aluminum foil cap was removed and the mouth of the flask flamed with an alcohol lamp. The liquid medium was withdrawn from the donor flask with a sterile 50 ml syringe. Cells were transferred with a sterile transfer loop consisting of several coils of wire. The fully covered loop held a fresh weight of approximately 0.5 grams of cells. The cells were grown in the dark at 25 C on a New Brunswick Model V rotary action shaker, operating at 180 rpm.

After a growth period of either 14 or 21 days, cells were collected with a filtering flask fitted with a buchner funnel lined with miracloth. Cells were transferred to a preweighed aluminum foil tare and the fresh weight was

determined immediately with a Mettler Model H6T analytical balance. The cells were then allowed to dry at 80 C in a dry heat oven for 72 hr. The dry weight was then recorded.

Determination of Required Organic Compounds

The requirement for each of the organic compounds present in the control medium was studied by comparing the growth (dry weight and fresh weight) of cultures in control medium with growth in medium minus a single organic compound. The influence of each compound on sustained growth was determined by making measurements after each of several successive passages in test medium from which the compound had been omitted. In these studies the inoculum used for the first passage was taken from a culture grown in control medium, but the subsequent transfers were made with cells which had been previously grown in the medium being tested.

The optimal concentrations of required organic compounds were established by comparing fresh and dry weights of cultures grown in media with varying concentrations of the compound being examined.

CHAPTER 3

RESULTS

The control medium (Table I-1) contained four vitamins: calcium panthenate, nicotinic acid, pyridoxine, and thiamin. The requirement for each of these was studied individually (Table I-2). Maximum growth in all of the media tested occurred by day 14, after which time there was a slight decrease in fresh weight and a marked decrease in dry weight. At 14 days the fresh and dry weights of all of the test cultures closely approximated that of the cells grown in control medium, indicating that none of the vitamins were required for growth.

The influence of two sugars, sorbitol and myo-inositol, is shown in Table I-3. The omission of sorbitol had no influence on growth. However, when myo-inositol was omitted, the increase in fresh weight after 14 days was reduced by 69% and the dry weight by 61%. The maximum growth in both media occurred by day 14, after which time there was a slight decrease in fresh weight and a pronounced decrease in dry weight. This indicated that the curtailment of growth brought on by the omission of myo-inositol was due to a reduction in total growth and not just the growth rate. The

TABLE I-2.--Fresh weight and dry weight of cells following 14 or 21 days of growth in media with various vitamins omitted.

COMPOUND OMITTED	FRESH WT. (g) on Day		DRY WT. (g) on Day	
	14	21	14	21
Ca. Pantothenate	21.3	20.8	.744	.683
Nicotinic Acid	22.5	20.2	.756	.667
Pyridoxine	21.6	19.8	.705	.688
Thiamin	21.8	20.2	.728	.713
All Vitamins	20.1	19.3	.752	.665
Control (All Vit. Present)	21.2	20.3	.760	.686

Each value represents an average of 9 flasks. Three flasks were harvested at the end of 3 consecutive growth periods.

TABLE I-3.--Fresh weight and dry weight of cells following 14 or 21 days of growth in media with various sugars omitted.

COMPOUND OMITTED	FRESH WT. (g) on Day		DRY WT. (g) on Day	
	14	21	14	21
Sorbitol	21.4	20.9	.751	.691
myo-Inositol	7.0	6.5	.280	.269
Control	22.3	20.87	.727	.684

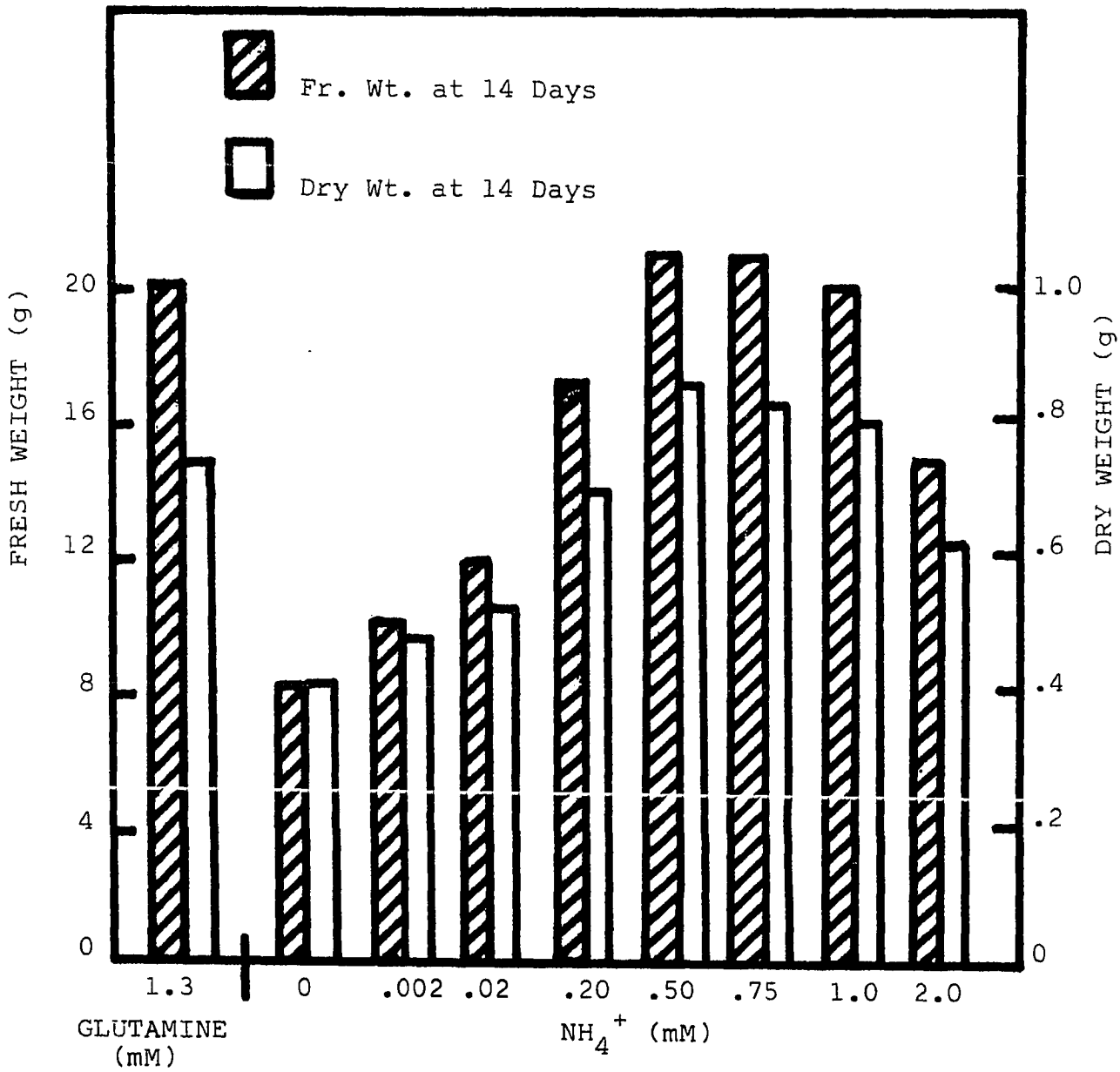
Each value represents an average of 6 flasks. Two flasks were harvested at the end of 3 consecutive growth periods.

reduction in growth was slight after the first passage in inositol free medium (7% of the dry weight) and more pronounced following the second and third transfers (90% of the dry weight).

Control medium contained nitrogen in two forms, NO_3^- (24mM) and glutamine (1.3mM). Figure I-1 shows a study conducted to determine if glutamine was required. No sorbitol or vitamins were in the media used in this series of experiments. A comparison of dry weights after 14 days showed that growth in medium possessing only NO_3^- as a nitrogen source was 44% of that occurring in medium containing both NO_3^- and glutamine. However, the enhanced growth observed on glutamine medium was also achieved by replacing glutamine with $(NH_4)_2SO_4$ at concentrations ranging from .5 to 1.0 mM (Fig. I-1). Concentrations of ammonium ions above 1 mM inhibited growth, and attempts to grow cultures with NH_4^+ as the sole nitrogen source were unsuccessful.

In addition to influencing growth, the source of nitrogen also had an effect on the overall morphology of the cells. Suspension cultures of rose cells cultured continuously in control medium normally grew as undifferentiated cell aggregates ranging from less than a hundred cells to several thousand cells (15,21). When cells were grown in medium with NO_3^- serving as the only nitrogen source the clumping was much more pronounced. Cells grown in this medium developed into large masses of tightly packed cells

Figure I-1.--Influence of supplementary amounts of glutamine or ammonium on growth.



All medium contained NO_3^- (24 mM). Each value represents an average of three flasks.

similar in appearance to callus cultures grown on agar medium (Fig. I-2). The clumps ranged from 0.5 to 2 cm in diam, and there were no free cells. In contrast to this, cultures grown in medium possessing NO_3^- plus NH_4^+ grew as dispersed cells and small clumps of cells.

Having established that myo-inositol and NH_4^+ were required for maximum growth, a study was then conducted to establish the optimal concentrations for these components (Table I-4). In this experiment the medium did not contain glutamine, sorbitol, or vitamins. Maximum growth occurred at concentrations of 0.1 g/l of inositol and 0.2 mM NH_4^+. Because of the reported heat lability of myo-inositol (44), this compound had formerly been added with a millipore filter syringe. However, autoclaving of inositol during these experiments had no adverse effect on its stimulatory influence.

A study was also conducted with NAA and kinetin to establish whether these compounds were essential, and if so their optimal concentrations (Table I-5). Very little growth occurred in the absence of these compounds. Increased amounts of either one of these hormones stimulated growth, but maximum growth required the simultaneous provision of both. The optimal concentrations were 1×10^{-3} g/l for NAA and 5×10^{-4} g/l for kinetin.

The information gained in the preceding studies was used to formulate the minimal organic medium (MPR-medium) shown in Table I-6. Suspension cultures have been maintained

Figure I-2.--Cells grown for 3 consecutive generations in media containing NO_3^- as the only source of nitrogen.

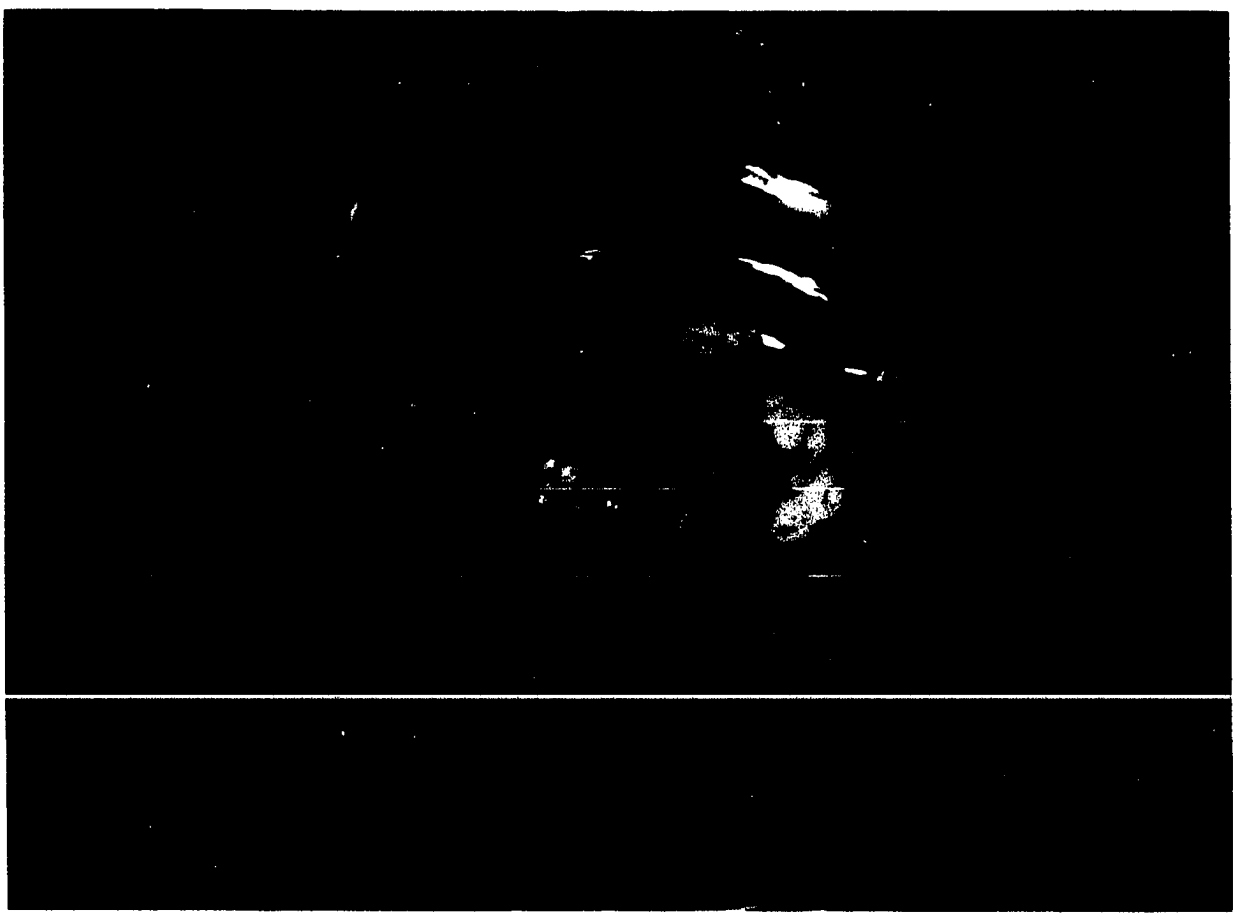


TABLE I-4.--Fresh weight and dry weight of cells following 14 days of growth in media with varying concentrations of myo-inositol and ammonium.

NH_4^+ mM	myo-INOSITOL (g/l) 0.05	0.1	0.2
0.002	12.68	10.87	10.11
	0.77	0.75	0.71
0.02	10.25	11.13	10.74
	0.74	0.76	0.73
0.2	16.58	18.12	15.37
	0.79	0.83	0.78
2.0	8.75	13.22	10.35
	0.66	0.72	0.70

Each value represents an average of 3 flasks harvested at the end of a 14 day growth period. Upper figure is g fresh weight; lower figure is g dry weight.

TABLE I-5.--Fresh weight and dry weight of cells following 14 days of growth in media with varying concentrations of napthaleneacetic acid and kinetin.

KINETIN (g/l)	NAA (g/l)						
	0	$1x10^{-6}$	$1x10^{-5}$	$1x10^{-4}$	$1x10^{-3}$	$1x10^{-2}$	$1x10^{-1}$
0	0.78	0.63	0.72	1.28	3.11	6.36	5.24
	0.069	0.052	0.065	0.092	0.2333	0.243	0.233
$5x10^{-7}$	0.69	0.70	0.78	3.44	13.54	6.25	5.19
	0.052	0.063	0.060	0.210	0.531	0.252	0.231
$5x10^{-6}$	0.73	1.53	2.89	5.15	16.70	5.22	3.98
	0.058	0.097	0.113	0.260	0.669	0.218	0.159
$5x10^{-5}$	0.83	2.56	4.41	7.09	16.51	0.81	0.77
	0.061	0.092	0.218	0.357	0.801	0.051	0.049
$5x10^{-4}$	7.12	7.93	14.88	15.77	21.43	0.68	0.56
	0.301	0.332	0.653	0.691	0.831	0.044	0.055
$5x10^{-3}$	5.16	5.69	5.84	15.33	15.10	0.79	0.74
	0.211	0.272	0.269	0.673	0.655	0.059	0.062
$5x10^{-2}$	3.58	2.84	3.21	0.89	0.80	0.79	0.63
	0.162	0.128	0.155	0.063	0.068	0.071	0.059

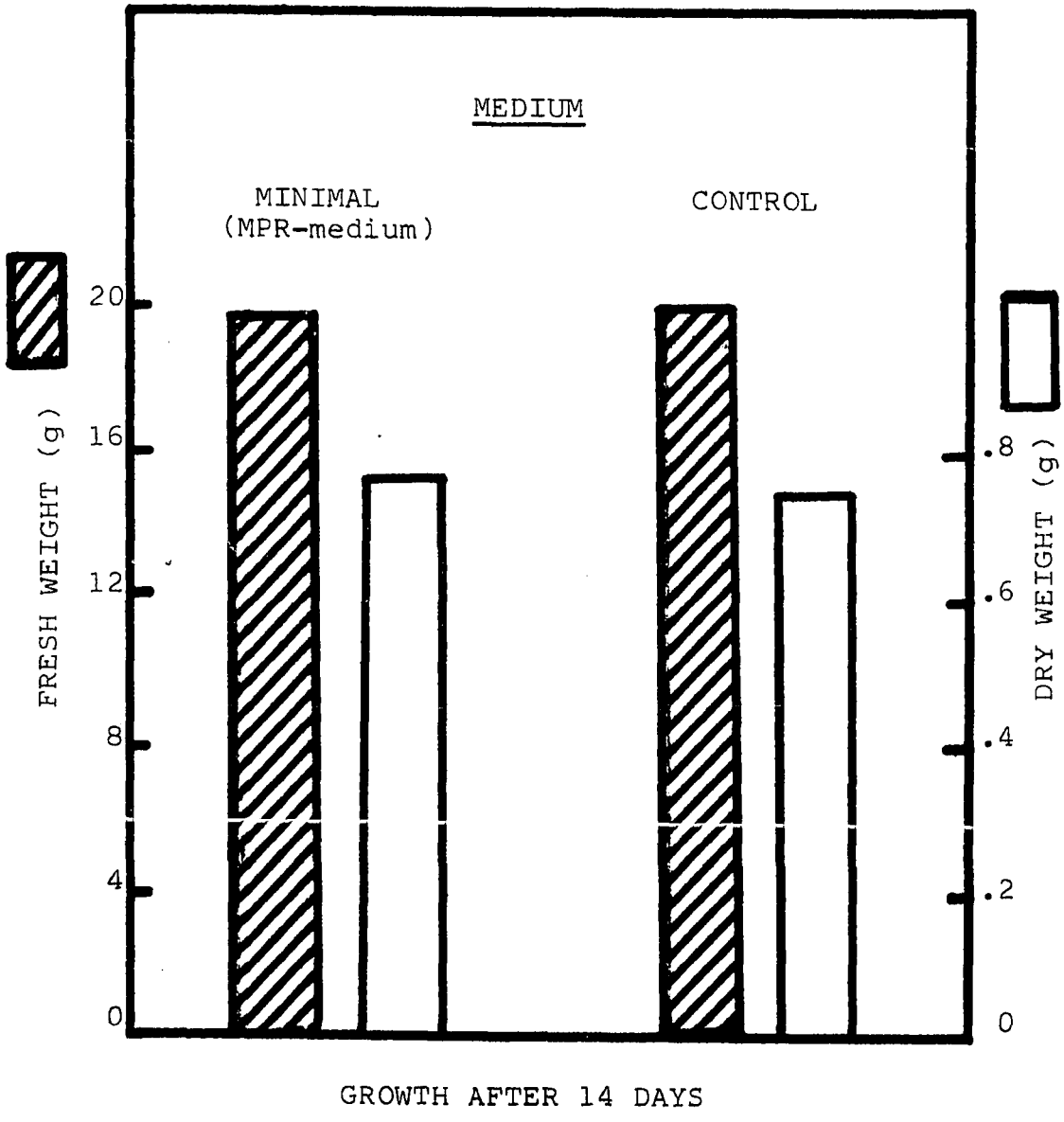
Each value represents an average of 3 flasks harvested at the end of a 14 day growth period. Upper figure is g fresh weight; lower figure is g dry weight.

TABLE I-6.--Minimal organic medium for maximum growth of suspension cultures of Paul's Scarlet rose MPR.

	AMOUNT (mg/l)	mM
INORGANIC SALTS		
Macronutrients		
Ammonium sulfate	60.00	0.47
Calcium nitrate (.4 H_2O)	280.00	1.19
Ferrous sulfate (.7 H_2O)	6.00	0.02
Magnesium sulfate (.7 H_2O)	760.00	6.31
Potassium chloride	900.00	12.07
Potassium nitrate	80.00	0.79
Sodium nitrate	1800.00	21.18
Sodium dihydrogen phosphate (.H_2O)	300.00	2.17
Sodium sulfate (.10 H_2O)	453.00	1.40
Micronutrients		µM
Boric acid	0.20	3.226
Cobalt chloride (.6 H_2O)	0.01	0.042
Copper sulfate (.5 H_2O)	0.02	0.125
Manganese sulfate (.H_2O)	0.80	4.730
Potassium iodide	0.50	2.778
Molybdic acid	0.01	0.062
Zinc sulfate (.7 H_2O)	0.50	1.739
ORGANIC COMPOUNDS		
Kinetin (6-furfurylaminopurine)	0.50	2.326
Naphthaleneacetic acid	1.00	5.326
		mM
myo-Inositol	100.00	0.560
Sucrose	20 g/l	58.48

on this medium through 20 transfers. Growth in this medium was essentially identical to that achieved with the control medium (Fig. I-3). Callus cultures grown on complete PN-25 medium have been successfully transferred to liquid MPR-medium. However, the growth rate was much slower than that achieved when inoculums were taken from suspension cultures. At the present time we are maintaining callus cultures grown on MPR-medium containing agar. These were established by placing suspension tissue grown in liquid MPR-medium on MPR-medium containing agar. They have been maintained on solid MPR-medium for three generations of growth. Each generation grew for approximately eight weeks.

Figure I-3.--Growth in minimal organic medium for Paul's Scarlet rose (MPR-Medium) as compared to growth in control medium.



Each value represents an average of 9 flasks harvested at the end of 3 consecutive growth periods.

CHAPTER 4

DISCUSSION

Although intact higher plants are metabolically self sufficient and their cells display genetic totipotency, the fact remains that most cultured organs and cells must be provided with an exogenous supply of organic compounds. Very often suspension cultures have more requirements than callus or organ cultures (35). It follows that this feature has restricted the usefulness of suspension cultures in nutritional and metabolic studies. Cultures started from different plants have different requirements; therefore, a minimal medium must be established for each (35). In previous studies conducted by several workers (8,10-16,21,52), Paul's Scarlet rose has been grown on PN-25 medium which contains 14 organic compounds. In the present study sustained growth was demonstrated on a minimal organic medium containing only four organic compounds: myo-inositol, napthaleneacetic acid, kinetin, and sucrose (Table I-6).

Myo-inositol was not essential for the growth of the cells, but it had a pronounced stimulatory influence. Although such an influence has been noted on several tissue cultures, the exact role played by inositol in tissue culture

growth is not certain. It may serve as a regulator of mitosis, as demonstrated by Davidson and Webster (7), or it may act as a cofactor in metabolism as suggested by Tanner and Kandler (37) in their work on the biosynthesis of stachyose. The high concentrations required for optimal growth (100-fold greater than hormone levels) suggests that the compound was used as a substrate, perhaps in cell wall synthesis, as shown by Loewus (23).

When both NAA and kinetin were omitted from the medium, no growth occurred. The provision of either one of these by itself permitted growth, but maximum growth required the presence of both at concentrations of 1×10^{-3} g/liter for NAA and 5×10^{-4} g/liter for kinetin. None of the media tested induced differentiation in rose cultures as demonstrated by Skoog et al in their synergistic studies on tobacco callus cultures (35).

Rose cells grew on medium with only NO_3^- serving as a nitrogen source, but maximal growth required NO_3^- plus a supplemental amount of either NH_4^+ or glutamine. Excessive amounts of NH_4^+ (1mM) inhibited growth, and attempts to grow cells solely on NH_4^+ were unsuccessful. Similar results have been obtained with several callus and root cultures (35). Gamborg showed a similar growth requirement for suspension cultures of soybean and suggested that a trace amount of ammonium was required for full utilization of provided nitrate. In support of this idea, Schrader (3) showed that an

appreciable induction of nitrate reductase by nitrate in corn seedlings was only achieved if the seedlings had been grown in medium containing ammonium.

The source of nitrogen provided to rose cultures also had a pronounced influence on the degree of cell aggregation. Cells grown in medium possessing only NO_3^- grew as large firm cell masses. However, when a supplemental amount of either glutamine or NH_4^+ was provided along with NO_3^-, then mature cultures were comprised primarily of small cell aggregates. This suggests that the source of nitrogen, in some manner, influences the makeup of the cell wall, or the cross linkages between adjacent cell walls.

PART II

THE EFFECT OF A CO_2 DEFICIENT ENVIRONMENT ON SUSPENSION CULTURES OF PAUL'S SCARLET ROSE TISSUE

CHAPTER 1

INTRODUCTION

Nonautotrophic CO_2 fixation (39), also termed heterotrophic (53) and dark CO_2 fixation (32), has been demonstrated in plant, animal, and microbial cells. The rapid growth of some microorganisms (47) and animal tissue culture cells (17,30) was stopped when they were incubated in a CO_2 deficient atmosphere. Similar experiments conducted with excised root tips resulted in only a partial reduction in growth (32,34).

Thus, it appears that nonautotrophic CO_2 fixation was an essential requirement for animal and some microbial cells but not for higher plant cells. However, the failure of CO_2 privation to inhibit growth of plant cells might have been due to the different growth rates of the cells studied. The doubling time (dry weight increase) of the plant tissue previously examined was approximately 10 days whereas, the doubling time for microbial and animal cell cultures was

20 min and 48 hr respectively. In slow growing plant cells the requirement for dark CO_2 fixation may have been minimal and was satisfied by the residual CO_2 remaining in the systems used (32,34).

The work included in Part II was initiated to examine the influence of a CO_2 deficient environment on the growth of suspension cultures of Paul's Scarlet rose which have a normal doubling time of approximately 24 hours. Since the growth of these cells (15) is characterized by a division phase (rate of division exceeds rate of expansion) followed by an expansion phase (rate of expansion exceeds rate of division), it was possible to explore the influence of exogenous CO_2 on these two phases of cell development.

CHAPTER 2

MATERIALS AND METHODS

Growth of Tissue

Suspension cultures of Paul's Scarlet rose were grown on MPR medium (Table I-6). Medium was prepared in 1 to 4 liter batches just prior to use. One liter of medium was prepared by mixing 950 ml of water, 50 ml of stock solution (inorganic salts except for $(NH_4)_2SO_4$ and $FeSO_4$), kinetin, and NAA. This mixture was stirred for 8 hr on a warm hot plate to dissolve the kinetin. Sucrose, inositol, and $FeSO_4$ were added and the pH adjusted to 5.5 with 0.1 N NaOH. The medium was placed into 250 ml flasks (80 ml per flask), $(NH_4)_2SO_4$ was added (1 ml of a 0.038 M solution), and the flasks were autoclaved for 17 min at 250 F and 15 lbs. pressure. All of the chemicals were added in the dry form except for the inorganic stock solution and the $(NH_4)_2SO_4$ solution. The final concentration of each chemical added is shown in Table I-6.

General information pertaining to cell transfers, growth conditions, harvest procedures, and weight determinations were described in Part I. In all of the growth studies reported in this Part, each treatment represented an average

of at least five replicate cultures.

pH Studies

The pH studies were performed with MES (2-[N-morpholino] ethane sulfonic acid), a buffer introduced by Good et al. (18). The buffer was purchased from Nutritional Biochemicals Corporation.

CO_2 Privation Studies

The provision of CO_2 deficient air to cultures was accomplished by placing a CO_2 trap into the mouth of the culture flasks (17). A CO_2 trap consisted of a rubber stopper with a 15 x 127 mm test tube projected through it. The bottom one-third of the test tube had five 15 x 2 mm slits in it. Two Whatman no. 5 filters (9 cm diam) were rolled and placed inside the test tube to provide a large surface area for CO_2 absorption. The open end of the test tube was stoppered with cotton and the assembly was autoclaved. A syringe was then used to aseptically saturate the filter paper with 2.5 ml of 30% KOH. The traps were changed every 5 days to insure adequate trapping of CO_2 and avoid excessive accumulation of water in the bottom of the test tube.

PEP Carboxylase Extraction and Assay

The procedure used for the extraction and assay of phosphoenolpyruvate carboxylase (PEP carboxylase) was a modification of that used by Slack and Hatch (31). The activity

of crude extracts was determined by measuring the incorporation of $H^{14}CO_3^-$ in the presence of phosphoenolpyruvate. A cell extract was prepared by grinding 2.5 g of cells in 15 ml of 0.1 M tris-HCl buffer (pH 7.8), containing 10 mM 2-mercaptoethanol and 1.25 g of polyvinylpyrrolidone. The cells were ground at 0 C for 2 min on a Virtis grinder set at high speed. The homogenate was filtered through miracloth. The PEP carboxylase activity was then determined by adding 0.2 ml of the crude extract to a reaction mixture of: 50 μ moles of tris-HCl buffer (pH 8.3), 10 μ moles of 2-mercaptoethanol, 5 μ moles of $MgCl_2$, 2 μ moles sodium phosphoenolpyruvate, giving a total volume of 0.8 ml. The reaction was carried out in a liquid scintillation vial kept at 30 C. After 3 min the reaction was stopped by adding 0.8 ml of 20% TCA. The sample was dried overnight in an air stream, 10 ml of scintillation fluid (15) was added, and the radioactivity was then measured on a Beckman LS-100 liquid scintillation counter.

Light Studies

Tissue grown in the presence of light were grown continuously under a bank of five fluorescent lights producing 500 foot candles.

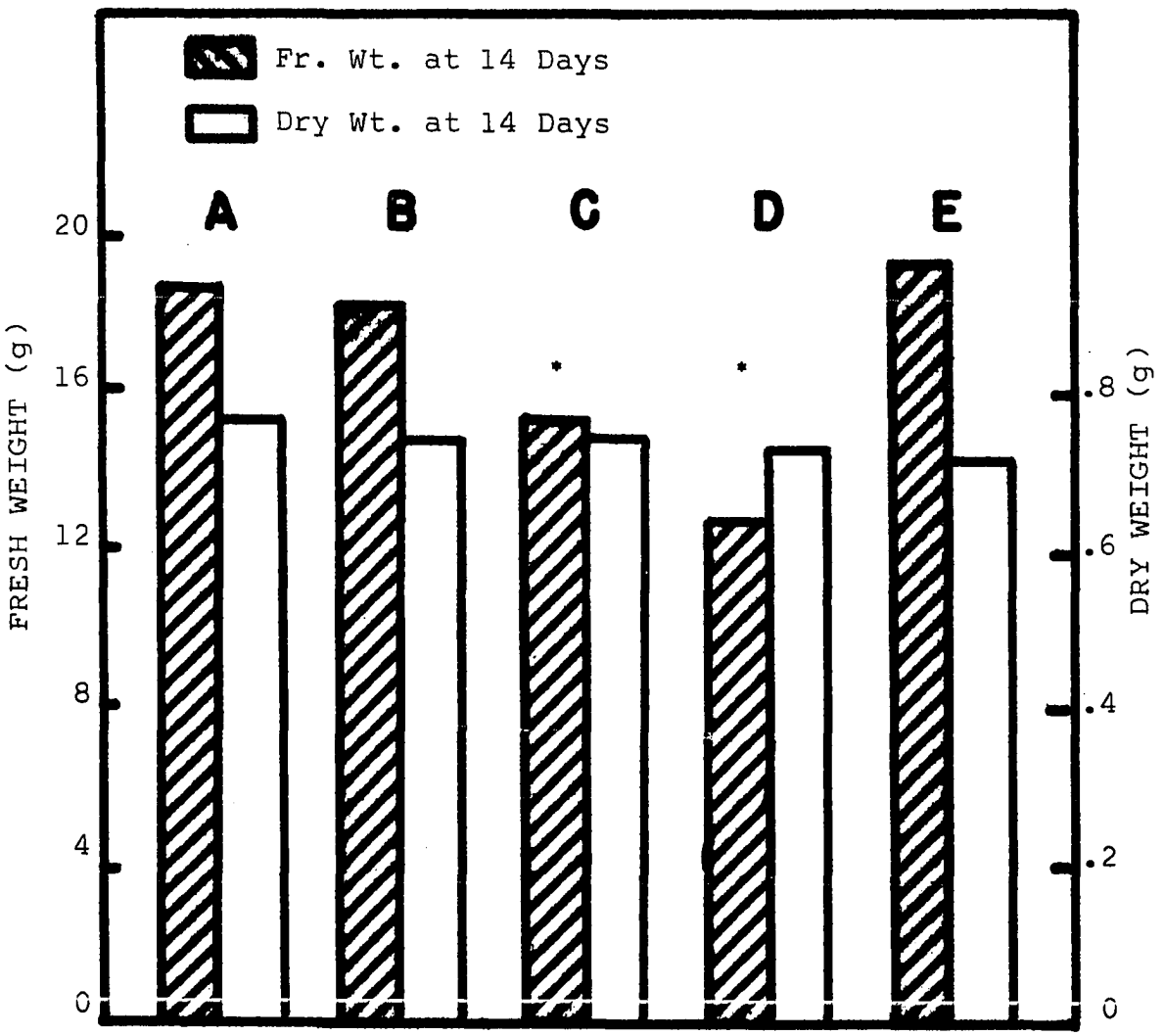
CHAPTER 3

RESULTS

The influence of CO_2 deficient air on cell growth was examined by growing cells in nonbuffered medium held in flasks fitted with and without CO_2 traps. Cell growth in medium exposed to CO_2 deficient air was the same as that of cells grown in medium exposed to normal air (Fig. II-1 A and B). However, these data did not rule out a requirement of CO_2 for growth, since bicarbonate ions present in the medium could also serve as a source of CO_2.

The initial pH of the medium was too low (pH 5.5) to allow for large quantities of bicarbonate ions to be present (46). However, pH measurements showed that during cell growth (Fig. II-2) the pH of the medium shifted upwards to a final pH of approximately 6, a change which would lead to increased quantities of soluble HCO_3^-. To eliminate bicarbonate ions from the medium it was desirable to grow cells at the lowest possible pH, and thereby shift the equilibrium between CO_2 and HCO_3^- in the direction of CO_2 (46). After the medium was saturated with CO_2 any excess would be evolved into the air where it could be removed with a NaOH trap for CO_2. A brief survey of available buffers showed that a 0.05 M

Figure II-1.--Influence of CO_2 and pH of medium on the growth of rose cells.

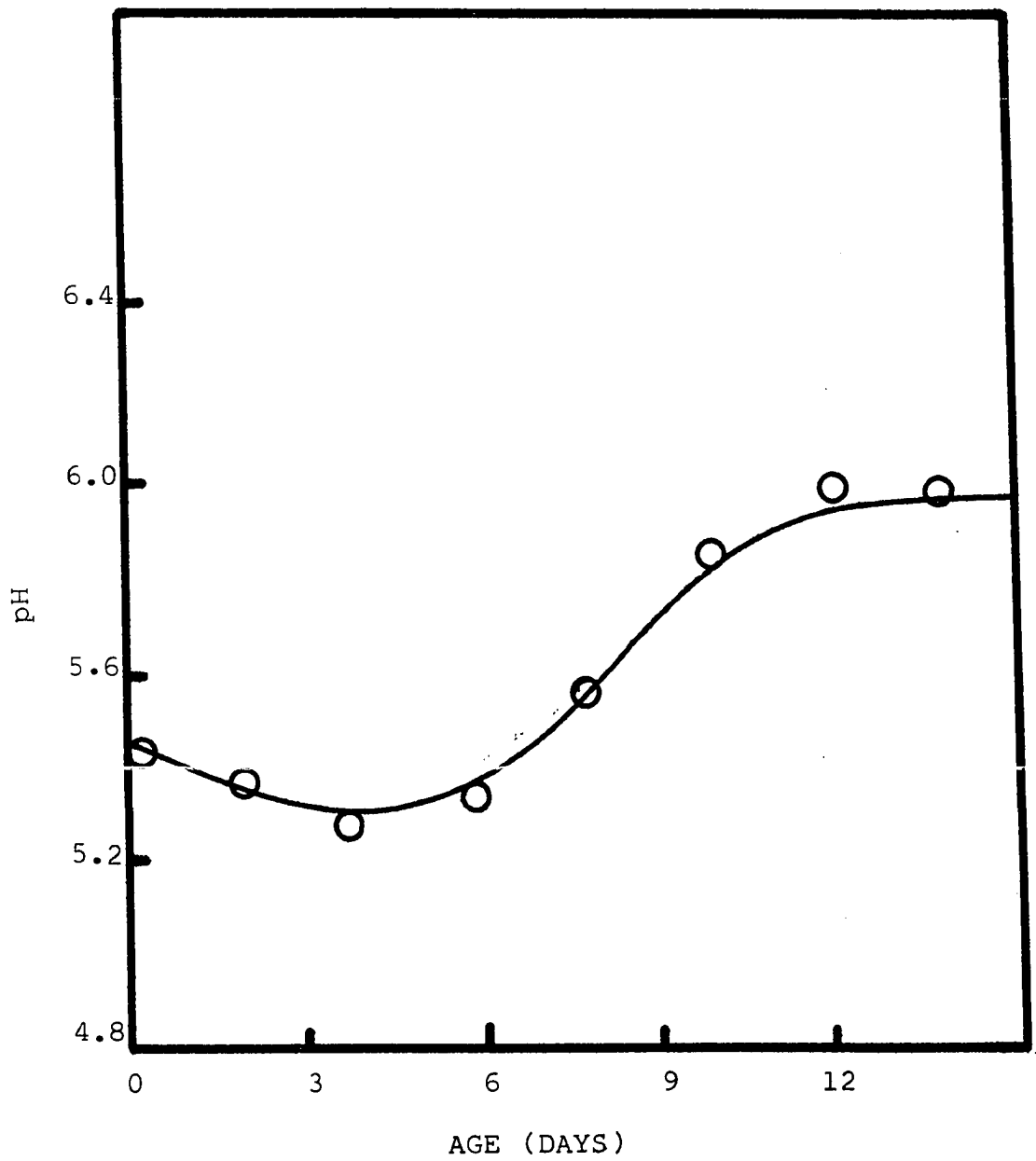


Buffered medium contained 0.05 molar MES. Treatments administered were as follows:

A. Control; no buffer; no CO_2 trap
B. No buffer; with CO_2 trap (0-14 days)
C. With buffer (pH 5.2, 0-14 days); no CO_2 trap
D. With buffer (pH 5.2, 0-14 days); with CO_2 trap (0-14 days)
E. With buffer (pH 5.2, 0-7 days) (pH 6.0, 7-14 days); no CO_2 trap

(*) Fresh weight significantly different from control at 0.05 level or better

Figure II-2.--The pH changes which occurred in the medium of normally grown cells.



concentration of MES (2-[N-morpholino] ethane sulfonic acid) stabilized the pH throughout the 14 day growth period to within 0.1 units of the starting pH.

The growth of cells for 14 days in media buffered at different fixed pH values gave the results shown in Table II-1. Growth was inhibited below pH 5.2 and above pH 5.4. The inhibition appeared to be a function of the pH and not the presence of the organic buffer, MES, since inhibition occurred at both high and low pH values. The inhibition at the low pH could have been due in part to the total absence of bicarbonate ions in the medium (46). It was surprising to find that when the pH was kept at 6.0, the final pH in nonbuffered medium (Fig. II-2), growth was inhibited by 85%. The optimal pH range of the medium for cell growth was 5.2 to 5.4, but even in this pH range growth only amounted to approximately 16 g, compared to 18.5 g when the pH was allowed to drift in nonbuffered medium (Fig. II-1 A and C). This suggested that the pH shift which occurred between days 3 and 7 in nonbuffered medium enhanced cell growth or perhaps the presence of the organic buffer, MES, was slightly inhibitory. To resolve this question, cells were inoculated in medium buffered at 5.2 with 0.05 M MES and after 7 days NaOH was added to raise the pH to 6.0, a pH shift similar to that occurring in nonbuffered medium (Fig. II-2). The growth of these cells was equal to that of cells grown in nonbuffered medium (Fig. II-1 A and B).

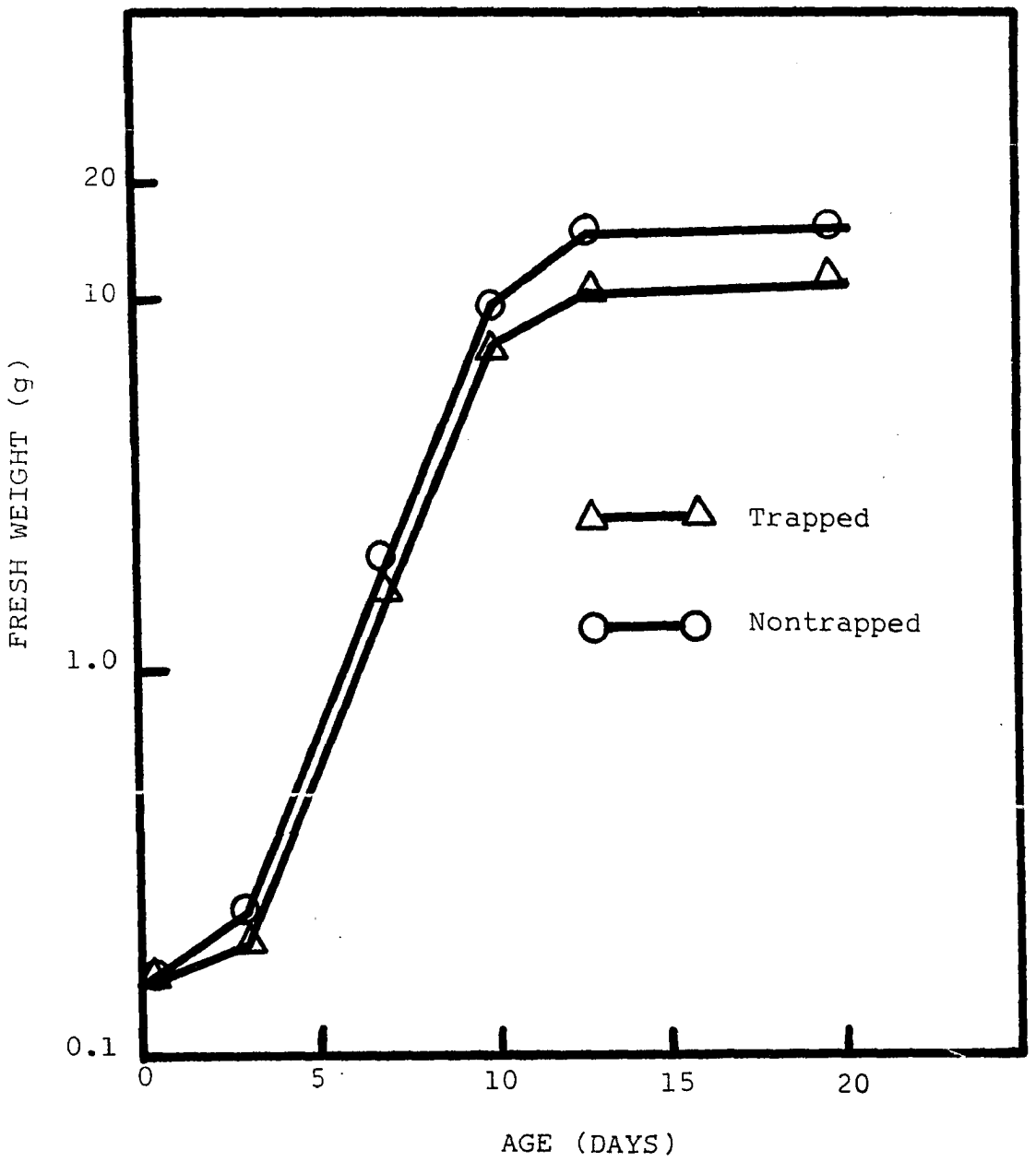
TABLE II-1.--Fresh weight of cells following 14 days of growth in media with different fixed pH. A 0.05M concentration of MES buffer was used.

pH	4.2	4.4	4.8	5.2	5.4	5.8	6.0	6.2	6.6	6.8
Fresh Wt. (g)	5.9	7.4	12.9	16.6	16.4	11.4	4.3	2.7	0.4	0.4

The information gained in the pH study described in the preceeding paragraph was used to examine more closely the CO_2 requirement for cell growth. A more CO_2 deficient environment was now provided by reducing the bicarbonate ion concentration in the medium by buffering it at a low pH (5.2) and simultaneously removing CO_2 from the air above the medium with a NaOH trap. The influence of this CO_2 deficient environment on cell growth was studied by comparing the fresh weights and dry weights of cultures grown for 14 days in medium with a fixed pH of 5.2, held in flasks either with or without CO_2 traps. When the CO_2 trap was present, the fresh weight increase was reduced by approximately 22%, but the dry weight increase was only slightly influenced (Fig. II-1 C and D), and in some experiments there was no influence on dry weight.

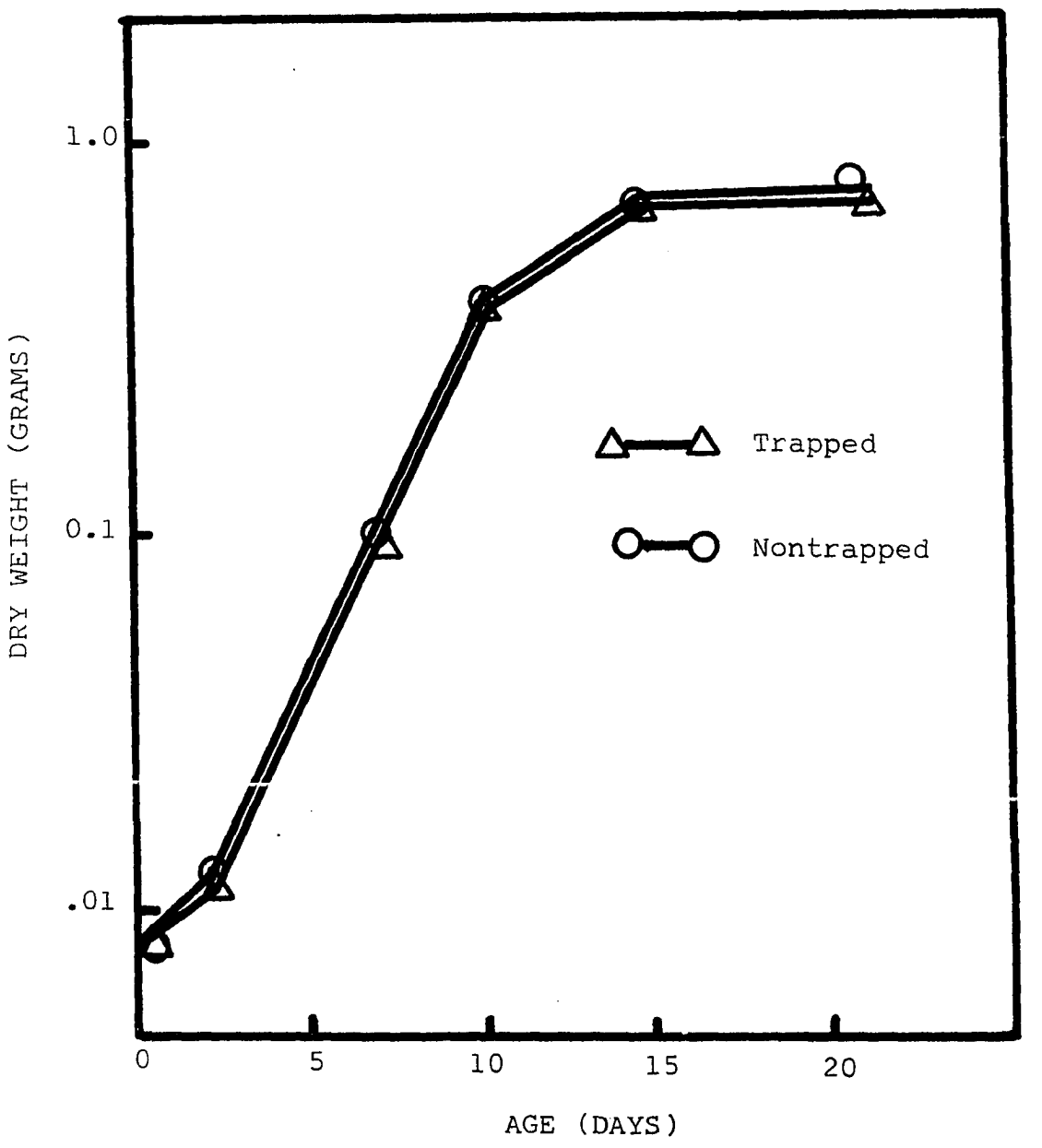
A study of growth kinetics under CO_2 deficient conditions (low pH plus CO_2 trap) showed that the inhibition of fresh weight increase occurred primarily between days 7 and 12 (Fig. II-3), a period which has been previously described as one of cell expansion accompanied by minimal cell division (15). A similar study of dry weight increases showed no differences (Fig. II-4). Thus it appeared that a CO_2 deficient environment inhibited growth due to cell expansion, since the fresh weight did not increase, but the synthesis of organic constituents appeared to continue since the dry weight increased.

Figure II-3.--A comparison of growth in normal versus CO_2 deficient air.



Medium used in both treatments was buffered at 5.4 with 0.05 molar MES.

Figure II-4.--A comparison of growth in normal versus CO_2 deficient air.

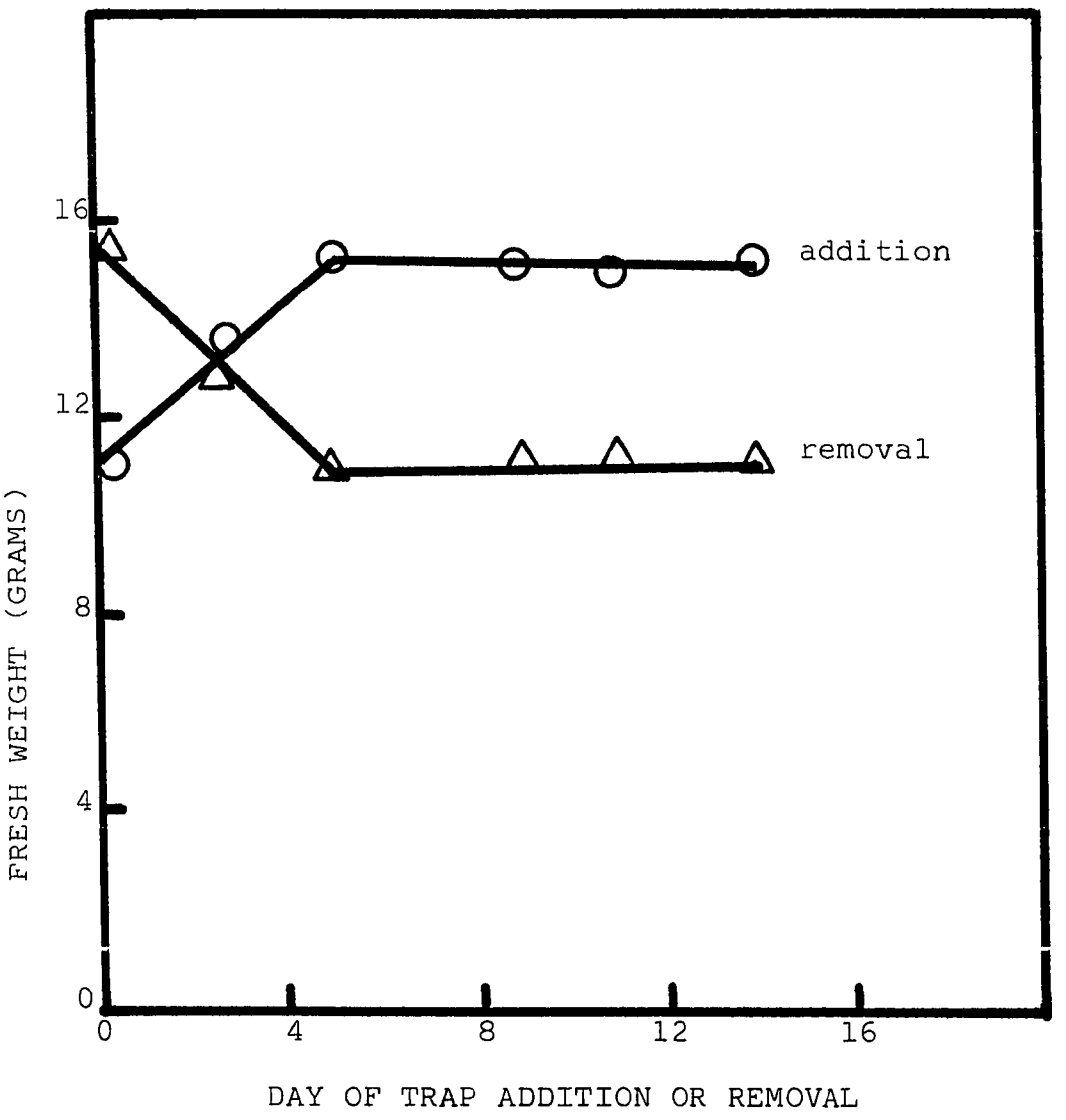


Medium used in both treatments was buffered at 5.4 with 0.05 molar MES.

It was not clear whether inhibition imposed by a CO_2 deficient environment required continuous privation of CO_2 or privation only during that period when inhibition occurred (day 7 to 12). To examine this, CO_2 traps were added or removed on different days during the growth cycle, and, after 14 days, the cells were harvested and weighed. Figure II-5 shows that when the trap was added for the entire growth period (from day 0), the yield was 10.8 g fresh weight. If the trap was added after 3 days, the growth was 13.7 g. However, if the trap was added on day 5 or thereafter, the yield was 15 g, approximately equal to that obtained when the trap was removed all the time (from day 0). If the trap was removed after 3 days, the yield was 12.3 g; if it was removed on day 5, or thereafter, the yield was approximately 11.0 g fresh weight, a value equal to that obtained when the trap was present continuously. Thus a CO_2 deficient environment inhibited growth only if it was provided within the first 5 days of growth.

Initial assays for PEP carboxylase were performed on crude extracts from 14 day old cells grown in nonbuffered medium without a CO_2 trap. PEP carboxylase activity was present and the fixation of $H^{14}CO_3^-$ was linear with time (Table II-2). A developmental study of PEP carboxylase activity was performed (Table II-3). The activity of this enzyme increased ten fold during the first three days, stayed at an extremely high level (0.50×10^6 cpm$\cdot$min$^{-1}\cdot$g^{-1} fresh weight) for the

Figure II-5.--The effect on growth of adding and removing the CO_2 trap at various times during browth.



Medium was buffered at 5.4 with 0.05 molar MES.

TABLE II-2.--Total amount of ^{14}C fixed by PEP carboxylase in 12 day old tissue after different incubation periods.

TIME (min)	^{14}C FIXED ($cpm \cdot min^{-1} \cdot g^{-1}$)
1	364
2	624
3	1001
4	1443

TABLE II-3.--Total amount of ^{14}C fixed by PEP carboxylase extracted from tissue of different ages.

	DAY					
	0	3	4	5	9	12
ACTIVITY (10^6 cpm·min^{-1}·g^{-1})	0.04	0.40	0.50	0.36	0.22	0.05

subsequent two day period, and then declined gradually. The developmental pattern was the same when a CO_2 trap was present.

The influence of light and kinetin on PEP carboxylase synthesis was studied by assaying for PEP carboxylase activity in extracts removed from cells grown under different light and kinetin treatments. Four treatments studied were: (1) light plus 0.00235 M kinetin, (2) light plus no kinetin, (3) dark plus 0.00235 M kinetin, (4) dark plus no kinetin. Enzyme assays were run on days 4½ and 6½. In all treatments the activity increased between days 0 and 4½ and decreased between days 4½ and 6½ (Table II-4). The highest activity was present in cells grown for 4½ days in light with no kinetin present.

TABLE II-4.--Total amount of ^{14}C fixed by PEP carboxylase extracted from tissue growth with or without kinetin and in the presence or absence of light for 4½ or 6½ days.

DAY	^{14}C FIXED ($cpm \cdot min^{-1} \cdot g^{-1}$ fresh weight)			
	With Kinetin		Without Kinetin	
	Light	Dark	Light	Dark
0	1630	1630	1630	1630
4½	58100	47700	38200	26000
6½	35300	29600	22600	17400

CHAPTER 4

DISCUSSION

The influence of fixed pH on the growth of suspension cultures of plant cells was studied for the first time by using a buffer (MES) which was nontoxic to growth and was not depleted from the medium. Maximum growth of suspension cultures (fresh weight increase) of Paul's Scarlet rose required a low pH (5.2-5.4) during the division phase of growth (day 0-7) and a higher pH (5.8 to 6.0) during the expansion phase (day 7-14) (15). Either a gradual shift of nonbuffered medium or an abrupt change of buffered medium (Fig. II-1) led to maximum growth. The stimulatory influence of a low pH was probably because it favored the uptake of NAA (28), an essential growth requirement for rose cells (26). The enhanced growth associated with a slightly higher pH during the expansion phase was surprising since high pH (>5.5) has been shown to inhibit cell expansion during coleoptile growth (29).

CO_2 privation studies conducted with aqueous cultures of animal and bacterial cells resulted in complete inhibition of growth (17,47). However, in the present study, the fresh weight increase of rapidly growing plant cells was reduced by only 22% and the dry weight increase was not influenced when

cells were grown for 14 days in a CO_2 deficient environment. Thus, rose cells showed a partial requirement for exogenous CO_2 whereas, animal and some bacterial cells have been shown to have an absolute requirement (17,47). This major difference in cell metabolism was most likely due to the high level of phosphoenolpyruvate carboxylase in rose cells whereas, it was absent from animal cells (50) and present in only a few species of bacterial cells (38,48). Since PEP carboxylase has a low Km for CO_2 (1,51), its presence in rose cells allowed for an efficient use of endogenously generated CO_2 coming from respiration. Thus, nonautotrophic CO_2 fixation may be an essential requirement for all cells, but plant cells have a greater capacity to use endogenously generated CO_2.

Cells grown in medium buffered at pH 5.2 appeared to require CO_2 during their division phase (15), but growth was curtailed primarily during the expansion phase. This may have been due to a reduction in cell division during early growth; thereby, reducing the number of cells available for expansion during the later stages of growth. An alternative is that cell division was normal but expansion was reduced. Microscopical studies conducted in an attempt to resolve this proved unsuccessful because of the heterogeneity of cell size and extensive clumping present in a single culture.

PART III

METABOLISM OF ENDOGENOUSLY GENERATED $^{14}CO_2$ VERSUS EXOGENOUSLY PROVIDED BICARBONATE-^{14}C

CHAPTER 1

INTRODUCTION

Radioisotope studies with procaryotic and eucaryotic cells showed that $^{14}CO_2$ provided in the dark was assimilated into a host of cell constituents, including amino acids, organic acids, and nitrogen bases (2,5,6,20,32,33,40,41,49). Several authors have proposed that nonautotrophic CO_2 fixation was necessary to replenish carbon skeletons removed from the TCA cycle (4,30,33), but others have felt that it contributed directly to carbon skeletons which were further metabolized to amino acids (19,54). Although the later has been suggested as an important role played by nonautotrophic CO_2 fixation in plants, an analysis of its contribution to protein synthesis has never been made. The present study was conducted to evaluate the contribution of nonautotrophic CO_2 fixation to the synthesis of amino acid and their subsequent use in protein formation.

CHAPTER 2

MATERIALS AND METHODS

Growth of Tissue

The cultures were grown on buffered MPR medium as previously described in Part I.

Extraction and Separation of Fractions

Approximately one gram samples were ground in 15 ml of hot 80% ethanol for 3 min. The ethanol homogenate was separated into chloroform soluble compounds, soluble amino acids, organic acids and alcohol insoluble residue, and the protein present in the insoluble residue was hydrolyzed as described by Fletcher and Beevers (15). Individual amino acids recovered from either the soluble phase or the protein hydrolysate were separated and identified by the chromatographic technique of Morris and Thompson (24). The organic acids were bound to Dowex 1-X10 (formate form) and eluted with a formic acid gradient (0-4 N). The amount of each individual organic acid was estimated by titration of the various fractions with 0.05 N NaOH to a phenolphthalein endpoint.

Isotopes Used

The glutamate-1-^{14}C used was purchased from New England Nuclear Laboratories. The bicarbonate-^{14}C and acetate-^{3}H used were purchased from International Chemical and Nuclear Laboratory.

Determination of ^{14}C and ^{3}H Activity

The presence and amount of ^{14}C and ^{3}H in the various fractions were determined with a Beckman LS-100 liquid scintillation counter. The samples were placed into a scintillation vial containing 10 ml of scintillation fluid which consisted of 6 g of 2.5 diphenyloxazol and 100 g of napthalene per liter of 1,4 dioxane.

Uptake of ^{14}C or ^{3}H from Medium

The depletion of either bicarbonate-^{14}C, glutamate-1-^{14}C or acetate-^{3}H from the medium was determined by removing 10 ul of the incubation medium at various time intervals and assaying for radioactivity. The uptake of bicarbonate-^{14}C by cells was corrected for the direct loss of $^{14}CO_2$ to the atmosphere by running controls which consisted of duplicate amounts of bicarbonate-^{14}C in medium containing no cells.

Continuous Feeding Experiments

One gram samples of cells were incubated in 25 ml erlenmeyer flasks containing 5 ml of the medium (pH 7.5). The medium contained 4 μc of $NaH^{14}CO_3$ (specific activity 10 μc/μ mole). At the end of the incubation period the cells

were filtered through miracloth, washed thoroughly with deionized water, placed into 15 ml of hot 80% ethanol, and homogenized for 3 min on a Virtis grinder set at medium speed. In the time course study the cells were treated as above, except one gram samples were collected at various times. A similar experiment was conducted with glutamate-1-^{14}C (specific activity 1.3 μc/μ mole) except the pH of the medium was left at 5.5.

Pulse Label Experiments

Pulse experiments were performed with bicarbonate-^{14}C (specific activity 10 μc/μ mole) or glutamate-1-^{14}C (specific activity 1.3 μc/μ mole). Five grams of cells were incubated in 50 ml erlenmeyer flasks containing 25 ml of medium plus either 40 μc of bicarbonate-^{14}C or 50 μc of glutamate-1-^{14}C. The bicarbonate-^{14}C medium was kept at pH 7.5 to reduce loss of $^{14}CO_2$ to the atmosphere (46) whereas, the glutamate-1-^{14}C medium was kept at pH 5.4 to enhance the uptake of the substrate by the cells (22). Cells were incubated for 15 min and then the medium was sucked off. The cells were rinsed three times with nonradioactive medium. One gram of cells was harvested for a 0 time sample, and the remaining cells were divided equally among five 25 ml erlenmeyer flasks containing 5 ml of nonradioactive medium. The pH was the same for all of the medium used. During the incubation period and the time after pulse period, the flasks were rotated gently

on a rotary shaker. At times 0, 30, 60, 120, and 180 minutes after pulse one gram samples were collected, washed, and homogenized, as previously described.

Double Labeling Experiments

Double labeling experiments were performed by exposing cells to either 10 μc of glutamate-1-^{14}C and 20 μc of acetate-^{3}H, or 4 μc of bicarbonate-^{14}C and 200 μc of acetate-^{3}H. One gram samples were incubated for 60 minutes in 25 ml erlenmeyer flasks containing 5 ml of medium. The pH of the medium was 5.4 for the glutamate-acetate treatment and 7.5 for the bicarbonate-acetate treatment. After 60 minutes the cells were harvested, washed, and homogenized as previously described. The specific activities of the substrates provided in these experiments were: glutamate-1-^{14}C (1.3 μc/μ mole), acetate-^{3}H (1.0 μc/μ mole), and bicarbonate-^{14}C (10 μc/μ mole).

CHAPTER 3

RESULTS

CONTINUOUS FEEDING OF BICARBONATE-^{14}C TO 5 AND 11 DAY OLD CELLS

The utilization of ^{14}C-bicarbonate by five and eleven day old cells, which had been previously grown in either air or CO_2 deficient air was studied. At appropriate ages specially grown cells were transferred to aerated medium containing $NaH^{14}CO_3$ (specific activity of 10 $\mu c/\mu$ mole). The uptake of bicarbonate-^{14}C by the cells and the incorporation of ^{14}C into cell constituents were determined.

Privation of CO_2 during growth, or differences in cell age had little influence on the uptake of bicarbonate-^{14}C (Table III-1). In all samples bicarbonate ions were taken up rapidly during the first 30 min and more slowly thereafter. At the end of 90 min approximately 80% of the available bicarbonate-^{14}C had been taken up by the cells, except for eleven day old cells, previously grown in CO_2 deficient air where only 65% was taken up. After 90 min the amount of radioactivity taken up by all samples was approximately 1.75 X 10^6 CPM/g of cells.

TABLE III-1.--The percent of available bicarbonate-^{14}C taken up by the cells.

	5 DAYS OLD		11 DAYS OLD	
TIME (min)	AIR	CO_2 DEFICIENT AIR	AIR	CO_2 DEFICIENT AIR
30	72	73	68	55
60	80	79	71	60
90	80	81	77	65

After 90 minutes of incubation in bicarbonate-^{14}C, cell extracts were prepared and the distribution of ^{14}C among key cell constituents was determined (Table III-2). In five day old cells, a higher percent of the ^{14}C was in the organic acids of the cells taken from air (Table III-2). Fractionation of the organic acids showed that the majority of the ^{14}C was in malate (Table III-3). In eleven day old cells the distribution of ^{14}C was approximately the same in cells grown under both conditions. A comparison between cells of different ages showed the eleven day old cells to have a higher percent of ^{14}C in organic acids and a lower percent in protein amino acids and the chloroform soluble fraction.

Examination of the individual soluble amino acids showed that aspartate and glutamate were the most heavily labeled amino acids in cells grown for either five or eleven days under both conditions. However, ^{14}C was not restricted to these amino acids. Eleven of the fourteen amino acids examined, each had ^{14}C in excess of 1% of the total cpm (Table III-4). All eleven of these newly synthesized amino acids were available for protein synthesis (Table III-5). This suggested that nonautotrophic CO_2 fixation was an important source of carbon skeletons for amino acid synthesis and subsequent protein formation.

The percent distribution of ^{14}C among the soluble or protein bound amino acids was very similar for both ages and treatments. However the amount of incorporation was much

TABLE III-2.--Total cpm and the percent distribution of ^{14}C in cell constituents after a 90 minute incubation of cells in bicarbonate-^{14}C.

CELL CONSTITUENT	5 DAYS OLD		11 DAYS OLD	
	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)
CHLOROFORM SOLUBLE	22000(7.6)	22100(18.9)	4200(1.1)	3200(0.8)
PROTEIN AMINO ACIDS	63000(21.9)	52200(21.1)	20500(5.5)	23100(6.1)
SOLUBLE AMINO ACIDS	41100(14.2)	46800(18.9)	52300(14.3)	45200(11.3)
ORGANIC ACIDS	162300(56.3)	126000(51.0)	288000(79.1)	310000(81.8)

Numbers in parenthesis represent the percent of the total ^{14}C.

TABLE III-3.--The total cpm present and the percent distribution of ^{14}C in selected organic acids after a 90 minute incubation of cells in bicarbonate -^{14}C.

	5 DAYS OLD		11 DAYS OLD	
ORGANIC ACID	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)
MALATE	155200(95.3)	113500(89.7)	280800(97.2)	303000(97.5)
CITRATE	7300(4.5)	13100(10.3)	8200(2.8)	7700(2.5)
SUCCINATE	200(0.2)	---(0.0)	---(0.0)	---(0.0)

Numbers in parenthesis represent the percent of the total ^{14}C.

TABLE III-4.--The total cpm present and percent distribution of ^{14}C in soluble amino acids after a 90 minute incubation of cells in bicarbonate-^{14}C.

	5 DAYS OLD		11 DAYS OLD	
AMINO ACID	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)
ASPARTATE	8500(37.0)	10500(42.0)	5950(41.0)	10510(40.9)
THREONINE	700(3.0)	650(2.6)	620(4.3)	850(3.3)
METHIONINE	550(2.4)	250(1.0)	400(2.8)	100(0.4)
LEUCINE/ISO-LEUCINE	200(0.9)	250(1.0)	75(0.5)	110(0.4)
GLUTAMATE	9800(42.6)	10500(42.0)	3920(27.0)	9400(36.6)
PROLINE	400(1.7)	200(0.1)	675(4.6)	380(1.5)
SERINE	650(2.8)	510(2.0)	435(3.0)	2160(8.4)
GLYCINE	300(1.3)	650(2.6)	800(5.5)	575(2.2)
ALANINE	550(2.4)	450(1.8)	500(3.4)	850(3.3)
TYROSINE	150(0.7)	50(0.2)	0(0.0)	0(0.0)
VALINE	50(0.2)	100(0.4)	0(0.0)	0(0.0)
PHENYLALANINE	50(0.2)	0(0.0)	0(0.0)	150(0.6)
ASPARAGINE	400(1.7)	350(1.6)	600(4.1)	300(1.3)
GLUTAMINE	600(2.6)	350(1.5)	550(3.8)	220(1.1)

Numbers in parenthesis represent the percent of the total ^{14}C.

TABLE III-5.--Total cpm and percent distribution of ^{14}C in protein bound amino acids after a 90 minute incubation of cells in bicarbonate-^{14}C.

	5 DAYS OLD		11 DAYS OLD	
AMINO ACID	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)	AIR cpm (%)	CO_2 DEFICIENT AIR cpm (%)
ASPARTATE	14100(43.0)	14000(42.4)	4600(44.3)	5900(45.9)
THREONINE	3500(10.8)	2200(6.7)	490(3.9)	470(3.5)
METHIONINE	1500(4.6)	1200(3.6)	90(0.7)	50(0.4)
LEUCINE/ISO-LEUCINE	1000(3.1)	500(1.5)	150(1.2)	40(0.3)
GLUTAMATE	8000(24.7)	11000(33.3)	4100(32.5)	4230(31.1)
PROLINE	1600(4.9)	1400(4.2)	580(4.6)	420(5.3)
SERINE	400(1.2)	400(1.2)	360(2.9)	360(2.6)
GLYCINE	900(2.8)	1000(3.0)	710(5.6)	940(6.9)
ALANINE	900(2.8)	900(2.7)	500(4.0)	480(3.5)
TYROSINE	0(0.0)	0(0.0)	0(0.0)	0(0.0)
VALINE	400(1.2)	300(0.9)	40(0.3)	100(0.7)
PHENYLALANINE	300(0.9)	100(0.5)	0(0.0)	0(0.0)

Numbers in parenthesis represent the percent of the total ^{14}C.

greater in the five day old cells. Therefore all subsequent labeling studies were conducted with five day old cells grown in air.

TIME COURSE STUDY DURING CONTINUOUS PROVISION OF BICARBONATE-^{14}C TO FIVE DAY OLD CELLS

In the preceeding experiment it was shown that carbon 14 originating from bicarbonate ions was readily incorporated into a host of metabolites including eleven amino acids. It was presumed that the first compound into which bicarbonate-^{14}C was fixed was oxaloacetate (19). Labeling periods of shorter duration were provided in an attempt to follow the newly fixed ^{14}C from oxaloacetate to the ^{14}C labeled amino acids. Of special interest was the relationship between: oxaloacetate and the TCA cycle, oxaloacetate and malate, oxaloacetate and aspartate.

Most of the ^{14}C incorporated into the organic acids (Table III-6) was in malate (Table III-7). There was a large increase in malate-^{14}C from 0-35 minutes and a slight increase thereafter. The incorporation of ^{14}C into citrate, synthesized in the TCA cycle, followed a much different pattern. Carbon-14 accumulated in citrate during the first 15 minutes when bicarbonate-^{14}C was most plentiful. After 15 minutes ^{14}C disappeared from citrate, even though large amounts of malate-^{14}C remained. It was concluded that some of the oxaloacetate-^{14}C formed by $^{14}CO_2$ fixation, entered the TCA cycle, but if it were converted to malate-^{14}C then it did not

TABLE III-6.--Total cpm of ^{14}C in cell constituents at various time intervals during a 90 minute incubation of cells in bicarbonate-^{14}C.

CELL CONSTITUENT	TIME (min) 5	10	15	35	60
CHLOROFORM SOLUBLE	9490	6000	22080	31440	48128
ORGANIC ACIDS	102300	130500	228800	417600	396500
SOLUBLE AMINO ACIDS	21120	50050	109600	217200	131600
PROTEIN AMINO ACIDS	2310	4550	11200	56400	90300

TABLE III-7.--Total cpm of ^{14}C in selected organic acids at various time intervals during a 90 minute incubation of cells in bicarbonate-^{14}C.

ORGANIC ACID	TIME (min) 5	10	15	35	60
MALATE	84000	100000	198000	384000	404000
CITRATE	16000	20000	50000	33000	29000
SUCCINATE	231	720	570	500	890

enter the cycle. Furthermore the accumulation of ^{14}C in malate indicated that malate-^{14}C was not readily converted to oxaloacetate-^{14}C which could then enter the cycle. The pattern of ^{14}C incorporation into aspartate (Table III-8) was more similar to that of malate-^{14}C than to citrate-^{14}C; therefore, it was concluded that oxaloacetate-^{14}C coming from nonautotrophic $^{14}CO_2$ fixation was converted directly to either malate-^{14}C or aspartate-^{14}C. However, the conversion of malate-^{14}C to oxaloacetate-^{14}C and on to aspartate-^{14}C did not occur readily during the later stages of the experiment since the activity in malate-^{14}C persisted while it disappeared from aspartate-^{14}C.

The ^{14}C incorporated into the soluble amino acids increased during the first 35 minutes and decreased thereafter (Table III-6). The ^{14}C labeled amino acids in protein increased steadily during the entire 60 minutes, thus indicating that the soluble amino acids formed were used in protein synthesis. The distribution of ^{14}C among the individual soluble and protein bound amino acids (Tables III-8 and III-9) was essentially the same as the previous experiment with bicarbonate-^{14}C (Tables III-4 and III-5).

CONTINUOUS PROVISION OF GLUTAMATE-1-^{14}C TO FIVE DAY OLD CELLS

Endogenously generated CO_2 arising from decarboxylation reactions may also serve as a source for nonautotrophic CO_2 fixation. Labeling studies were performed to: (1)

TABLE III-8.--Total cpm of ^{14}C in soluble amino acids at various time intervals during a 90 minute incubation of cells in bicarbonate-^{14}C.

AMINO ACID	TIME (min) 5	10	15	35	60
ASPARTATE	6347	8010	30568	68760	46389
THREONINE	143	150	560	2520	3480
METHIONINE	65	150	160	600	470
LEUCINE/ISO-LEUCINE	0	660	640	714	611
GLUTAMATE	5990	14925	42160	64080	44086
PROLINE	33	495	388	1362	1172
SERINE	205	225	624	1980	1598
GLYCINE	374	615	1320	1740	3055
ALANINE	124	4710	6104	4638	3318

TABLE III-9.--Total cpm of ^{14}C in protein amino acids at various time intervals during a 90 minute incubation of cells in bicarbonate-^{14}C.

AMINO ACID	TIME (min) 5	10	15	35	60
ASPARTATE	371	1550	4072	16980	23115
THREONINE	0	0	152	2556	5161
METHIONINE	0	210	72	372	686
LEUCINE/ISO-LEUCINE	0	300	144	648	808
GLUTAMATE	150	300	1136	8256	14852
PROLINE	46	420	216	1032	2162
SERINE	86	30	88	348	827
GLYCINE	26	210	304	1500	1683
ALANINE	72	60	512	2124	2667

determine what compounds are synthesized from endogenously generated CO_2; (2) compare the use of endogenously generated CO_2 with exogenously provided bicarbonate-^{14}C.

One endogenous source of CO_2 is glutamic acid, which is readily decarboxylated to CO_2 and gamma-aminobutyric acid by glutamic acid decarboxylase (9), or glutamic acid may be deaminated to form alpha-ketoglutaric acid, which, upon entry into the TCA cycle is immediately decarboxylated, giving rise to CO_2 and succinyl-CoA. In both cases, the carbon atom in the number one position of glutamic acid is released. Therefore glutamic acid-1^{14}C was used as an endogenous source of $^{14}CO_2$, and its appearance in metabolites not synthesized directly from glutamic acid could only be accounted for by nonautotrophic CO_2 fixation.

One gram samples were provided with either $NaH^{14}CO_3$ (specific activity of 10 μc/μ mole) or glutamate-1-^{14}C (specific activity of 1.3 μc/μ mole) for 70 min. The glutamate-1-^{14}C was taken up slowly as compared to the bicarbonate-^{14}C (Table III-10). After 70 min the distribution of ^{14}C in the bicarbonate-^{14}C treated cells (Table III-11) was comparable to previous studies (Table III-6). Carbon-14 recovered from the cells provided with glutamate-1-^{14}C was widely distributed (Table III-11). The large amount of radioactivity in the soluble amino acid fraction was due to glutamate-^{14}C (Table III-13) which presumably was not decarboxylated.

TABLE III-10.--The percent of available bicarbonate-^{14}C and glutamate-1-^{14}C taken up by 5 day old cells.

TIME (min)	GLUTAMATE-1-^{14}C	BICARBONATE-^{14}C
15	9	67
30	15	84
70	39	85

TABLE III-11.--Total cpm of ^{14}C in cell constituents after a 70 minute incubation of cells in bicarbonate-^{14}C or glutamate-1-^{14}C.

CELL CONSTITUENT	GLUTAMATE-1-^{14}C	BICARBONATE-^{14}C
CHLOROFORM SOLUBLE	85800	13200
PROTEIN AMINO ACIDS	190600	42000
SOLUBLE AMINO ACIDS	1022400	28800
ORGANIC ACIDS	69625	148018

TABLE III-12.--Total cpm and percent distribution of ^{14}C in selected organic acids after a 70 minute incubation of cells in bicarbonate-^{14}C or glutamate-1-^{14}C.

ORGANIC ACID	GLUTAMATE-1-^{14}C cpm (%)	BICARBONATE-^{14}C cpm (%)
MALATE	46347(66)	147752(92)
CITRATE	8048(12)	8010(8)
SUCCINATE	15230(22)	256(.01)

Numbers in parenthesis represent the percent of the total ^{14}C.

TABLE III-13.--Total cpm and percent distribution of ^{14}C in soluble and protein bound amino acids after a 70 minute incubation of cells in bicarbonate-^{14}C or glutamate-1-^{14}C.

AMINO ACID	BICARBONATE-^{14}C cpm (%)	GLUTAMATE-1-^{14}C cpm (%)
	Soluble Amino Acids	
ASPARTATE	7950(50)	16000(14)
THREONINE	750(5)	5700(6)
METHIONINE	300(2)	46050(41)
LEUCINE/ISO-LEUCINE	800(5)	3600(4)
GLUTAMATE	8300(*)	475300(*)
PROLINE	2650(17)	13700(12)
SERINE	650(4)	2550(2)
GLYCINE	900(6)	19300(17)
ALANINE	2000(11)	4650(4)
	Protein Amino Acids	
ASPARTATE	11400(38)	2800(3)
THREONINE	2200(7)	2200(2)
METHIONINE	300(-)	300(1)
LEUCINE/ISO-LEUCINE	256(1)	13400(12)
GLUTAMATE	4000(*)	33000(*)
PROLINE	10300(34)	27900(26)
SERINE	1300(5)	4800(4)
GLYCINE	2800(10)	55800(52)
ALANINE	1300(4)	1000(1)

*Glutamate was not used to determine the percent distribution in this column.

A comparison between the labeling pattern of bicarbonate-^{14}C versus glutamate-1-^{14}C treated cells showed major differences. Examination of total ^{14}C in the organic acids (Table III-12) showed that cells provided with bicarbonate-^{14}C had 92% of the total in malate and .01% in succinate. In contrast to this when glutamate-1-^{14}C was provided, 66% of the total was in malate whereas only 22% was in succinate. The percent in citrate was approximately the same in both.

The pattern of ^{14}C distribution in the individual amino acids was also different. The percent distributions in Table III-13 were calculated without glutamate-^{14}C, since glutamate-1-^{14}C was used as one of the substrates. When tissue was supplied with bicarbonate-^{14}C, most of the ^{14}C present in the soluble and protein bound amino acids was in aspartate and glutamate (Table III-13). Tissue supplied with glutamate-1-^{14}C had a more even distribution of the ^{14}C among several amino acids, including the aspartate family (threonine, methionine, isoleucine). It was also noted that in the glutamate-1-^{14}C treatment, the aspartate family contained more ^{14}C in comparison to aspartate than in the bicarbonate-^{14}C treatment.

The extent to which each newly synthesized amino acid was available for protein synthesis was examined by determining the percent distribution of ^{14}C among soluble and protein bound amino acids (Table III-14). In the bicarbonate-^{14}C treatment more radioactivity was in the protein bound than

TABLE III-14.--The percent of total ^{14}C in the amino acids fixed into the protein fraction after a 70 minute incubation of cells in bicarbonate-^{14}C or glutamate-1-^{14}C.

AMINO ACID	BICARBONATE-^{14}C	GLUTAMATE-1-^{14}C
ASPARTATE	59	15
THREONINE	75	28
METHIONINE	50	1
LEUCINE/ISO-LEUCINE	24	79
GLUTAMATE	33	-
PROLINE	80	62
SERINE	67	65
GLYCINE	97	74
ALANINE	39	18

in the soluble form. For example, 59% of the ^{14}C in aspartate was in the protein fraction as compared to 15% in the aspartate protein fraction in glutamate-1-^{14}C treated tissue. This difference might have been due to the different sources of CO_2, or it might have stemmed from the different rates at which the bicarbonate and glutamate entered the tissue. To resolve this question a pulse study using the same substrates was performed.

PULSE LABEL OF GLUTAMATE-1-^{14}C AND BICARBONATE-^{14}C TO FIVE DAY OLD CELLS

Pulse label experiments were performed by providing tissue with either bicarbonate-^{14}C or glutamate-1-^{14}C for 15 min. The medium containing the label was then drawn off and replaced with nonradioactive medium. At various time intervals one gram samples of tissue were collected, and the distribution of ^{14}C among cell constituents was determined. During and immediately following the pulse the ^{14}C activity in the chloroform fraction of both treatments increased at first and then decreased (Table III-15).

In both treatments the ^{14}C content of the organic acids was highest immediately following the pulse. In the glutamate-1-^{14}C treated tissue the ^{14}C disappeared slowly from the organic acids; whereas, in the bicarbonate-^{14}C treated tissue a rapid decrease in activity occurred during the first 30 min. Examination of the ^{14}C in the individual organic acids showed a major difference in the distribution pattern.

TABLE III-15.--Total ^{14}C in cell constituents at various time intervals after a 15 minute pulse in glutamate-1-^{14}C or bicarbonate-^{14}C (cpm).

CELL CONSTITUENTS	SUBSTRATE	TIME AFTER PULSE (min) 0	30	60	120	180
CHLOROFORM SOLUBLE	Glut.	25600	88800	52800	35600	27000
	$H^{14}CO_3^-$	26800	30300	70000	69600	48600
PROTEIN AMINO ACID	Glut.	53800	82800	101400	116400	123200
	$H^{14}CO_3^-$	33000	46400	56000	40800	50800
SOLUBLE AMINO ACID	Glut.	655700	296000	196000	---	114400
	$H^{14}CO_3^-$	185800	39000	26000	18000	14200
ORGANIC ACID	Glut.	54100	52700	48000	---	51800
	$H^{14}CO_3^-$	442644	244478	298300	---	240900

A higher percent of the ^{14}C coming from glutamate-1-^{14}C entered succinate (Table III-16). In contrast to this, in the bicarbonate-^{14}C treated cells, at 0 time, there was 400 times more ^{14}C in malate than in succinate. These values were consistent with previously reported results collected when substrates were provided continuously (Table III-12). The malate formed from glutamate-1-^{14}C remained constant, while the malate formed from bicarbonate-^{14}C steadily declined (Table III-16).

The general pattern of ^{14}C changes in the amino acids was the same in both treatments (Table III-15). Immediately following the pulse large amounts of ^{14}C were present in the soluble amino acids and subsequently disappeared, while ^{14}C appeared in the protein. However, the decay of ^{14}C from the soluble amino acids was not matched by that appearing in protein. Analysis of the ^{14}C associated with individual amino acids (Table III-17 and Table III-18) showed that the source of ^{14}C, glutamate versus bicarbonate effected: (1) the percent distribution of ^{14}C among the soluble or protein bound amino acids, (2) the percent conversion of soluble ^{14}C labeled amino acids present at 0 time to protein bound ^{14}C at 60 min. Glutamate was not used in the calculations of percent distribution of ^{14}C in Tables III-17 and III-18 since glutamate-1-^{14}C was one of the substrates added. The ^{14}C in the individual soluble amino acids from both treatments (Table III-17) declined slowly during the 60 min after pulse. After 60 min the

TABLE III-16.--Total cpm and percent distribution of ^{14}C in selected organic acids after a 15 minute pulse in glutamate-1-^{14}C or bicarbonate-^{14}C.

ORGANIC ACID	SUBSTRATE	TIME AFTER PULSE (min) 0 cpm (%)	30 cpm (%)	60 cpm (%)	120 cpm (%)
MALATE	Glut.	20240(37)	22340(43)	19860(41)	21680(49)
	$H^{14}CO_3^-$	403529(91)	237278(97)	284900(97)	232464(96)
CITRATE	Glut.	4579(8)	2900(4)	1760(4)	3260(7)
	$H^{14}CO_3^-$	37964(8)	6360(2)	6920(2)	7420(3)
SUCCINATE	Glut.	29310(55)	27448(53)	26280(55)	18860(44)
	$H^{14}CO_3^-$	1151(1)	840(1)	1508(1)	1260(1)

TABLE III-17.--Total and percent distribution of ^{14}C in soluble amino acids after a 15 minute pulse in glutamate-1-^{14}C or bicarbonate-^{14}C.

AMINO ACID	SUBSTRATE	TIME AFTER PULSE (min) 0 cpm (%)	30 cpm (%)	60 cpm (%)
ASPARTATE	Glut.	3900(6)	2500(7)	2450(9)
	$H^{14}CO_3^-$	25400(21)	5500(27)	3950(32)
THREONINE	Glut.	5150(8)	2050(6)	1350(5)
	$H^{14}CO_3^-$	1500(1)	800(4)	600(5)
METHIONINE	Glut.	1050(2)	650(3)	300(2)
	$H^{14}CO_3^-$	400(0)	310(1)	200(2)
LEUCINE/ISO-LEUCINE	Glut.	23850(37)	12350(36)	11250(43)
	$H^{14}CO_3^-$	500(0)	500(2)	200(2)
GLUTAMATE	Glut.	348200(*)	83500(*)	40850(*)
	$H^{14}CO_3^-$	80200(66)	10500(51)	5050(41)
PROLINE	Glut.	11700(18)	7000(20)	4300(16)
	$H^{14}CO_3^-$	1100(1)	600(2)	400(3)
SERINE	Glut.	9100(14)	4900(14)	3450(13)
	$H^{14}CO_3^-$	2450(2)	1300(6)	950(8)
GLYCINE	Glut.	6800(11)	2850(8)	1500(6)
	$H^{14}CO_3^-$	2350(2)	700(4)	375(4)
ALANINE	Glut.	2750(4)	2050(6)	1500(6)
	$H^{14}CO_3^-$	8300(7)	500(2)	700(6)

*Glutamate was not used to determine the percent distribution in this column.

TABLE III-18.--Total and percent distribution of ^{14}C in protein amino acids after a 15 minute pulse in glutamate-1-^{14}C or bicarbonate-^{14}C.

AMINO ACID	SUBSTRATE	TIME AFTER PULSE (min) 0 cpm (%)	30 cpm (%)	60 cpm (%)
ASPARTATE	Glut.	700(5)	2800(15)	1200(3)
	$H^{14}CO_3^-$	11000(51)	13000(40)	14500(39)
THREONINE	Glut.	1800(13)	2900(15)	3200(8)
	$H^{14}CO_3^-$	1000(5)	1900(6)	2500(7)
METHIONINE	Glut.	300(2)	300(2)	500(1)
	$H^{14}CO_3^-$	100(1)	200(1)	600(2)
LEUCINE/ISO-LEUCINE	Glut.	2800(21)	2200(11)	19600(51)
	$H^{14}CO_3^-$	200(1)	900(3)	900(2)
GLUTAMATE	Glut.	38300(*)	24500(*)	23800(*)
	$H^{14}CO_3^-$	5600(26)	10200(31)	11200(30)
PROLINE	Glut.	3900(29)	5400(28)	8400(22)
	$H^{14}CO_3^-$	600(3)	1900(6)	2000(5)
SERINE	Glut.	1300(10)	1600(8)	2200(6)
	$H^{14}CO_3^-$	500(2)	700(2)	1000(3)
GLYCINE	Glut.	2400(18)	3700(19)	3000(8)
	$H^{14}CO_3^-$	700(3)	1600(4)	1600(4)
ALANINE	Glut.	300(2)	300(2)	500(1)
	$H^{14}CO_3^-$	1700(8)	2300(7)	2900(8)

*Glutamate was not used to determine the percent distribution in this column.

majority of the ^{14}C is still in aspartate and glutamate from cells fed bicarbonate-^{14}C; whereas, the ^{14}C from cells fed glutamate-1-^{14}C was more widely dispersed among several amino acids (isoleucine, proline, serine, glycine). The ^{14}C in all of the individual protein amino acids from both tissues (Table III-18) steadily increased during the 60 min after pulse. The distribution pattern of label was similar to that of the soluble amino acids.

The availability for protein synthesis of ^{14}C amino acids formed from either bicarbonate-^{14}C or glutamate-^{14}C was studied. This was examined by calculating: (1) the percent distribution of ^{14}C among soluble and protein bound amino acids at 60 minutes following the pulse (Table III-19), and (2) the percent of the ^{14}C in soluble amino acids at 0 time which was recovered as protein bound ^{14}C at 60 minutes (Table III-20). The later calculation was performed as follows:

$$100 \times \frac{^{14}C \text{ in protein amino acids at 60 min} - ^{14}C \text{ in protein at 0}}{^{14}C \text{ in sol. amino acid at 0} - ^{14}C \text{ in sol. amino acid at 60 min}}$$

The percent of the total ^{14}C labeled amino acids incorporated into protein 60 minutes after pulse was consistently higher in each individual amino acid of the bicarbonate-^{14}C treated tissue (Table III-19). Similar results occurred in a previously reported experiment in which these substrates were provided continuously (Table III-14).

TABLE III-19.--The percent distribution of ^{14}C between soluble and protein bound amino acids at 60 minutes following a 15 minute pulse of either glutamate-1-^{14}C or bicarbonate-^{14}C.

AMINO ACID	BICARBONATE-^{14}C	GLUTAMATE-1-^{14}C
ASPARTATE	79	33
THREONINE	81	70
METHIONINE	75	63
LEUCINE/ISO-LEUCINE	82	64
GLUTAMATE	69	36
PROLINE	83	66
SERINE	59	39
GLYCINE	81	67
ALANINE	81	21

TABLE III-20.--The percent of the ^{14}C in soluble amino acids at 0 time which was recovered as protein bound ^{14}C at 60 minutes after a 15 minute pulse in glutamate-1-^{14}C or bicarbonate-^{14}C.

AMINO ACID	BICARBONATE-^{14}C	GLUTAMATE-1-^{14}C
ASPARTATE	16	34
THREONINE	166	37
METHIONINE	250	27
LEUCINE/ISO-LEUCINE	233	133
GLUTAMATE	1	0
PROLINE	200	61
SERINE	33	16
GLYCINE	46	11
ALANINE	16	8

The second method of calculating the data (Table III-20) showed that the cells provided with bicarbonate-^{14}C had a lower percent conversion of aspartate-^{14}C and a higher percent conversion for all other amino acids studied. The aspartate family amino acids (threonine, methionine, isoleucine) from cells provided with bicarbonate-^{14}C had over 100% of the ^{14}C soluble amino acids recovered as protein bound. This meant that additional amounts of these amino acids had to be synthesized during the time after pulse period. In cells provided with glutamate-1-^{14}C, all of the amino acids, except for isoleucine and proline, had less than 40% of the ^{14}C in the soluble amino acids at 0 time recovered as protein bound ^{14}C at 60 minutes after pulse. Both methods of processing the data indicated that those amino acids synthesized from bicarbonate-^{14}C were used more efficiently in protein synthesis.

DOUBLE LABELING WITH ACETATE-^{3}H AND GLUTAMATE-1-^{14}C OR BICARBONATE-^{14}C

Since the distribution of ^{14}C among the organic acids and amino acids was different in tissue provided with either an external or internal source of CO_2, a double labeling experiment was performed to determine if there was any similarity between either of these patterns and the pattern produced when tritiated acetate was metabolized in the TCA cycle. Tissue was fed either $NaH^{14}CO_3$ (specific activity of 10 $\mu c/\mu$ mole) and Na acetate-^{3}H (specific activity of 1

μc/μ mole) or glutamate-1-^{14}C (specific activity of 1.3 μc/μ mole) and $Na^{3}H$-acetate (specific activity of 1 μc/μ mole) continuously for 60 minutes. After these treatments carbon 14 and tritium were present in all fractions of both tissues (Table III-21). When glutamate-1-^{14}C and acetate-^{3}H were provided the percent distribution of these two isotopes was similar. The distribution of radioactivity among citrate, succinate, and malate was 71, 9, 20 and 68, 15, 17 for ^{14}C and ^{3}H respectively (Table III-22). Also, the distribution of ^{14}C and ^{3}H were similar in the individual amino acids recovered from tissue fed glutamate-1-^{14}C and acetate-^{3}H. In contrast to this, the patterns of ^{14}C and ^{3}H were different in the individual amino acids from tissue fed bicarbonate-^{14}C and acetate-^{3}H (Table III-23). Most of the ^{14}C of both the soluble and protein bound amino acids coming from bicarbonate-^{14}C went into aspartate and glutamate. The ^{14}C and ^{3}H coming from either glutamate or acetate were more evenly distributed into several amino acids.

TABLE III-21.--Total ^{14}C and ^{3}H in cell constituents after a 60 minute incubation in glutamate-1-^{14}C and acetate-^{3}H, or bicarbonate-^{14}C and acetate-^{3}H.

CELL CONSTITUENT	Experiment 1		Experiment 2	
	^{14}C From Glut.	^{3}H From Acetate	^{14}C From HCO_3^-	^{3}H From Acetate
CHLOROFORM SOLUBLE	21800	298400	12600	20000000
PROTEIN AMINO ACID	285000	130470	75600	180000
SOLUBLE AMINO ACID	672500	189400	80400	904200
ORGANIC ACIDS	64932	48800	250900	616200

TABLE III-22.--Total and percent distribution of ^{14}C and ^{3}H in selected organic acids after a 60 minute incubation in glutamate-1-^{14}C and acetate-^{3}H, or bicarbonate-^{14}C and acetate-^{3}H.

	Experiment 1		Experiment 2	
ORGANIC ACID	^{14}C From Glut. cpm (%)	^{3}H From Acetate cpm (%)	^{14}C From HCO_3^- cpm (%)	^{3}H From Acetate cpm (%)
MALATE	46223(71)	33072(68)	239016(95)	369978(68)
CITRATE	5915(9)	7399(15)	11121(4)	58250(21)
SUCCINATE	12794(20)	8336(17)	791(1)	119011(11)

TABLE III-23.--Total and percent distribution of ^{14}C and ^{3}H in soluble and protein bound amino acids after a 60 minute incubation in glutamate-1-^{14}C and acetate-^{3}H, or bicarbonate-^{14}C and acetate-^{3}H.

AMINO ACID	^{14}C From Glut. cpm (%)	^{3}H From Acetate cpm (%)	^{14}C From HCO_3^- cpm (%)	^{3}H From Acetate cpm (%)
		Soluble Amino Acids		
ASPARTATE	5760(5)	3980(14)	7655(54)	8295(17)
THREONINE	2125(2)	1085(4)	1050(7)	1350(3)
METHIONINE	1600(1)	800(3)	285(2)	2800(6)
LEUCINE/ISO-LEUCINE	82800(74)	7355(27)	1375(9)	4775(10)
GLUTAMATE	203800(*)	60710(*)	17495(*)	106405(*)
PROLINE	13575(12)	7900(29)	700(5)	13645(28)
SERINE	2300(2)	3975(14)	1085(7)	13685(28)
GLYCINE	1985(2)	1775(6)	840(6)	1910(4)
ALANINE	2060(2)	700(3)	1465(10)	2055(4)
		Protein Amino Acids		
ASPARTATE	3590(9)	2000(6)	12080(57)	9800(25)
THREONINE	5060(12)	1640(5)	3610(17)	2310(6)
METHIONINE	760(2)	2650(8)	---(--)	4000(10)
LEUCINE/ISO-LEUCINE	7460(18)	5730(16)	1200(6)	7800(20)
GLUTAMATE	142660(*)	55990(*)	9510(*)	25400(*)
PROLINE	15650(38)	16550(47)	1200(6)	4700(12)
SERINE	1970(5)	3070(8)	460(1)	7800(20)
GLYCINE	6930(16)	3410(10)	1380(7)	1730(3)
ALANINE	100(-)	150(-)	1200(6)	1600(4)

*Glutamate was not used to determine the percent distribution in this column.

CHAPTER 4

DISCUSSION

The metabolic role played by nonautotrophic CO_2 fixation in plant tissue was studied by providing rose cells with a source of $^{14}CO_2$ and after various time periods determining the distribution of ^{14}C among cell constituents. Preliminary studies showed that the radioactive substrates used were taken up equally well by tissue which had been grown in either air or CO_2 deficient air. Therefore, cells grown in air were used in the subsequent studies.

When actively growing cells (5 days old) were incubated in bicarbonate-^{14}C, the majority of the ^{14}C was recovered in malate. Most likely, the bicarbonate-^{14}C was fixed into oxaloacetate-^{14}C (considered the first product of nonautotrophic CO_2 fixation [19]), and it was converted immediately to malate as many authors have reported (5,22,32,40,41,49). Radioactivity was also present in succinate and citrate.

A large portion of the ^{14}C fixed nonautotrophically in actively growing cells was found in the soluble amino acids with aspartate and glutamate being the most heavily labeled, as shown by other investigators (5,22,32). However, unlike previous studies, in the present work nine other amino

acids possessed ^{14}C and all of them were readily utilized for protein synthesis. This finding was consistent with the idea advanced by several authors (5,32,42) that nonautotrophic CO_2 fixation is important for amino acid and protein synthesis. It also suggested that the reduced growth noted earlier in this dissertation during CO_2 privation studies might be attributed to a restricted supply of amino acids for protein synthesis.

Provision of an internal or an external source of $^{14}CO_2$ showed that both were used for nonautotrophic CO_2 fixation, and the ^{14}C entered the same cell constituents. Thus, any essential requirement for nonautotrophic CO_2 fixation could possibly be satisfied by internally generated CO_2 and thereby allow for a limited amount of growth, as noted earlier in the CO_2 privation studies.

Major differences were noted in the percent distribution of ^{14}C among cell constituents, depending upon the source of $^{14}CO_2$. The most striking difference was in the ^{14}C present in the organic acids. When tissue was provided with bicarbonate-^{14}C, the percent distribution among citrate, succinate, and malate was 8, 0, and 92 whereas, when glutamate-1-^{14}C was provided, the distribution was 12, 22, and 66. The $^{14}CO_2$ coming from an internal source appeared to be fixed into a compound which was metabolized similar to that of acetate utilized in the TCA cycle whereas, the $^{14}CO_2$ from an external source was fixed into a compound that did not readily enter

the TCA cycle. This can be explained by hypothesizing that CO_2 may be nonautotrophically fixed at various sites within the cell, and oxaloacetate, the presumed product, was metabolized differently. Mukerzi and Ting have shown PEP carboxylase to exist as several isoenzymes which were localized in different cell compartments such as the cytoplasm, chloroplasts, and mitochondria (25). Perhaps the $^{14}CO_2$ from bicarbonate-^{14}C is fixed in the cytoplasm whereas, the $^{14}CO_2$ from glutamate-1-^{14}C is fixed in the mitochondria, where it is released when glutamic acid is deaminated to form alpha ketoglutaric acid, which is decarboxylated in the TCA cycle.

REFERENCES

1. Bandurski, R. S. 1955 Further studies on the enzymatic synthesis of oxalacetate from phosphorylenolpyruvate and carbon dioxide. Jour. Biol. Chem. 217: 137-150.

2. Baugh, C. L., J. W. Lanham, and M. J. Surgalla. 1964. Effects of bicarbonate on growth of Pasteurella pestis. J. Bacteriol. 88: 553-558.

3. Beevers, L. and R. H. Hageman. 1969. Nitrate reduction in higher plants. Ann. Rev. Plant Physiol. 20: 495-522.

4. Chang, R. S., H. Liepens, and M. Margolish. 1961. Replacement of CO_2 in HeLa cultures. Proc. Soc. Exp. Biol. and Med. 106: 149.

5. Clegg, C. J. and C. P. Whittingham. 1970. Dark CO_2 fixation by potato tuber tissue. Phytochem. 9: 279-287.

6. Danner, J. and I. P. Ting. 1967. CO_2 metabolism in corn roots. Plant Physiol. 42: 719-724.

7. Davidson, D. and P. L. Webster. 1967. Effects of IAA and myo-inositol on mitotic cycles. In: F. Whightman and G. Setterfield, eds., Proceedings of the 6th International Conference on Plant Growth Substances. Runge Press, Ottawa, pp. 543-551.

8. Davies, M. E. 1971. Multisample enzyme extraction from cultured plant suspensions. Plant Physiol. 47: 38-42.

9. Dixon, R. O. D. and L. Fowden. 1961. ϒ-Aminobutyric acid metabolism in plants. Annals of Botany. 25: 513-530.

10. Dougall, D. K. 1966. Biosynthesis of protein amino acids in plant tissue culture II. Further isotope competition experiments using protein amino acids. Plant Physiol. 41: 1411-1415.

11. Dougall, D. K. 1965. The biosynthesis of protein amino acids in plant tissue culture I. Isotope competition experiments using glucose-u-C^{14} and the protein amino acids. Plant Physiol. 40: 891-897.

12. Dougall, D. K. 1970. Threonine deaminase from Paul's Scarlet rose tissue cultures. Phytochem. 9: 959-964.

13. Dougall, D. K. and M. M. Fulton. 1967. Biosynthesis of protein amino acids in plant tissue culture III. Studies on the biosynthesis of arginine. Plant Physiol. 42: 3 87-390.

14. Dougall, D. K. and M. M. Fulton. 1967. Biosynthesis of protein amino acids in plant tissue culture IV. Isotope competition experiments using glucose-U-C^{14} and potential intermediates. Plant Physiol. 42: 941-945.

15. Fletcher, J. S. and H. Beevers. 1970. Acetate metabolism in cell suspension cultures. Plant Physiol. 45: 765-772.

16. Fletcher, J. S. and H. Beevers. 1971. Influence of cycloheximide on the synthesis and utilization of amino acids in suspension cultures. Plant Physiol. 48: 261-264.

17. Geyer, R. P. and R. S. Chang. 1958. Bicarbonate as an essential for human cells in vitro. Arch. Biochem. and Biophys. 73: 500-506.

18. Good, N. E., G. D. Winget, T. N. Winter, S. Connally, R. Izawa, and R. M. M. Singh. 1966. Hydrogen ion buffers for biological research. Biochem. 5: 467-477.

19. Lachica, R. V. F. 1968. Carbon dioxide fixation. Enzymologia. 34: 281-298.

20. Lachica, R. V. F. and P. A. Hartman. 1968. Carbon dioxide fixation and the synthesis of aspartic acid by S. faecuim var. Durans. Biochemical and Biophysical Research Communications. 32: 691-695.

21. Lamport, D. T.A. 1964. Cell suspension cultures of higher plants: Isolation and growth energetics. Exp. Cell Res. 33: 195-206.

22. Lips, S. H. and H. Beevers. 1966. Compartmentation of organic acids in corn roots. I. Differential labeling of 2 malate pools. Plant Physiol. 41: 709-712.

23. Loewus, F. 1971. Carbohydrate interconversions. Ann. Rev. Plant Physiol. 22: 337-364.

24. Morris, C. J. and J. F. Thompson. 1965. Conversion of m-carboxyphenylalanine to m-carboxyphenylglycine in Wedgewood iris leaves. Arch. Biochem. Biophys. 110: 506-510.

25. Mukerji, S. K. and I. P. Ting. 1969. Phosphoenolpyruvate carboxylase isoenzymes. Arch. Biochem. Biophys. 131: 336-340.

26. Nesius, K. and J. S. Fletcher. 1973. Carbon dioxide and pH requirements of nonphotosynthetic tissue culture cells. Physiol. Plant. 28: 259-263.

27. Nesius, K., L. E. Uchytil, and J. S. Fletcher. 1972. Minimal organic medium for suspension cultures of Paul's Scarlet rose. Planta. 106: 173-176.

28. Norris, R. F. and M. J. Bukovac. 1972. Effect of pH on penetration of naphthaleacetic acid and naphthaleneacetamide through isolated pear leaf cuticle. Plant. Physiol. 49: 615-618.

29. Rayle, D. L. and R. Cleland. 1970. Enhancement of wall loosening and elongation by acid solutions. Plant. Physiol. 46: 250-255.

30. Runyan, W. S. and R. P. Geyer. 1967. Partial replacement of CO_2 in strain L and HeLa cultures. P.S.E.B.M. 125: 1301-1304.

31. Slack, C. R. and M. D. Hatch. 1967. Comparative studies on the activity of carboxylases and other enzymes in relation to the new pathway of photosynthetic carbon dioxide fixation in tropical grasses. Biochem. 103: 660-668.

32. Splittstoesser, W. E. 1966. Dark CO_2 fixation and its role in the growth of plant tissue. Plant Physiol. 41: 755-759.

33. Stallknecht, G. F. and C. J. Mirocha. 1970. Carbon dioxide fixation by uredospores of Uromyces phaseoli and its incorporation into cellular metabolites. Phytopathology. 60: 1338-1342.

34. Stolwijk, J. A. J. and K. V. Thimann. 1957. On the uptake of carbon dioxide and bicarbonate by roots and its influence on growth. Plant Physiol. 32: 513-520.

35. Street, H. E. 1966. Growth, differentiation and organogenesis in plant tissue and organ cultures. In: E. N. Willmer, ed., Cells and Tissue in Culture, Vol. III. Academic Press, New York.

36. Street, H. E. and G. G. Henshaw. 1966. Introduction and methods employed in plant-tissue culture. In: E. N. Willmer, ed., Cells and Tissue in Culture, Vol. III. Academic Press, New York.

37. Tanner, W. and O. Kandler. 1968. Myo-inositol, a co-factor in the biosynthesis of stachyose. Eur. J. Biochem. 4: 233-239.

38. Theodore, T. S. and E. Englesberg. 1964. Mutant of Salmonella typhimurium deficient in the carbon dioxide fixing enzyme phosphoenolpyruvic carboxylase. Bacteriol. 88: 946-955.

39. Ting, I. P. 1971. Nonautotrophic CO_2 fixation and crassulacean acid metabolism. In: M. D. Hatch, C. B. Osmond, and R. O. Slayter, eds., Photosynthesis and Photorespiration. Wiley--Interscience, New York, pp. 169-185.

40. Ting, I. P. and I. W. Sherman. 1966. Carbon dioxide fixation in malaria. I. Kinetic studies in Plasmodium lophurae. Comp. Biochem. Physiol. 19: 855-869.

41. Ting, I. P. and W. C. Zschoche. 1970. Asparagine biosynthesis by cotton roots. Carbon dioxide fixation and cyanide incorporation. Plant Physiol. 45: 429-434.

42. Ting, I. P. and W. M. Dugger. 1967. CO_2 metabolism in corn roots. I. Kinetics of carboxylation and decarboxylation. Plant Physiol. 42: 712-718.

43. Tulecke, W. 1964. A haploid tissue from the female gametophyte of Ginkgo biloba L. Nature. 203: 94-95.

44. Tulecke, W. 1963. Research on tissue cultures of higher plants. Technical Report. Aerospace Medical Research Laboratory. Wright-Patterson Air Force Base, Ohio, pp. 63-124.

45. Tulecke, W. and L. G. Nickell. 1960. Methods, problems, and results of growing plant cells under submerged conditions. Trans. N.Y. Acad. Sci. 22: 196.

46. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric Techniques, Burgess Press, Minneapolis, pp. 18-45.

47. Valley, G. and L. F. Rettger. 1927. Influence of carbon dioxide on bacteria. J. Bacteriol. 14: 101-123.

48. Victor, R. and F. Lachia. 1968. Carbon dioxide fixation. Enzymologia. 34: 281-298.

49. Wagner, H. G. 1967. The incorporation of $^{14}CO_2$ into green peas in the dark. J. Exp. Bot. 18: 672-691.

50. Walker, D. A. 1962. Pyruvate carboxylation and plant metabolism. Biol. Rev. 37: 215-256.

51. Waygood, E. R., R. Mache, and C. K. Tan. 1969. Carbon dioxide, the substrate for phosphoenolpyruvate carboxylase from leaves of maize. Can. Bot. 47: 1455-1458.

52. Williams, B. L. and T. W. Goodwin. 1965. The terpenoids of tissue cultures of Paul's Scarlet Rose. Phytochem. 4: 81-88.

53. Wood, H. G. and C. H. Werkman. 1935. The utilization of CO_2 by the propionic acid bacteria in dissimilation of glycerol. Bacteriol. 30: 332.

54. Wood, H. G., Vennesland, B. and E. A. Evans. 1945. The mechanism of CO_2 fixation by cell free extracts of pigeon liver. J. Biol. Chem. 159: 153-158.