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PHOTOSYNTHETIC CARBON METABOLISM IN PRIMARY AND SECONDARY LEAVES OF *Zea mays* L.

by

GAIL MAN-WAH LAW

A Thesis

Submitted to

The Faculty of Graduate Studies

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PHOTOSYNTHETIC CARBON METABOLISM

IN

PRIMARY AND SECONDARY LEAVES OF *Zea mays* L.

by

GAIL MAN-WAH LAW

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

This thesis is affectionately dedicated to Harold and Helena, my brother and sister-in-law, whose unfailing love, wise counsel, moral and prayer support have enabled me to find myself, and to experience the joy, peace and the liberating power of the Gospel of Jesus Christ.

"In the day of my trouble I will call upon Thee:
For Thou wilt answer me.
Among the gods there is none like unto Thee, O Lord;
Neither are there any works like unto Thy works.
All nations whom Thou has made shall come
and worship before Thee, O Lord;
And shall glorify Thy Name.

For THOU ART GREAT, and DOEST WONDROUS THINGS:
THOU ART GOD ALONE."

Psalm 86: 7 - 10

ABSTRACT

Kinetic experiments of $^{14}CO_2$ incorporation into leaf discs of Zea mays. L. var. Earliking and identification of the metabolic products have shown that the photosynthetic carbon pathway of primary leaves from 6 - 8 day old seedlings differs from that of secondary leaves from 16 - 18 day old seedlings. The kinetic pattern of photosynthetic intermediates in primary leaves indicated that the Calvin cycle was predominant, whereas in secondary leaves the major route of carbon flow was via the C_4-dicarboxylic acid cycle as shown by the early production of malate which decreased with time.

These results were in spite of the fact that phosphoenolpyruvate carboxylase (EC 4.1.1.31), the key enzyme of the C_4 cycle was just as active in the chloroplast and cytoplasmic fractions of both primary and secondary leaves. However, it was observed that the phosphoenolpyruvate carboxylase of primary leaves had a higher apparent Km for its substrate, phosphoenolpyruvate, in comparison to that of the secondary leaves, which may be partly responsible for the differences.

Chloroplasts isolated from both primary and secondary leaves by the 'laceration technique' of Mache and Waygood (FEBS Letters 3: 89 - 92, 1969) were incapable of fixing CO_2 without the addition of a CO_2 acceptor, although some very young, undifferentiated chloroplasts from primary leaves possessed a small but significant capacity for endogenous CO_2 fixation.

Chloroplasts isolated from either primary or secondary leaves

were capable of fixing CO_2 into photosynthetic intermediates in the presence of 3.2 mM of phosphoenolpyruvate (its approximate K_{app}). This capacity was shared by the corresponding cytoplasmic (supernatant) fractions which showed greater activity, but gave a somewhat different pattern of photosynthetic intermediates.

For CO_2 fixation, the apparent Km values for phosphoenolpyruvate, HCO_3^- and Mg^{+2} were respectively: 4.00, 0.56 and 2.00 mM for primary leaf chloroplasts; 7.40, 0.89 and 0.30 mM for primary leaf supernatant; 2.04, 0.67 and 2.22 mM for secondary leaf chloroplasts and 2.44, 0.40 and 0.35 mM for secondary leaf supernatant.

These values corresponded reasonably well with the kinetic parameters in the enzymatic assay of phosphoenolpyruvate carboxylase of the corresponding fractions being respectively: 10.00, 0.43 and 4.00 mM for the enzyme from chloroplasts of primary leaves; 10.00, 0.44 and 1.14 mM for the primary leaf supernatant enzyme; 1.40, 0.27 and 5.71 mM for the enzyme from chloroplasts of secondary leaves and 3.85, 1.33 and 0.21 mM for the secondary leaf supernatant enzyme.

The response to temperature of the CO_2 fixation capacity and the phosphoenolpyruvate carboxylase activity of the chloroplast and supernatant fraction of primary leaves was not severely affected by subnormal temperature conditions (10 - 15^oC) during growth, but these activities were almost completely arrested in secondary leaf chloroplasts and extracts under the same conditions.

From these results it is apparent that phosphoenolpyruvate carboxylase participates in the regulation of CO_2 fixation capacity

of secondary leaf chloroplasts, and their capacity for CO_2 fixation is not merely due to contamination by the phosphoenolpyruvate carboxylase of its corresponding supernatant (cytoplasmic) enzyme.

The distribution of ^{14}C in the products of CO_2 fixation in the presence of 3.2 mM phosphoenolpyruvate in the chloroplast fractions of both leaf types was also different from that obtained in the supernatant fractions of the corresponding leaf types. The former were more heavily labelled in the amino acid and sugar fractions as compared to the latter which favoured incorporation of ^{14}C into the organic acid fraction. More specifically, in the chloroplast fraction of both leaf types the major portion of the radioactivity was recovered in glycerate, dihydroxyacetone and glyceraldehyde. The supernatant fraction showed significant label in glycerate only. Malate was not found as a product of CO_2 fixation in any of the fractions at this concentration (3.2 mM) of phosphoenolpyruvate. The absence of malate and the formation of the three 3-C compounds in chloroplast fractions was taken to be evidence in support of a transcarboxylation reaction resulting from the product of β-carboxylation as first envisaged by Hatch and Slack (Biochem. J. 101: 103-111, 1966) and corroborated biochemically by Pan (Ph. D. Thesis, University of Manitoba, 1974).

Investigations have shown that the 'laceration technique' is superior to the more commonly employed methods of 'grinding' and 'blending' for the isolation of chloroplasts. Although the chloroplast

'pellet' obtained is predominantly mesophyll in origin, the chloroplasts retain more of their in vivo physical and biochemical integrity. Bundle sheath chloroplasts generally account for 10% of the total and they can be separated by differential centrifugation. The method is recommended for the isolation of mesophyll chloroplasts from monocotyledonous plants for studies on CO_2 fixation and enzyme activities.

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TABLE OF CONTENTS

	Page
ABSTRACT	i
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF PLATES	xvii
LIST OF ABBREVIATIONS	xviii
INTRODUCTION	1
LITERATURE REVIEW	5
CARBON DIOXIDE ASSIMILATION - THE C_4 HYPOTHESES	5
A. Radiotracer Studies with Leaf Tissues	6
B. Radiotracer and Enzyme Studies with Isolated Chloroplasts and Differentially Enriched Plant Extracts	10
C. Radiotracer and Enzyme Studies with Isolated Cell Types	14
D. Radiotracer Studies with Tissue Culture	17
MATERIALS AND METHODS	18
RESULTS	
I. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO PRIMARY LEAF TISSUE OF MAIZE	32
A. Kinetic Experiments	32
1. Rate of Incorporation	32
2. Distribution of ^{14}C in the Methanol-water Soluble Fraction	32
3. Distribution of ^{14}C in the Various Products of the Organic Acid, Amino Acid and Sugar Fractions	33

RESULTS (continued)

Page

B. Pulse-Chase Experiments 34

1. Distribution of ^{14}C in the Methanol-water Soluble Fraction 35

2. Distribution of ^{14}C in the Various Products of the Organic Acid, Amino Acid and Sugar Fractions 35

II. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO SECONDARY LEAF TISSUE OF MAIZE 38

A. Kinetic Experiments 38

1. Rate of Incorporation 38

2. Distribution of ^{14}C in the Methanol-water Soluble Fraction 38

3. Distribution of ^{14}C in the Various Products of the Organic Acid, Amino Acid and Sugar Fractions 39

B. Pulse-Chase Experiments

1. Distribution of ^{14}C in the Methanol-water Soluble Fraction 41

2. Distribution of ^{14}C in the Various Products of the Organic Acid, Amino Acid and Sugar Fractions 41

III. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO ISOLATED CHLOROPLASTS FROM MAIZE LEAVES IN THE ABSENCE OF PHOSPHOENOLPYRUVATE 43

A. Isolated Chloroplasts from Primary Leaves 43

B. Isolated Chloroplasts from Secondary Leaves 43

IV. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO ISOLATED CHLOROPLASTS AND SUPERNATANT OF MAIZE LEAVES IN THE PRESENCE OF PHOSPHOENOLPYRUVATE 45

A. Rate of Incorporation 46

1. Isolated Chloroplasts from Primary Leaves 46

2. Supernatant from Primary Leaves 46

3. Isolated Chloroplasts from Secondary Leaves 47

4. Supernatant from Secondary Leaves 47

RESULTS (continued) Page

B. Determination of Apparent Km Values for PEP, HCO_3^- and Mg^{+2} for CO_2 Fixation 48

1. Isolated Chloroplasts from Primary Leaves 48

2. Supernatant from Primary Leaves 49

3. Isolated Chloroplasts from Secondary Leaves 49

4. Supernatant from Secondary Leaves 49

C. Products of $NaH^{14}CO_3$ Fixation 50

D. Effect of Temperature 53

1. Isolated Chloroplasts from Primary Leaves 54

a) Normal temperature treatment 54

b) Cold treatment 54

2. Supernatant from Primary Leaves 54

a) Normal temperature treatment 54

b) Cold treatment 55

3. Isolated Chloroplasts from Secondary Leaves 55

a) Normal temperature treatment 55

b) Cold treatment 55

4. Supernatant from Secondary Leaves 56

a) Normal temperature treatment 56

b) Cold treatment 56

V. STUDIES OF PHOSPHOENOLPYRUVATE AND RIBULOSE DIPHOSPHATE CARBOXYLASESIN THE CHLOROPLAST AND SUPERNATANT OF MAIZE LEAVES 57

A. Determination of Apparent Km Values for PEP, HCO_3^- and Mg^{+2} of Phosphoenolpyruvate Carboxylase 57

1. Chloroplastic PEP Carboxylase from Primary Leaves 57

2. Supernatant PEP Carboxylase from Primary Leaves 58

RESULTS (continued) Page

3. Chloroplastic PEP Carboxylase from Secondary Leaves 59

4. Supernatant PEP Carboxylase from Secondary Leaves 59

B. Effect of Temperature on the Activities of Phosphoenolpyruvate and Ribulose Diphosphate Carboxylases in Maize Leaves 60

1. Chloroplastic PEP and RuDP Carboxylases from Primary Leaves 60

a) Normal temperature treatment 60

b) Cold treatment 61

2. Supernatant PEP and RuDP Carboxylases from Primary Leaves 61

a) Normal temperature treatment 61

b) Cold treatment 62

3. Chloroplastic PEP and RuDP Carboxylases from Secondary Leaves 62

a) Normal temperature treatment 62

b) Cold treatment 63

4. Supernatant PEP and RuDP Carboxylases from Secondary Leaves 63

a) Normal temperature treatment 63

b) Cold treatment 63

VI. EVALUATION OF THE 'LACERATION TECHNIQUE' 65

A. Physical Integrity of Isolated Chloroplasts as Observed with Light Microscope 65

B. Recovery of Phosphoenolpyruvate and Ribulose Diphosphate Carboxylases by Different Techniques 67

C. Comparison of CO_2 Fixation Capacity of Secondary Leaf Chloroplasts Isolated by Different Techniques 68

D. Separation of Bundle Sheath and Mesophyll Chloroplasts by Differential Centrifugation 70

	Page
DISCUSSION	71
SUMMARY	93
BIBLIOGRAPHY	95
APPENDIX	107
Tables	108
Figures	127
Plates	217

LIST OF TABLES

Table No.		Page
I.	Distribution of radioactivity in extracts from maize primary leaf discs. Kinetic experiments.	108
II.	Distribution of radioactivity in various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs. Kinetic experiments.	109
III.	Distribution of radioactivity in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs. Kinetic experiments.	110
IV.	Distribution of radioactivity in various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs. Pulse-chase experiments.	111
V.	Distribution of radioactivity in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs. Pulse-chase experiments.	112
VI.	Distribution of radioactivity in extracts from maize secondary leaf discs. Kinetic experiments.	113
VII.	Distribution of radioactivity in various fractions of the methanol-water soluble fraction of the plant extracts from maize secondary leaf discs. Kinetic experiments.	
VIII.	Distribution of radioactivity in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs. Kinetic experiments.	115
IX.	Distribution of radioactivity in various fractions of the methanol-water soluble fraction of the plant extracts from maize secondary leaf discs. Pulse-chase experiments.	116
X.	Distribution of radioactivity in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs. Pulse-chase experiments.	117

Table No.		Page
XI.	Distribution of radioactivity in various fractions of maize leaf chloroplast and supernatant extracts after 2 minutes of $NaH^{14}CO_3$ fixation in the presence of 3.2 mM PEP.	118
XII.	Distribution of radioactivity in organic acid, amino acid and sugar fractions of maize leaf chloroplast and supernatant extracts after 2 minutes of $NaH^{14}CO_3$ fixation in the presence of 3.2 mM PEP.	119
XIII.	Distribution of radioactivity in products of maize leaf chloroplast and supernatant extracts after 2 minutes of $NaH^{14}CO_3$ fixation in the presence of 3.2 mM PEP.	120
XIV.	Comparison of methods of isolation for chloroplasts from maize primary and secondary leaves.	121
XV.	Distribution of intact chloroplasts between mesophyll and bundle sheath chloroplasts by the 'laceration technique'.	122
XVI.	Levels of phosphoenolpyruvate and ribulose diphosphate carboxylases by different extraction methods from maize primary and secondary leaves.	123
XVII.	Comparison of CO_2 fixation capacity of chloroplasts isolated by different techniques from maize secondary leaves.	124
XVIII.	Separation of bundle sheath and mesophyll chloroplasts by differential centrifugation.	125
XIX.	Summary of apprent Km values for PEP, HCO_3^- and Mg^{+2} for the CO_2 fixation capacity and PEP carboxylase activity of maize leaf cellular fractions.	126

LIST OF FIGURES

Figure No.		Page
1.	Incorporation of $^{14}CO_2$ into the various fractions of plant extracts from maize primary leaf discs. Kinetic experiments.	128
2.	Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs. Kinetic experiments.	130
3.	Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs. Kinetic experiments.	132
4.	Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs. Pulse-chase experiments.	134
5.	Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs. Pulse-chase experiments.	136
6.	Incorporation of $^{14}CO_2$ into the various fractions of plant extracts from maize secondary leaf discs. Kinetic experiments.	138
7.	Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of the plant extracts from maize secondary leaf discs. Kinetic experiments.	140
8.	Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs. Kinetic experiments.	142
9.	Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of the plant extracts from maize secondary leaf discs. Pulse-chase experiments.	143
10.	Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs. Pulse-chase experiments.	144

Figure No.		Page
11.	Incorporation of $^{14}CO_2$ into isolated chloroplasts of maize primary leaves in the absence of PEP.	148
12.	Incorporation of $^{14}CO_2$ into cellular fractions of maize primary leaves as a function of time.	150
13.	Incorporation of $^{14}CO_2$ into cellular fractions of maize secondary leaves as a function of time.	152
14.	Effect of PEP concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize primary leaves.	154
15.	Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize primary leaves.	156
16.	Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize primary leaves.	158
17.	Effect of PEP concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize primary leaves.	160
18.	Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize primary leaves.	162
19.	Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize primary leaves.	164
20.	Effect of PEP concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize secondary leaves.	166
21.	Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize secondary leaves.	168
22.	Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize secondary leaves.	170
23.	Effect of PEP concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize secondary leaves.	172

Figure No.		Page
24.	Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize secondary leaves.	174
25.	Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize secondary leaves.	176
26.	Effect of temperature on CO_2 fixation capacity of primary leaf cellular fractions of maize grown under 25°C day and 20°C night temperatures.	178
27.	Effect of temperature on CO_2 fixation capacity of primary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.	180
28.	Effect of temperature on CO_2 fixation capacity of secondary leaf cellular fractions of maize grown under 25°C day and 20°C night temperatures.	182
29.	Effect of temperature on CO_2 fixation capacity of secondary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.	184
30.	Effect of PEP concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize primary leaves.	186
31.	Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize primary leaves.	188
32.	Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize primary leaves.	190
33.	Effect of PEP concentration on the initial velocity of PEP carboxylase in supernatant fraction of maize primary leaves.	192
34.	Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in supernatant fraction of maize primary leaves.	194
35.	Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in supernatant fraction of maize primary leaves.	196

Figure No.		Page
36.	Effect of PEP concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize secondary leaves.	198
37.	Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize secondary leaves.	200
38.	Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize secondary leaves.	202
39.	Effect of PEP concentration on the initial velocity of PEP carboxylase in supernatant fraction of maize secondary leaves.	204
40.	Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in supernatant fraction of maize secondary leaves.	206
41.	Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in supernatant fraction of maize secondary leaves.	208
42.	Effect of temperature on PEP and RuDP carboxylase activities in primary leaf cellular fractions of maize grown under 25°C day and 20°C night temperatures.	210
43.	Effect of temperature on PEP and RuDP carboxylase activities in primary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.	212
44.	Effect of temperature on PEP and RuDP carboxylase activities in secondary leaf cellular fractions of maize grown under 25°C day and 20°C night temerpatures.	214
45.	Effect of temperature on PEP and RuDP carboxylase activities in secondary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.	216

LIST OF PLATES

Plate No.		Page
1.	Sample radioautogram of the products in the organic acid fraction of leaf disc extracts.	218
2.	Sample radioautogram of the products in the amino acid fraction of leaf disc extracts.	220
3.	Sample radioautogram of the products in the sugar fraction of leaf disc extracts.	222
4.	Sample radioautogram of the products in the organic acid fraction of chloroplast extracts.	224
5.	Sample radioautogram of the products in the amino acid fraction of chloroplast extracts.	226
6.	Sample radioautogram of the products in the sugar fraction of chloroplast extracts.	228

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DTT	Dithioerythritol
EDTA	Ethylene diamine tetra-acetic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid
MES	2-(N-morpholino) ethanesulfonic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PEP	Phosphoenolpyruvate
PGA	Phosphoglyceric acid
POPOP	1,4-Bis (2-5) phenyloxazoylbenzene
PPO	2,5-Diphenyloxazole
PVP	Polyvinyl pyrolidone
RuDP	Ribulose diphosphate
TES	N-tris (Hydroxymethyl) Methyl-2-aminoethanesulfonic acid
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

The view has long been held that higher plants assimilate CO_2 through the Calvin cycle via RuDP carboxylase (Benson and Calvin, 1947) in which the initial product is 3-PGA. However, it was shown in the 1960's that in some plants the initial product of CO_2 assimilation was a four-carbon organic acid. For this reason, the former group of plants are referred to as C_3 plants and the latter C_4 plants (Downton and Tregunna, 1968). After the proposal of the new pathway of CO_2 assimilation for C_4 plants by Hatch and Slack (1966), considerable work has been done on many C_4 species (Slack et al., 1969; Bucke and Long, 1971; Latzko et al., 1971) with various newly developed techniques in an attempt to elucidate the details of the pathway. As some areas have been clarified, more problems have arisen and many questions remain unanswered.

Maize is one of the C_4 plants initially studied. While the majority of workers agree that maize is a C_4 plant (Slack et al., 1969; Berry et al., 1970; Samejima and Miyachi, 1971), it has also been demonstrated that RuDP serves as primary CO_2 acceptor in maize leaves (Latzko et al., 1971) which is a characteristic feature of C_3 plants; and the products of CO_2 fixation in vitro by isolated maize chloroplasts are those of the C_3 rather than the C_4-pathway (Gibbs et al., 1970; O'Neal et al., 1971, 1972). The latter group of workers used either primary leaves or whole seedlings of 2 to 4 day old maize plants. But the majority of the other investigators used more mature

plants over two weeks old. It is doubtful that results obtained from very young developing leaf tissue could be representative for mature, fully developed tissue as assumed by some of these authors.

The C_4-pathway as proposed by Hatch and Slack (1966) involves the concerted effort of both mesophyll and bundle sheath chloroplasts. It is believed that the initial site of CO_2 fixation is in the mesophyll chloroplasts resulting in the formation of a C_4-dicarboxylic acid. This initial product has to be either decarboxylated or transcarboxylated before the carbon can be further metabolized by the bundle sheath chloroplasts. If the former takes place, the decarboxylated CO_2 is believed to be refixed by the bundle sheath chloroplasts. If the latter takes place, the carboxyl group is supposed to be 'transferred' onto a carboxyl acceptor with the subsequent formation of regular C_3 photosynthetic intermediates. The enzyme believed to be involved with decarboxylation reaction, NADP-malic enzyme, has been found to be located in the bundle sheath cells (Slack et al., 1969; Hatch and Kagawa, 1973). Bundle sheath cells of maize have also been shown to decarboxylate malate in vitro (Huber et al., 1973). On the other hand, evidence for a transcarboxylation reaction has also been obtained with maize extracts (Pan, 1974). However, decarboxylation and/or transcarboxylation reactions with subsequent formation of regular C_3 photosynthetic intermediates have yet to be demonstrated.

Although isolated chloroplasts from some C_3 plants have been shown to possess the ability of fixing CO_2 without the addition of

substrate (or CO_2 acceptor) (Kalberer et al., 1967), very little work of this nature had been reported on C_4 plants. The only successful reports were all investigations on very young maize seedlings of 2 to 4 day old (Gibbs et al., 1970; Poincelot, 1972). Two reports on CO_2 fixation by chloroplasts isolated from mature C_4 plants were both on studies of chloroplast systems fortified with PEP (Baldry et al., 1969; Waygood et al., 1971). Interestingly enough, this capacity of CO_2 fixation by isolated mesophyll protoplasts of mature maize leaves was also dependent on the addition of PEP (Kanai and Edwards, 1973a). Phosphoenolpyruvate carboxylase is very active in C_4 plants and has been found by most workers to be located in the cytoplasm of the mesophyll cells (Andrews et al., 1971; Lyttleton, 1971). In the light of the activity and location of this enzyme, two questions one would naturally ask are (a) whether the capacity for CO_2 fixation by isolated chloroplasts in the presence of PEP is an artifact due to unavoidable contamination of the inactive chloroplast by the cytoplasm during isolation and (b) if this is the case, what further role the mesophyll chloroplasts are assuming if they are not involved with the β-carboxylation of PEP.

Most work on isolation of C_4 chloroplasts has been criticized on the basis of technical difficulties in eliminating contamination by cytoplasmic enzymes and separation of the two types of chloroplasts (Black, 1973; Laetsch, 1974). Accordingly, whenever a new method is developed the research worker has to be familiar with both the advantages and limitations of the technique and interpret the results

within the practical limits of the technique employed. Most commonly employed methods are blending of leaf tissues with a blender or grinding them in a mortar. A less popular technique, the 'laceration technique', first developed by Mache and Waygood (1969) and later modified by Arya and Waygood (1971, unpublished) has never been studied in comparison with the more popular isolation methods with regards to physical and biochemical integrity of the chloroplasts obtained.

With these problems in mind, the present investigation was undertaken to answer the following questions:

1. Are the discrepancies cited from literature on maize photosynthetic pathway the result of failure on the investigators' part to employ plant tissues of comparable developmental stages?
2. Is it possible to distinguish a transcarboxylation or a decarboxylation reaction by studying the products of CO_2 fixation of isolated chloroplasts?
3. Is CO_2 fixation by isolated chloroplasts in the presence of PEP an artifact due to unavoidable contamination by the cytoplasmic enzyme PEP carboxylase?
4. What is the role of mesophyll chloroplasts in addition to production of the initial CO_2 acceptor, PEP?
5. Is the 'Laceration Technique' desirable for the isolation of chloroplasts? What are some of its advantages and limitations?

LITERATURE REVIEW

The distinguishing feature in the basic architecture of a C_4 leaf is the radial arrangement of chlorenchyma around the vascular bundles. The chlorenchyma is differentiated into an inner layer of large thick-walled, cylindrical cells containing prominent chloroplasts and one or more outer layers of palisade-like cells. The wide occurrence of C_4 plants in tropical regions mostly subjected to wet and arid conditions led many workers to believe that the structure of C_4 leaves was related to their physiological and biochemical characteristics (Laetsch, 1974). The following is a summary of the past research related to the metabolism of C_4 plants.

CARBON DIOXIDE ASSIMILATION - THE C_4 CYCLE HYPOTHESES

Radioisotopes have proved to be useful in the study of CO_2 assimilation in C_4 plants. While earlier investigations were conducted with leaf tissues, more recent ones have been facilitated by the development of different techniques of isolation of chloroplasts and cell types. Although mesophyll and bundle sheath chloroplasts have never been isolated in their purest forms, non-aqueous and aqueous methods of extraction of plant tissues together with density fractionation, and sequential grinding of leaf tissues have yielded fractions of leaf extracts differentially enriched with mesophyll or bundle sheath cell contents, or with mesophyll or bundle sheath chloroplasts. Recently, protoplasts of mesophyll cells and bundle sheath cells were successfully separated from C_4 plants such as crabgrass (Edwards *et al*,

1970, 1971; Edwards and Black, 1971) and maize (Kanai and Edwards, 1973a; 1973b). This technique further helps in clarifying the picture of carbon metabolism in C_4 plants.

A. Radiotracer Studies with Leaf Tissues

It was observed that photosynthetic fixation of CO_2 by sugar cane leaves resulted in a primary labelling of the organic acids, malate and aspartate, following a short term exposure of the leaf tissue to radioactive carbon dioxide (Kortschak et al., 1965). This finding was confirmed by Hatch and Slack (1966) who proposed a cycle linking CO_2 fixation into C_4 acids with a carboxyl transfer reaction to RuDP producing 3PGA. After a series of perceptive experiments (Slack and Hatch, 1967; Hatch and Slack, 1968; 1969) and successful location of various enzymes by a non-aqueous stepwise method of density fractionation of leaf homogenate they revised the first proposed scheme (Slack et al., 1969; Hatch and Slack, 1970). The revised pathway consists of two interconnected metabolic cycles. Carbon dioxide is introduced into the first cycle by the carboxylation of PEP, and the oxaloacetate so formed is rapidly interconverted into pools of malate and aspartate. The C-4 carboxyl of one or both of the dicarboxylic acids is transferred to an acceptor (RuDP) provided by the second cycle, appearing as C-1 of 3PGA. The pyruvate, derived from C-1, C-2 and C-3 of the dicarboxylic acid is then phosphorylated to regenerate the primary carbon dioxide acceptor, PEP. In the second cycle, 3PGA is converted to hexose phosphates

and then to photosynthetic end products by a series of reactions similar to those operative in the Calvin cycle. This cycle is completed by the reaction leading to the formation of the C_5 carboxyl acceptor that provides C-2 and C-3 of 3PGA. The first cycle operates in the mesophyll cells whereas the second cycle in the bundle sheath cells.

This C_4-dicarboxylic acid pathway was supported by the work of Johnson and Hatch (1970) who found, in kinetic and pulse-chase experiments on maize and sugar cane leaves, that the route via C-4 of the dicarboxylic acids was essentially the sole route of entry of carbon to 3PGA. That a C_4 acid was the initial product of this pathway has been confirmed by several workers. Berry et al. (1970) found that 90% of the radioactivity could be recovered from C_4 acids in a 2 second exposure of maize leaves to radioactive carbon dioxide. Samejima and Miyachi (1971) also reported a light enhanced dark fixation of CO_2 into aspartate and malate in maize leaves. Their transient experiments provided further evidence that the step of transferring radioactivity from C_4 acid to 3PGA was one that required light. This finding was confirmed by Farineau (1971) whose similar transient experiments provided evidence that light was required for the decarboxylation of C_4 acids. Furthermore it was observed that the changes in pool sizes of PEP and aspartate under the same transient conditions supported the involvement of these compounds as substrate and product for the operation of a β-carboxylation pathway in mesophyll cells and the C_4 acids were the source of CO_2

for the operation of the Calvin cycle in maize.

The transfer of radioactivity from the C-4 carboxyl of the C_4 acid onto an acceptor appearing as C-1 of 3PGA calls for either a transcarboxylation or a decarboxylation plus a refixation reaction. The evidence for a decarboxylation reaction was provided by Hatch (1971) who reported that the internal pool size of CO_2 in Amaranthus and maize leaves was ten fold greater in light than in darkness. He suggested that this large pool of CO_2 was derived from malate, and the C_4-pathway served as a CO_2 concentrating mechanism.

Another less popular model to C_4-pathway states that the initial carboxylation reaction takes place in the cytoplasm of mesophyll cells and that C_4 acids probably are decarboxylated in the chloroplasts and CO_2 incorporated via the photosynthetic reductive pentose phosphate pathway (Calvin cycle). Photosynthetic intermediates are transported from mesophyll cell chloroplasts to bundle sheath cell chloroplasts where they are converted to starch. Some CO_2 could be fixed directly in the bundle sheath cells. This model states that photorespiration goes on primarily in bundle sheath cells, and that the evolved CO_2 is reassimilated in the cytoplasm of mesophyll cells. In this model, the mesophyll cells act as a CO_2 trap rather than as the pump indicated in Hatch and Slack's model (Laetsch, 1974). All the evidence which support the first model could be used as evidence for the second model as well. Furthermore, it has been found that bundle sheath cells of Digitaria sanguinalis (L.) Scop. (Edwards and Black, 1971) and maize (Chollet and Ogren, 1972) are able to fix CO_2 in vitro.

The presence of RuDP carboxylase in fractions derived from mesophyll cells was also reported (Slack et al., 1969: Berry et al., 1970; Bucke and Long, 1971; Poincelot, 1972). Some enzymes of the photosynthetic reductive pentose phosphate pathway, e.g. NADP-specific glyceraldehyde phosphate dehydrogenase, were found to have no differential distribution between the mesophyll and bundle sheath cell layers (Slack et al., 1969; Hatch and Slack, 1970; Edwards and Black, 1971; Hatch and Kagawa, 1973). Farineau (1971) also pointed out that the Calvin cycle might well be operative in mesophyll chloroplasts as well as in bundle sheath chloroplasts.

The favoured view for carboxyl transfer step at present is one of decarboxylation for which theory the isotopic composition of the photosynthetic intermediates in C_4 plants would have to be determined at the step of RuDP carboxylase. However, the studies on enzymatic fractionation of carbon isotopes for these C_4 intermediates did not support this postulation, but rather that of a transcarboxylation (Whelan et al., 1972). Evidence for such a transcarboxylation reaction has also been obtained with maize extract (Pan, 1974).

Contrary to the mostly accepted model which states that the β-carboxylation takes place in the mesophyll chloroplasts, it has been shown that RuDP can serve as primary CO_2 acceptor in maize primary leaves, and PEP appears to be a product of CO_2 fixation (Latzko et al., 1971). These investigators suggested that a C_4-dicarboxylic acid was not required as a carrier of CO_2 in C_4 plants.

B. Radiotracer and Enzyme Studies with Isolated Chloroplasts and Differentially Enriched Plant Extracts

Sequential grinding of plant tissues and non-aqueous density fractionation of plant extracts are methods which have helped to clarify the picture of compartmentation especially with reference to enzyme location and properties of individual enzymes.

It is important to note the localization of various enzymes involved in the C_4-dicarboxylic acid pathway for a better understanding of the operation of the pathway. Slack et al. (1969) first reported that PEP carboxylase demonstrated an occasional association with the chlorophyll fraction (i.e. chloroplasts) in non-aqueous density fractionation experiments. Baldry et al. (1969), Gibbs et al. (1970) and Lyttleton (1971) reported that it was not necessarily associated with chloroplasts and was probably localized in the cytoplasm. However, it is generally agreed that it is located predominantly in the mesophyll cells (Lyttleton, 1971; Huang and Beevers, 1972; Hatch and Kagawa, 1973) rather than in the bundle sheath cells of C_4 plants.

The localization of other enzymes involved in the C_4-dicarboxylic acid pathway have been investigated by many workers (Slack et al., 1969; Hatch and Kagawa, 1973). It is generally agreed that pyruvate Pi dikinase, NADP-specific malate dehydrogenase, adenylate kinase are of mesophyll origin and fructose diphosphate aldolase, alkaline fructose diphosphatase, ribulose phosphate kinase, ribulose-diphosphate carboxylase are of bundle sheath origin. Distributed equally between the mesophyll and bundle sheath cells are 3PGA kinase,

NADP-specific glyceraldehyde phosphate dehydrogenase, triose phosphate isomerase, phospho-hexoisomerase and phospho-glucomutase.

Variations among C_4 species had been noticed. The initial product of CO_2 fixation in some species is malate, while in others, aspartate. The former species are referred to as 'malate formers' and the latter 'aspartate formers'. Downton (1970) observed a high malic enzyme content in 'malate formers' which did not have a postillumination burst of CO_2, and he proposed that in these species carboxyl transfer from C-4 of C_4 acid was catalysed by malic enzyme which not only enabled the decarboxylation of this C_4 acid, but also the refixation of the decarboxylated CO_2 mediated by the NADPH transferred with the malate. Thus the malic enzyme functions as a catalyst for the decarboxylation reaction as well as producing reducing power.

The carboxyl transfer step in 'aspartate formers' had been a puzzle to many research workers until recently. Johnson et al.(1971) and Andrews et al.(1971) reported on an inverse relationship between the content of malic enzyme and that of aspartate and alanine amino transferases in C_4 plants. The two amino transferases were equally distributed between mesophyll and bundle sheath cells of 'aspartate formers'. They proposed that aspartate, instead of malate, was the carrier of CO_2, and was transported from the mesophyll cells into the bundle sheath cells, in which it was converted into oxaloacetate, and the latter decarboxylated to produce CO_2 for 3PGA formation via RuDP. Hatch and Mau (1973) and Hatch (1973) investigated the

localization of these amino transferases. They reported that in Atriplex spongiosa, an 'aspartate former', there were three isoenzymes for each of the amino transferases. One of the aspartate amino transferases was a constituitive enzyme, and was very low in activity. The other two were induced by light and were high in activity, one being localized in mesophyll cytoplasm and the other in the bundle sheath mitochondria. Of the three isoenzymes of alanine amino transferases, one was associated with mesophyll cells and one with bundle sheath cells, while the third lost its activity during isolation. Hatch and Kagawa (1973, 1974) have demonstrated the presence of an NAD-specific malic enzyme localized in mitochondria of bundle sheath cells of Atriplex spongiosa and some other 'aspartate formers'. The enzyme activity was high and comparable to photosynthetic rates, and the activity was enhanced by light. They proposed a scheme for the decarboxylation of aspartate formers which was mediated through the co-operation of mesophyll cytoplasmic aspartate aminotransferase, bundle sheath mitochondrial aspartate aminotransferase, malate dehydrogenase and NAD-specific malic enzyme. The aspartate formed in cytoplasm of mesophyll cell was transported into the bundle sheath mitochondria, where it was deaminated and converted into oxaloacetate, which was in turn reduced to malate, and the latter decarboxylated by the NAD-specific malic enzyme to give CO_2 for the formation of 3PGA via RuDP carboxylase. The properties of these enzymes have been studied by these workers.

Other variations in the carboxyl transfer step are possible.

Edwards et al. (1971) reported relatively high activities of PEP carboxykinase in C_4 species which were low in malic enzyme, and proposed that this enzyme could serve to decarboxylate oxaloacetate in these species.

By using the technique of differential grinding of sugar cane leaves, Baldry et al. (1971) and Bucke and Long (1971) were able to obtain enzymes of the Calvin cycle, the bulk of which were recovered in grana-containing chloroplasts. PEP carboxylase was released with the breakage of the non-chlorophyllous bulliform cells and chlorophyllous mesophyll cells. They suggested that the site of β-carboxylation of CO_2 was in the bulliform cells, and the C_4 acid would then be translocated to the mesophyll cells where decarboxylation would take place. The CO_2 released would be photoassimilated by the grana containing chloroplasts in the mesophyll cells, mediated by the Calvin cycle enzymes. The resulting sugars or sugar-phosphates might then be translocated to the bundle sheath chloroplasts for storage as starch.

Carbon dioxide fixation by isolated chloroplasts has been reported by Baldry et al. (1969) with sugar cane and Waygood et al. (1971) with maize. In both cases the fixation by chloroplasts was light and phosphoenolpyruvate dependent. The products of fixation by sugarcane chloroplasts were mainly C_4 acids while those of maize chloroplasts were a whole array of intermediates and products of the Calvin cycle and C_4 acids. The work of these investigators tend to support the fact that the C_4-pathway was operative in chloroplasts of C_4 plants. Contrary to their work, Gibbs et al. (1970) and O'Neal et al. (1971, 1972) reported successful isolation of chloroplasts from

primary leaves of maize, which could fix CO_2 without exogenous PEP. The products of fixation were those of the Calvin cycle and not of C_4-pathway.

C. Radiotracer and Enzyme Studies with Isolated Cell Types

The operation of the C_4-dicarboxylic acid pathway of photosynthesis relies on the physical juxtaposition of cell types, and the intracellular alignment of different organelles especially chloroplasts, mitochondria and possibily microbodies. Sequential grinding, non-aqueous density fractionation and differential centrifugation methods, though proven to be useful in elucidating the mechanism of this C_4-pathway, are not without limitations. The major problems lie in the unavoidable contamination of one type of chloroplasts or differentially enriched plant extracts by their chlorophyllous counterpart, and the impossibility of isolating organelles in their natural state. A more reliable method for studying this mechanism would therefore call for a separtion of cell types. Although there had been earlier reports of the isolation of mesophyll cells from higher plants, such attempts had never been successful with C_4 plants until Edwards *et al.* (1970; 1971) and Edwards and Black (1971) reported the separation of bundle sheath cells from mesophyll cells of *Digitaria sanguinalis* (L.) Scop. leaves. They proposed another hypothesis for crabgrass photosynthesis which is essentially similar to the C_4-pathway proposed by Hatch and Slack (1966) except for the fact that atmospheric CO_2 could get into contact with bundle

sheath cells and was directly assimilated by the Calvin cycle enzymes operative in these cells. Supporting evidence for this hypothesis is that the functional Calvin cycle enzymes, including RuDP carboxylase were found in bundle sheath cells, and these were capable of fixing CO_2 when supplied with ribose-5-phosphate, adenosine diphosphate or ribulose diphosphate. Microscopic studies also showed that 15% of the internal surface area exposed to gases was probably located in the bundle sheath cells. This direct fixation could account for 15% of the total fixation by the plant, while the other 85% was fixed via the currently accepted C_4-pathway.

Edwards and Gutierrez (1972) were able to isolate mesophyll and bundle sheath cells from Panicum miliaceum (L.) by using the method of sequential grinding and filtration. Chollet and Ogren (1972) also isolated bundle sheath strands from maize by employing similar method. In both cases, it was shown that bundle sheath strands or cells were capable of fixing CO_2 without exogenous substrate in light. However, the addition of ribose-5-phosphate did enhance the fixation. The products of fixation were found to be mainly phosphosugars, 3PGA, and malate (Chollet and Ogren, 1973). Contamination of bundle sheath cells by mesophyll cell content was most probable in their experiments. Isolated mesophyll cells were not capable of fixing CO_2 without the addition of phosphoenolpyruvate or pyruvate. In this case, light was not an absolute requirement for, but it did enhance fixation.

A new method was recently developed by Kanai and Edwards (1973a,

1973b). By using an aqueous dextran polyethylene glycol two-phase system, they were able to isolate and purify mesophyll protoplasts and bundle sheath protoplasts of maize. It was found that the mesophyll protoplasts were unable to fix CO_2 without the addition of substrate. But at 10 mM phosphoenolpyruvate they were able to fix CO_2 up to 500 - 700 μmoles/mg chlorophyll/hr, both in light and in darkness. On the other hand, purified mesophyll protoplasts from C_3 plants and plants with the Crassulacean acid type of metabolism were able to fix CO_2 up to 30 - 50 μmoles/mg chlorophyll/hr without exogenous substrate in the light.

With the studies of these workers, it is very clear that the site of CO_2 fixation by β-carboxylation is in the mesophyll cells, and that the Calvin cycle is operative in the bundle sheath cells. The location of various enzymes were also confirmed: PEP carboxylase, NADP malate dehydrogenase and carbonic anhydrase are localized in mesophyll cells in the case of maize, while Ribose-5-phosphate isomerase, ribulose-5-phosphate kinase, RuDP carboxylase and fructose diphosphate aldolase are localized in bundle sheath cells. In case of malate formers, malic enzyme is located in bundle sheath cells, and NADP glyceraldehyde-phosphate dehydrogenase is equally distributed between the two cell types. In the case of aspartate formers, aspartate and alanine transaminases and glyceraldehyde 3-phosphate dehydrogenases are found about equally distributed between the photosynthetic cell types.

D. Radiotracer Studies with Tissue Culture

Cultures from stem explant of mature Froelichia gracilis (Hook) Mog. (Laetsch and Kortschak, 1972) and from sugar cane stalk (Kortschak and Nickell, 1970) had been fed with radioactive bicarbonate. Though both species had typical C_4 type of leaf anatomy, the products of fixation by the callus cells in these C_4 plant cultures demonstrated a C_3 type of fixation. It was suggested that both C_3 and C_4 types of fixation could operate in the same plant. The hypothesis was supported by Kortschak's work with sugar cane tissue culture (Kortschak, 1971). He showed that the products of CO_2 fixation in chloroplasts of such culture were that of the Calvin cycle.

MATERIALS AND METHODS

PLANT TISSUE

Seeds of Zea mays var. Earliking were sown in flats of sterilized soil/sand/peat mixture in the proportion of 2:1:1. The flats were placed in the greenhouse at a 25°C day and 20°C night temperature and a photoperiod of 13 hour light with 11 hours of darkness. The soil was kept moist by watering daily with tap water. Six to eight days after planting, the primary leaves were fully unrolled and secondary leaves had just started to develop. Sixteen days to eighteen days after planting, the oldest secondary leaves were expanded and the primary leaves were without any apparent trace of senescence. For different experiments, fully unrolled primary leaves of 6 to 8 day old seedlings and fully expanded secondary leaves of 16 to 18 day old seedlings were excised. The leaves were washed with distilled water, placed on moist paper towels to prevent wilting and leaf discs were then cut out with a clean, sharp cork borer of 1 cm. in diameter. The leaf discs were used immediately in the experiments.

CHLOROPLAST PREPARATION

Chloroplasts were isolated by the 'laceration technique' of Mache and Waygood (1969) from primary leaves of 6 to 8 day old seedlings and secondary leaves of 16 to 18 day old seedlings. After harvesting, the leaves were washed with distilled water and placed

in a glass trough (3.5 cm x 1.5 cm x 42 cm) with 25 ml of Solution H (Shepherd et al., 1968). The leaves were gently lacerated parallel to the veins with five small closely spaced (1 mm apart) scalpels (Cat. no. 412, Irex German Surgicals 788, Adelaide Street, Toronto) with their ends embedded in a lucite block to form a handle. About 10 g of leaves were used each time. After laceration, the leaves were removed and the suspension was filtered through two layers of Kleenex tissue and was centrifuged at $0^{o}C$ for 50 seconds at 2,000 g. The pellet was then suspended in 1 ml of Solution A (Shepherd et al., 1968) and was used immediately. The supernatant was then centrifuged at 10,000 g for 15 minutes to get rid of smaller organelles and membranes. The pellet was discarded and the supernatant was used for the experiments.

For experiments in which the temperature effect was tested, some chloroplast preparations were isolated from plants grown at subnormal temperature. Seeds were sown in flats in the greenhouse. When the seedlings were about one inch above soil level the flats were transferred into a low temperature growth chamber at a $15^{o}C$ day and $10^{o}C$ night temperature and a photoperiod of 12 hour light with 12 hours of darkness. Primary leaves were harvested 4 to 5 days after the transfer when the leaves were fully unrolled. Secondary leaves were harvested about three weeks after the transfer when the leaves were fully expanded. Chloroplasts were isolated from these leaf tissues in the same way as described before.

For experiments in which different methods of chloroplast isolation

were compared two additional methods were employed. Ten gram of leaf tissue was sliced into 1 mm strips and were either ground gently in a chilled mortar with 25 ml of Solution H or blended in a waring blender with 25 ml of Solution H for 40 seconds at 10 second pulses and 100% line voltage. The suspension was filtered through two layers of Kleenex tissue and centrifuged, and the pellet was resuspended as described before.

For experiments in which bundle sheath chloroplasts were to be released from the residual leaf tissue after laceration, the lacerated tissue was sliced and transferred into a chilled mortar containing 5 ml of Solution H and ground gently. Filtration, centrifugation and resuspension were as described before.

For experiments in which the endogenous CO_2 fixation capacity was tested, the chloroplasts were isolated by the 'laceration technique' in Solution 1 (Gibbs et al., 1970) instead of Solution H, and the chloroplast pellet was resuspended in Solution 2 (Gibbs et al., 1970) instead of Solution A. Filtration and centrifugation procedures were as described before.

Solution H, pH 7.8

Mannitol	0.6M
EDTA	1mM
BSA	0.1%
TES	0.1M
DTT	1mM
$MgCl_2$	5mM

Solution A, pH 7.2

Mannitol	0.6M
TES	5mM
KCl	0.01M
$MgCl_2$	5mM
KH_2PO_4	1mM

Solution 1, pH 6.8	
Sorbital	0.35M
MES	0.04M
$MgCl_2$	5mM
$MnCl_2$	0.3mM
KH_2PO_4	0.25mM
EDTA	3.0mM
PVP 40	5%

Solution 2, pH 7.8	
Sorbital	0.35M
HEPES	0.04M
$MgCl_2$	0.3mM
$MnCl_2$	0.2mM
KH_2PO_4	0.25mM
EDTA	1.0mM
DTT	5mM

For experiments in which the effect of magnesium ion concentration was tested, $MgCl_2$ was omitted from both the isolation and resuspension media.

PHOTOSYNTHETIC $NaH^{14}CO_3$ FIXATION AND EXTRACTION OF RADIOACTIVE COMPOUNDS

Leaf Tissue

A Gilson differential manometer system was used with 1.5 ml of 0.1M potassium phosphate buffer (pH 7.2) in the main compartment of a Warburg flask fitted with a single side arm. Six leaf discs (1 cm diameter) threaded together with a piece of cotton thread, and an average weight of 0.1 g, were then carefully placed in the flask and distributed so that they formed a single layer with the underside of the leaf tissue in contact with the solution. 5 µCi of $NaH^{14}CO_3$ (s.a. 59 µCi/mM, Amersham/Searle, England) were added to the side arm.

The flasks were then equilibrated for 5 minutes in the Gilson Differential Respirometer at 30°C and 4,000 ft c at the base of the flask with a constant shaking rate of 105 oscillations per minute. At the end of the equilibration period the $NaH^{14}CO_3$ was tipped into the main compartment and the flasks were shaken at 105 oscillations per minute for different incubation time periods. The leaf discs were removed and killed by immersion in boiling 80% ethanol, and were then extracted in 80%, 60%, 40% 20% ethanol and water respectively according to the method of Wang (1960). After each alcoholic-water extraction, the samples were centrifuged at 10,000 rpm for 10 minutes, and the residue was quantitatively transferred to a scintillation vial. The supernatants from each extraction were pooled together and dried under an air jet. The samples were then dissolved in 2 ml of 20% methanol and extracted 4 times with equal volume of chloroform. The chloroform soluble fraction was washed twice with 2 ml aliquots of distilled water, and was pooled together with the residue fraction. This was dried down and resuspended in 1 ml of 20% methanol and the radioactivity determined. The methanol-water soluble fraction was dried down, redissolved in 1 ml of distilled water and an aliquot was taken for determination of radioactivity. The rest of the methanol-water soluble fraction was then frozen for further analysis.

In pulse-chase experiments 30 μCi of $NaH^{14}CO_3$ (s.a.60.3 μCi/mM, Amersham/Searle, England) were added to the side arm instead of 5 μCi.

After the pulse period, the leaf discs were transferred to a similar manometer flask with an identical concentration of unlabelled compounds. After incubating for different chase periods, the leaf discs were removed and killed in boiling ethanol (80% v/v). Extraction procedures were identical to that described previously.

Chloroplasts and Supernatant

Experiments with chloroplasts and supernatant fractions were carried out in 6 ml volume manometer flasks fitted with single side-arms in an illuminated Gilson Differential manometric system set at 30°C and 4,000 ft. c at the base of the flasks. Except where otherwise stated, each flask contained approximately 0.6 mg chloroplast protein, 3.2 μM of Na-phosphoenolpyruvate, and enough suspension medium to make up a volume of 0.48 ml in the main compartment and 20 μl of $NaH^{14}CO_3$ (s.a. 5 μCi/15mm) in the side arm. The contents of the flasks for supernatant experiments were essentially the same except that they contained 0.4 mg supernatant protein instead of chloroplasts.

The flasks were equilibrated for 3 minutes with shaking at 105 oscillations per minute. At zero time the $NaH^{14}CO_3$ was tipped into the main compartment. After different time periods of incubation, the reaction was stopped by the addition of 0.5 ml of 2N HCl and flushed for 5 minutes with air. The solution was extracted several times with a mixture of water, chloroform and methanol (12:5:3 v/v) according to the method of Cook and Bieleski (1969). The methanol-water soluble and chloroform soluble fractions were separated. The

chloroform soluble fractions were washed twice with water. The washings were added to the methanol-water soluble fractions. Both fractions were dried down and their radioactivity determined. Samples were frozen for further analysis.

For experiments in which endogenous CO_2 fixation capacity of isolated chloroplasts were tested the chloroplasts were suspended in Solution 2 according to the method of Gibbs et al. (1970) instead of Solution A. Extraction procedures were the same as described previously.

ION EXCHANGE CHROMATOGRAPHY OF METHANOL-WATER SOLUBLE FRACTION

The methanol-water soluble fraction was separated into three main fractions by means of ion exchange resins according to the method of Canvin and Beevers (1961) and Cossins and Beevers (1963). The three fractions were namely basic fraction (largely amino acids), acidic fraction (largely organic acids and sugar phosphates) and neutral fraction (mainly sugars).

The following two types of resins (Bio-Rad Labs; Richmond, California) were employed:

1. Cation exchange resin Dowex 50W-8 (hydrogen form, 200-400 mesh),
2. Anion exchange resin Dowex Ag 1-X10 (chloride form, 200-400 mesh).

Both types were prepared in bulk according to Atkins and Canvin's method (1971). The cation exchange resin, Dowex 50W-8 (H^+; 200-400 mesh) was slurried into glass columns (1 cm x 16 cm) plugged at the

base with glass wool, where it was treated with 10 ml of 2N HCl per 5 ml resin and then washed with distilled water until the effluent approached neutrality. The anion exchange resin, Ag 1-X10 (Cl^-; 200 - 400 mesh) was slurried into a large column (3cm x 20 cm) plugged at the base with glass wool, where it was converted into the formate form by treatment with 1M sodium formate until the effluent gave a negative test for chloride ions. The resin was then slurried into small glass columns (1 cm x 16 cm) where it was treated with 0.1N formic acid (50 ml/5 ml resin), followed by distilled water until the effluent approached neutrality.

The methanol-water soluble extracts were loaded onto the Dowex 50W-8-H^+ columns and eluted with 100 ml of distilled water. The effluent was concentrated down to 1 ml by means of evaporation under an air jet and was designated as the organic acid-phosphosugar-sugar fraction. The amino acids retained in the column were then eluted with 80 ml of 2N NH_4OH followed by 20 ml of 4N NH_4OH. The effluent was evaporated to dryness as described previously, and was designated the amino acid fraction.

An aliquot of each of the amino acid and organic acid-phosphosugar-sugar fractions was transferred to a scintillation vial with 10 ml of scintillation liquid for radioactivity determination. The rest of the samples were frozen for further analysis.

The organic acid phosphosugar-sugar fraction was quantitatively loaded onto the Dowex Ag-X10-$HCOO^-$ columns and flushed with 100 ml of distilled water. The effluent containing mainly sugars was collected and concentrated by evaporation down to 1 ml. The organic acids and

phosphosugars retained by the columns were eluted with 80 ml of 4N formic caid, followed by 20 ml of 6N formic acid. The effluents were evaporated to dryness and redissolved in 1 ml of distilled water.

An aliquot of each of the sugar and organic acid-phosphosugar fractions was used for the determination of radioactivity. The samples of these three main fractions were frozen for further analysis by means of thin-layer chromatography.

THIN-LAYER CHROMATOGRAPHY (TLC)

The Thin-Layer Plates

The cellulose powder MN300 (Machery, Nagel and Co.) was prepared by a modified method as described by Cook and Bieleski (1969). Fifteen ml of washed, dried cellulose was suspended in 100 ml of distilled water and homogenized at 16,000 rpm for 40 seconds, allowed to stand for 40 seconds, and homogenized again for 40 seconds. The slurry was then allowed to stand for 90 seconds before spreading with a Desaga spreader (Desaga, Heidelberg, West Germany). Plates, 20 x 20 cm in dimension and 250 microns thick of cellulose, were prepared and allowed to age for 2 days after spreading to ensure stability.

Aliquots containing approximately 2,000 to 5,000 dpm were loaded as a fine spot 2 cm away from either edge of the plate. Four sets of plates were chromatographed for each sample, two of which were for the determination of radioactivity, and another set of which was co-chromatographed with unlabelled authentic chemicals. In the last

set of plates the radioactive spots were scraped off and co-chromatographed with unlabelled authentic compounds.

The plates were allowed to stand overnight after the first direction run to ensure evaporation of the solvents.

Separation of Amino Acid and Organic Acid Fractions

Two dimensional TLC was used to separate the various products using the solvent system described by Bieleski and Young (1963). The first direction run was achieved in 100 ml of n-propanol: ammonium hydroxide: water (6:3:1 v/v). The second solvent system consisted of 100 ml of n-propyl acetate: formic acid: water (11:5:3 v/v).

Separation of Sugars

The sugars were separated by two dimensional TLC in 100 ml of n-propanol:water:n-propyl acetate:acetic acid:pyridine (120:60:20:4:1 v/v) in the first direction and 100 ml of n-butanol: acetic acid:water (12:5:3 v/v) for the second direction as described by Cook and Bieleski (1969).

DETECTION OF COMPOUNDS AND MEASUREMENT OF RADIOACTIVITY

Radioautograms were made by exposing the dried plates to "Kodak Medical X-ray Film, No-Screen" from Eastman Company, Rochester, New York. After an appropriate exposure period (1 week/5,000 dpm) the films were developed to locate radioactive spots. The spots were scraped off the plates and were quantitatively transferred into a

scintillation vial for radioactivity determination. The spots from another set of plates were also scraped off and were transferrred to a Pasteur pipette plugged at the tip with cotton wool, eluted with 10% butanol, evaporated to 1 ml volume and co-chromatographed with unlabelled authentic compounds. The set of plates with both radioactive extract and cold authentic chemicals were sprayed with appropriate reagents for the identification of radioactive compounds (Smith, 1960; Mezzetti et al., 1972):

1. Amino acids were detected by a solution of 0.2% ninhydrin in acetone,
2. Organic acids by anilin-xylose (1 ml anilin, 1 g xylose, 10 ml water, 10 ml ethanol, 30 ml butanol),
3. Sugar phosphates by ammonium molybdate (25 ml 4% ammonium molybdate, 5 ml 60% perchloric acid, 10 ml 1N HCl and 60 ml water),
4. Sugars by a solution of napthoresorcinal (20 mg napthoresorcinal, 10 ml ethanol and 0.5 ml of concentrated H_2SO_4).

Measurement of Radioactivity

Radioactivity was determined by means of Picker Nuclear Liquimat Scintillation Counter and corrected for quenching by using a quench curve prepared with picric acid and ^{14}C-toluene.

Aliquots of the aqueous samples obtained were placed in scintillation vials and made up to 0.5 ml with 20% methanol. Ten ml of

scintillation cocktail (0.03 g POPOP, 7.0 g PPO and 100 g naphthalene dissolved in a total volume of 1 litre of p-dioxane) was added to each sample and measured for radioactivity.

ENZYME PREPARATION

Enzyme Preparation from Whole Leaf

Five grams of leaves were cut into 1 mm wide strips and was blended in a waring blender for 5 minutes at 15 second pulses in 15 ml of hypotonic medium containing 40mM Tris-HCl, pH 7.8; 5mM DTT and 5mM $MgCl_2$.

Another method employed was grinding 5 g of leaves (already cut into 1 mm wide strips) in a chilled mortar with acid washed sand and 15 ml of hypotonic medium.

The brei obtained from both methods were passed through 4 layers of cheese cloth and the filtrate centrifuged at 10,000 g for 20 minutes. The pellet was discarded and the supernatant was used as enzyme preparation.

Enzyme Preparation from Chloroplasts and Supernatant

Chloroplasts were isolated by the 'laceration technique' as previously described. After filtration and centrifugation, the chloroplasts were ruptured by suspending the chloroplast pellet in hypotonic medium according to the method of Bahr and Jensen (1974). The suspension was used as chloroplastic enzyme preparation.

The supernatant obtained subsequent to centrifugation procedure was recentrifuged at 10,000 g for 15 minutes to get rid of membranes and small organelles. The pellet was discarded and the supernatant was used as enzyme preparation for supernatant (cytoplasm).

For experiments in which the effect of magnesium ion concentration on enzyme activity was tested $MgCl_2$ was omitted from the isolation medium.

ENZYME ASSAY

Phosphoenolpyruvate Carboxylase (EC 4.1.1.31)

Phosphoenolpyruvate carboxylase was assayed according to a modified method of Tan (1974). Except otherwise stated, the incubation mixture contained in μmoles: PEP (trisodium salt), 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); $NADH_2$, 2; $MgCl_2$, 5; Tris HCl, pH 7.8, 100; malate dehydrogenase, excess and crude enzyme (approximately 400 μg of supernatant protein or 600 μg chloroplast protein) in a final volume of 0.5 ml. The reaction mixture was preincubated at 30°C for 10 minutes. The reaction mixture was initiated by the addition of PEP. Control samples were without PEP. The reaction was terminated after 6 minutes by the addition of 1 ml of 2N HCl. Separation of the samples into methanol-water soluble and chloroform soluble fractions and determination of radioactivity from each fraction were as previously described for ^{14}CO fixation by isolated chloroplasts and supernatant.

Ribulose Diphosphate Carboxylase (EC 4.1.1.f)

Ribulose diphosphate carboxylase was assayed according to the method of Paulsen and Lane (1966). The incubation mixture contained in μmoles: RuDP (tetrasodium salt), 0.35; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); glutathione (reduced), 3; EDTA, 0.03; $MgCl_2$, 5; Tris-HCl, pH 7.8, 100; and crude enzyme (approximately 400 μg of supernatant protein or 600 μg of chloroplast protein) in a total volume of 0.5 ml. The reaction mixture was preincubated at 30°C for 10 minutes, and the reaction was initiated by the addition of RuDP. Control samples were without RuDP. Termination of reaction, separation of samples into methanol-water soluble and chloroform soluble fractions and determination of radioactivity were as described previously.

PROTEIN AND CHLOROPHYLL DETERMINATION

Protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Chlorophyll was determined by the method of Whatley and Arnon (1963) using 80% acetone.

CHLOROPLAST COUNT

Chloroplast counts were obtained using an Haemocytometer. Twenty fields were counted per sample.

RESULTS

I. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO PRIMARY LEAF TISSUE OF MAIZE

A. Kinetic Experiments

1. Rate of Incorporation

Figure 1 shows that the total incorporation of CO_2 into primary leaf tissue of maize plant was linear with incubation time up to 20 minutes. The rate of CO_2 fixation was as high as 53.3 μg CO_2 dm^{-2} hr^{-1} during this period. The rate of incorporation into the methanol-water soluble fraction was also linear with time within the same period, while that into the residue and chloroform soluble fraction exhibited a mildly exponential increase with time. More than 80% of the radioactivity was recovered from the methanol-water soluble fraction at all times (Table I).

2. Distribution of ^{14}C in the Methanol-Water Soluble Fraction

Column chromatography was employed to separate the methanol-water soluble fraction into organic acid, amino acid and sugar fractions. Table II and Figure 2 summarize the distribution of ^{14}C in these fractions after different periods of incubation time of the primary leaf tissue with $NaH^{14}CO_3$.

At the end of the 1 minute incubation period the bulk of radioactivity, about 80%, was recovered from the organic acid fraction. After two minutes of incubation, it gradually decreased with incubation time till it accounted for about 50% of the total label found in the methanol-water soluble fraction at the end of the 20 minute incubation period. The amino acid fraction accounted for 20% of the ^{14}C incorporated into this fraction from 1 to 5 minutes of fixation time, thereafter it gradually declined to around 12% at the end of the 20 minute period. Meanwhile, no radioactivity was detected from the sugar fraction till the end of the 5 minute period, accounting for 10% of the total label, then it increased steadily with incubation time up to 20 minutes.

3. Distribution of ^{14}C in the Various Products of the Organic Acid, Amino Acid and Sugar Fractions

Thin layer chromatography and autoradiography were employed to detect the distribution of ^{14}C among various products in these three fractions. The results were shown in Table III and summarized in Figure 3.

At the end of the 1 minute incubation time, more than 30% of the radioactivity was recovered from 3PGA and sugar monophosphates, and around 20% was recovered from malate. While the former decreased rapidly with incubation time after reaching the maximum at the end of 2 minutes, the latter

decreased only very gradually and remained at a high level at all times. The decrease in label percentage in 3PGA and sugar monophosphates was accompanied by an increase of ^{14}C content in the sugar fraction. The amount of label recovered from isocitrate lactone was considerable initially and it rapidly decreased with incubation time. Alanine, glycine and serine, glutamate and aspartate were the major amino acids detected. Alanine was heavily labelled at the end of 1 minute fixation, but the label decreased steadily with incubation time. This may reflect that alanine was formed via 3PGA. The level of glycine and serine was low at the beginning but gradually rose with incubation time and remained at a considerable level up to the 20 minute incubation time. Considerable amount of ^{14}C was also detected in aspartate at the end of the 5 minute incubation period, which also gradually decreased with time. This amino acid could have been derived from the relatively large pool of malate in the primary leaf tissue.

B. Pulse-Chase Experiments

To determine whether the major flow of carbon was through 3PGA or malate into sugars a pulse-chase experiment was undertaken as described in Materials and Methods. Only the methanol-water soluble fraction was analysed as it contained over 80% of the incorporated ^{14}C as can be seen previously (Fig. 1).

1. Distribution of ^{14}C in the Methanol-Water Soluble Fraction

Results of column chromatography on the methanol-water soluble fraction are shown in Table IV and Figure 4.

At the end of the 15 second pulse period about 90% of the radioactivity was recovered in the organic acid fraction which decreased with time indicating a relatively rapid turnover of compounds in this fraction. At the end of the 6 minute chase period this fraction accounted for 60% of the total label. Ten percent of the ^{14}C was recovered in the amino acid fraction at the end of the pulse period. This gradually increased to around 25% with the chase time accompanying the decrease in the organic acid fraction. Thereafter it levelled off. No ^{14}C was detected in the sugar fraction until the end of the 1.5 minute chase period. And this then increased gradually, though not linearly, with the chase time up to 6 minutes.

2. Distribution of ^{14}C into the Various Products of the Organic Acid, Amino Acid and Sugar Fractions

Figure 5 summarizes the results given in Table V. At the end of the 15 second pulse period around 30% of the label was recovered in 3PGA and 23% from malate. The former lost its radioactivity rapidly with time to 6% at the end of the 6 minute chase period. Malate also decreased rapidly in radioactivity for the first half minute of the chase period,

but then levelled off and remained at a constant level of around 14%. Sugar monophosphates increased in ^{14}C content very rapidly accompanying the sharp fall of label in 3PGA for the first minute of the chase period. Then they also gradually levelled off to around 18% at the end of the 6 minute chase period. Isocitrate lactone was heavily labelled initially at the end of the pulse period, but the radioactivity rapidly decreased indicating a very rapid turnover in this compound. The drop in ^{14}C content of isocitrate lactone was accompanied by a concomitant rise in label in glycine and serine. Alanine reached a maximum of 5% at 1.5 minutes, and then gradually decreased with chase period. Glutamate and aspartate remained insignificantly labelled at all times. No sugar was detected until the end of the 1.5 minute chase period at which the level of sugar monophosphates remained steady.

The results show that in primary leaves of maize both the Calvin cycle and C_4 pathway may be operating with the Calvin cycle playing a dominant role. This is shown by the high initial labelling of 3PGA and sugar monophosphates and the subsequent high level of malate in the kinetic experiments, and the rapid decrease of ^{14}C content in 3PGA in the pulse-chase experiments. Although the label in malate also decreased with time, it remained at a relatively high level at all times indicating that it was not rapidly utilized, although it may contribute to CO_2 fixation and was one of the initial products

of the process. It was also noted that the fall in 3PGA and malate level during the first half minute of the chase period was enough to account for the rise in sugar monophosphates and diphosphates level. The fall in isocitrate lactone level during the chase period was also enough to account for the rise in glycine and serine level. It is possible that isocitrate lactone was the precursor of these two amino acids in maize primary leaves.

II. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO SECONDARY LEAF TISSUE OF MAIZE

A. Kinetic Experiments

1. Rate of Incorporation

Figure 6 shows that the incorporation of ^{14}C into secondary leaf discs of maize was linear with time up to 20 minutes of incubation time. The rate of incorporation was as high as 63.1 μg CO_2 dm^{-2} hr^{-1} for this period, which was about 20% higher than that found in the primary leaf tissue. As in the primary leaf tissue, the radioactivity incorporated into the methanol-water soluble fraction was also linear with time for the same period, while that incorporated into the residue and chloroform soluble fraction was mildly exponential with time. Again over 80% (Table VI) of the ^{14}C was recovered in the methanol-water soluble fraction at all times.

2. Distribution of ^{14}C in the Methanol-Water Soluble Fraction

Separation of the methanol-water soluble fraction into organic acid, amino acid and sugar fraction was as described before.

Table VII and Figure 7 summarize the distribution of ^{14}C in the three different fractions. Over 60% of the radioactivity was recovered from the organic acid fraction,

which then decreased gradually with time to around 25% at the end of the 20 minute incubation time. This drop in the organic acid fraction was faster than that observed in the primary leaf tissue, and the final level at the end of 20 minutes was only 50% that of the primary leaf tissue. Amino acid fraction reached a maximum at the end of the 5 minute incubation period which accounted for 19% of the total radioactivity recovered from this fraction, thereafter, it decreased very gradually. Radioactivity in the sugar fraction was detected much earlier than in primary leaf tissue, and it increased rapidly with time accompanied by the rapid drop in the organic acid fraction. At the end of the 20 minute incubation period, it accounted for 60% of the total label recovered from the columns.

3. Distribution of ^{14}C in the Various Products of the Organic Acid, Amino Acid and Sugar Fractions

Figure 8 summarizes the results shown in Table VIII. At the end of the 1 minute incubation period malate accounted for nearly 25% of the total radioactivity incorporated into this fraction. As was found in the primary leaf tissue, this level dropped with time. However, the drop was much more drastic till it accounted for only 2% as compared to 14% observed from the primary leaf tissue at the end of the 20 minute experiment. This also explained the low level of

radioactivity recovered from the organic acid fraction as shown in Figure 7. Sugar monophosphates and 3PGA were also significantly labelled initially and reached a maximum of 31% at the end of the 5 minute experiment as compared to 40% maximum observed from the experiment with primary leaf tissue. Then it gradually decreased with time to around 9% which was higher than that observed from the primary leaf tissue. Some radioactivity was recovered from isocitrate lactone initially, then it gradually decreased as before. Considerable amount of ^{14}C was incorporated into aspartate at the end of the 1 minute incubation period, which subsequently dropped rapidly within the first five minutes of incubation, and then gradually levelled off to around 1% of the total ^{14}C content. This reflects that although maize has not been known as an 'aspartate former', the amino acid is synthesized in considerable amount. As observed before, glycine and serine rose with time till they accounted for about 8% of total ^{14}C content at the end of 5 minute incubation, thereafter it decreased. Differing from primary leaf tissue, alanine remained insignificantly labelled at all times.

B. Pulse-Chase Experiments

For the purpose of determining the major flow of carbon into sugars, a pulse-chase experiment was performed as for primary leaf tissue.

1. Distribution of ^{14}C in the Methanol-Water Soluble Fraction

Table IX and Figure 9 show that the distribution pattern of ^{14}C among the three fractions of the methanol-water soluble fraction was more or less the same as that found with primary leaf experiment. However, it was noted that the amino acid fraction dropped rapidly for the first 30 seconds of the chase period and was accompanied by a rapid rise in the ^{14}C content of the organic acid fraction.

2. Distribution of ^{14}C in the Products of the Organic Acid, Amino Acid and Sugar Fractions

Table X and Figure 10 summarize the results of the analysis. It can be seen that malate was highly labelled at the end of the pulse period accounting for 37% of the radioactivity incorporated as opposed to 23% observed with the primary leaf experiment. 3PGA accounted for only 14% of ^{14}C content as opposed to 38% found with primary tissue for the same period. Radioactivity was not recovered from the sugar monophosphates at the end of the pulse period. But a considerable amount was found at the end of the 30 second chase period, thereafter it increased rapidly with chase time reaching a maximum of 24% at 1 minute, and then gradually levelled off. From the variation of ^{14}C distribution with different periods of chase time, it is obvious that ^{14}C found entry into 3PGA and sugar monophosphates via

malate. Aspartate was significantly labelled at zero time and dropped rapidly with time. This accounted for the drop of amino acid fraction for the first 30 seconds of chase time shown in Figure 9. Isocitrate lactone was highly labelled at the end of the pulse period, amounting to 16% of the total radioactivity incorporated, and then it gradually declined with time accompanied by the concomitant rise in glycine and serine, as found with the primary leaf experiment. However, it was noted that the fall in isocitrate lactone was more than enough to account for the total increase in glycine and serine ^{14}C content. So part of the isocitrate lactone pool could be precursors for other photosynthetic intermediates. It is interesting to note that the fall in aspartate and malate could roughly account for the rise in 3PGA and sugar monophosphates in the first minute of the chase period.

From the results it can be seen that in secondary leaf discs of maize the flow of carbon into sugars is through the C_4 acids, with malate playing the major role as compared with that of aspartate.

III. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO ISOLATED CHLOROPLASTS FROM MAIZE LEAVES IN THE ABSENCE OF PHOSPHOENOLPYRUVATE

A. Isolated Chloroplasts from Primary Leaves

Carbon dioxide fixation by isolated chloroplasts from primary leaves of maize had been demonstrated by a few investigators (Gibbs et al., 1970; O'Neal et al., 1971; 1972). However, the preparation was difficult, the results variable and the system was very viscous and further product analysis was extremely difficult.

Attempts had been made to fix CO_2 with isolated chloroplasts from primary leaves of 6 to 8 day old maize seedlings. The results proved to be mostly negative. Figure 11 shows the best results obtained from more than ten trials. The fixation rate was very low (approximately 48 nmoles/mg protein/hr) though there was a remarkable difference between the fixation in darkness and in the light.

B. Isolated Chloroplasts from Secondary Leaves

Although CO_2 fixation capacity in leaf tissue of maize and isolated bundle sheath strands had been reported by a few workers (Samejima and Miyachi, 1971;

Latzko et al., 1971; Chollet and Ogren, 1972), such process with isolated mesophyll cells and isolated chloroplasts from seedlings 2 weeks or older had never been cited from literature. Attempts in fixing CO_2 with isolated chloroplasts from secondary leaves of 16 to 18 day old maize seedlings had proved to be all negative.

IV. PHOTOSYNTHETIC $NaH^{14}CO_3$ INCORPORATION INTO ISOLATED CHLOROPLASTS AND SUPERNATANT OF MAIZE LEAVES IN THE PRESENCE OF PHOSPHOENOLPYRUVATE

Carbon dioxide fixation by isolated chloroplasts of C_4 plants in the presence of PEP has been reported by some workers using sugarcane (Baldry et al., 1969) and maize (Waygood et al., 1971) leaves. Though CO_2 fixation by such preparations in the absence of PEP is low, in its presence the rate is greatly enhanced.

From the kinetic and pulse-chase experiments with leaf discs it was found that the CO_2 fixation pattern differs to certain extent between primary and secondary leaves of maize, therefore it would be interesting to see if this process by isolated chloroplasts from the two kinds of leaves would be different. The presence of PEP carboxylase in leaf cells often poses a problem in interpreting the results. It has generally been agreed that the enzyme is localized in mesophyll and not in bundle sheath cells (Slack et al., 1969; Bucke and Long, 1971; Hatch and Kagawa, 1973). However, it has not been resolved whether it is located strictly in the cytoplasm or loosely bound to chloroplast membrane. In order to resolve this problem, in experiments carried out with isolated chloroplasts another set was undertaken with the supernatant (cytoplasmic) fraction from the same preparation. Since the supernatant has very little chlorophyll, all results of CO_2 fixation are expressed on a per mg protein basis. The protein to chlorophyll ratio has been found to be 16 - 18 and 10 - 12 for primary and secondary leaf chloroplast preparations respectively. It was also found that fixation in the dark was always between 60 to 70% of that in the light. Results on dark experiments are not included in this section for it is beyond the scope of this thesis.

A. Rate of Incorporation

1. Isolated Chloroplasts from Primary Leaves

Figure 12a summarizes the results of $^{14}CO_2$ incorporation into isolated chloroplasts from primary leaves of maize. There was hardly any incorporation in the absence of PEP. Almost all of the ^{14}C was recovered from the methanol-water soluble fraction. The radioactivity recovered from the chloroform soluble fraction was negligible. The rate of incorporation was linear with time for about 10 minutes, after which it slowly declined. The initial rate of $^{14}CO_2$ fixation was about 2 μm CO_2/mg protein/hr. However, this rate varied with each preparation as had been found by other investigators (Gibbs et al., 1970; Waygood et al., 1971). The highest rate observed was over 20 μm/mg protein/hr. In all cases the rate of incorporation within the first 10 minutes was linear with time.

2. Supernatant from Primary Leaves

Figure 12b shows that in the absence of PEP there was again little fixation of $^{14}CO_2$ into the supernatant, which consisted of mainly cytoplasmic enzymes and enzymes from broken organelles. However, the incorporation was considerable in the presence of PEP. The rate of incorporation was linear for the first 6 minutes after

which it slowly declined. The initial rate of incorporation was approximately 5 μm CO_2/mg protein/hr. Again the rate varied with each preparation and the highest rate observed was 30 μm CO_2/mg protein/hr. And in all cases the rate was linear with time for the first 6 minutes.

3. Isolated Chloroplasts from Secondary Leaves

Figure 13a shows the rate of $^{14}CO_2$ incorporation into isolated chloroplasts from secondary leaves of maize was linear with time for the first 8 minutes, thereafter it slowly declined. As in isolated chloroplasts from primary leaves, there was no incorporation in the absence of PEP and very little ^{14}C was recovered from the chloroform soluble fraction. The rate of incorporation for the linear portion was around 7.5 μm CO_2/mg protein/hr. The rate also varied with each preparation, the highest observed was well over 40 μm CO_2/mg protein/hr.

4. Supernatant from Secondary Leaves

Figure 13b shows that the rate of incorporation of $^{14}CO_2$ into the supernatant fraction of secondary leaves of maize was linear with time for the first 6 minutes after which it slowly declined. The rate for the linear portion was as high as 60 μm CO_2/mg protein/hr. As observed

from primary leaf supernatant, there was very low incorporation into the supernatant in the absence of PEP.

B. Determination of Apparent Km Values for PEP, HCO_3^- and Mg^{+2} for CO_2 Fixation

Initial velocity of CO_2 fixation was measured from incubation mixture after 5 minutes of incubation. All initial velocity data were plotted versus the substrate or cofactor as well as in the double reciprocal form (1/v versus 1/S) according to the method of Lineweaver and Burk (1934), where v is the initial velocity and S is the concentration of the variable substrate.

Apparent Km values for various substrates were calculated from intercepts at the horizontal axis of the double reciprocal plots according to the following equation:

$$\frac{1}{v} = \frac{1}{V'} + \frac{Km}{V'}\left(\frac{1}{S}\right)$$

where Km is the apparent Km value for the variable substrate, S, and V' is the apparent maximum velocity.

1. Isolated Chloroplasts from Primary Leaves

The apparent Km values for PEP, HCO_3^- and Mg^{+2} for CO_2 fixation in this fraction were calculated to be 4.00, 0.56 and 2.00 mM respectively as shown in Figures 14 - 16 inclusive. Mg^{+2} up to 10 mM had not yet reached the saturation level for this process.

2. Supernatant from Primary Leaves

The apparent Km values for PEP, HCO_3^- and Mg^{+2} for CO_2 fixation in the supernatant of primary leaves were calculated to be 7.40, 0.89 and 0.30 mM respectively as shown in Figures 17 - 19. Differing from primary leaf chloroplasts saturation level for Mg^{+2} was reached at approximately 3mM. Further increase in Mg^{+2} concentration inhibited CO_2 fixation.

3. Isolated Chloroplasts from Secondary Leaves

The apparent Km values for PEP, HCO_3^- and Mg^{+2} for CO_2 fixation in the chloroplast fraction of secondary leaves were calculated to be 2.04, 0.67 and 2.22 mM respectively as shown in Figures 20 - 22, inclusive. CO_2 fixation was inhibited by PEP above 8 mM and Mg^{+2} above 7 mM. Apparent Km value for PEP was slightly lower than that for primary leaf chloroplast fraction.

4. Supernatant from Secondary Leaves

The apparent Km values for PEP, HCO_3 and Mg^{+2} for CO_2 fixation in this fraction were calculated to be 2.44, 0.40 and 0.35 mM respectively as shown in Figures 23 - 25. PEP concentration up to 16 mM did not produce any inhibition for the process. Mg^{+2} at a concentration of 1 mM reached saturation level and further

increase produced inhibition for CO_2 fixation. The apparent Km value for PEP in this fraction was considerably lower than that found in the primary leaf fractions.

C. Products of $NaH^{14}CO_3$ Fixation

It has been shown (Section IV.A) that both chloroplast and supernatant fractions from primary and secondary leaves of maize can fix CO_2 in the presence of PEP. Waygood et al. (1971) have shown that malate is the principal organic acid product of CO_2 fixation by maize leaf chloroplasts (mixed mesophyll and bundle sheath) at a concentration of 16 mM PEP. The present author has confirmed these results at 16 mM PEP.

One of the problems encountered with the 'laceration technique' of Mache and Waygood (1969) which is evaluated in Section VI is contamination by cytoplasmic (supernatant) PEP carboxylase and probably other enzymes. If the chloroplasts were completely inactive in CO_2 fixation, it would be expected that the distribution of ^{14}C in the products of the supernatant system and that of inactive chloroplasts contaminated with supernatant would be the same.

An experiment was undertaken to investigate this possibility. In this study it has been shown that saturation of CO_2 fixation could be attained at ca. 6 - 8 mM PEP (Fig. 20) and that 16 mM PEP was sometimes inhibitory. The concentration

of 3.2 mM PEP was used since it is approximately the Km of CO_2 fixation by the chloroplast fractions (Figs. 14, 20) with added PEP. It was thought that this may lower the relative proportion of supernatant fixation to chloroplast fixation in contaminated chloroplasts since the Km for CO_2 fixation by the primary leaf supernatant was 7.4 mM PEP. In these experiments 200 µCi of $NaH^{14}CO_3$ was used because of the low rate of CO_2 fixation. The reaction was terminated at the end of 2 minutes instead of 6 minutes in Section IV.B because it has been the author's experience that the major proportion of the ^{14}C was distributed in sugars with increasing time. The results of these experiments are reported in Tables XI to XIII inclusive.

Table XI shows that the supernatant fractions incorporated more $^{14}CO_2$ than the chloroplast fractions on a per mg protein basis. The major proportion of ^{14}C recovered from chloroplast fixation was located in the methanol-water soluble fraction. The amount of radioactivity in the chloroform soluble layer was negligible.

Table XII shows that in chloroplast fractions close to 60% of the radioactivity was recovered in the sugars and amino acids whereas in supernatant fractions over 50% of the label was recovered in the organic acid fraction. Primary leaf chloroplasts indicated a higher fixation into amino acid fraction as compared with secondary leaf chloroplasts.

The distribution of ^{14}C in products is shown in Table XIII. Aspartate was the only amino acid in which radioactivity was recovered in both fractions from both primary and secondary leaves. Considerable label was found in glycollate in both fractions for both leaf types. The highest amount of ^{14}C recovered from each cellular fraction was from the 3C (three carbon) compounds, glycerate, dihydroxy acetone and glyceraldehyde. These three compounds were more heavily labelled in the chloroplast fraction than in the supernatant fraction with the exception of glycerate which was more or less equal to the label found in the chloroplast fraction in both leaf types. This accounts for the fact that the percentage of label in 3C products was 32 - 35% in the chloroplast fractions as compared with 20 - 23% in the supernatant fractions.

Another difference between the supernatant and chloroplast fixation patterns lies in the labelling of sugar phosphates. The supernatant was more heavily labelled in sugar phosphates than the chloroplast fraction. On the other hand, the latter was slightly higher in pentose labelling than the former. ^{14}C content in sucrose, hexoses and polysaccharide was considerable. Chloroplast fraction from secondary leaves show a larger amount of radioactivity fixed into these compounds as compared with the primary leaf chloroplast fraction.

Although malate was heavily labelled in experiments with leaf discs as observed (Sections I, II) and isolated chloroplasts

when fortified with 16 mM PEP, surprisingly enough, it was not found labelled in these experiments with cellular fractions of primary and secondary leaves in the presence of 3.2 mM PEP.

D. Effect of Temperature

Plants using Calvin cycle as photosynthetic pathway thrive better in cooler climate while C_4 plants survive better in warmer regions. Effect of temperature on photosynthesis of higher plants is well documented (Jolliffe and Tregunna, 1968; 1973). Increasing pre-treatment day temperature resulted in decreased net photosynthesis in Agropyron smithii (C_3) while in Bouteloua gracilis (C_4) net photosynthesis was increased (Williams, 1974). If CO_2 enters cellular metabolism of primary leaves mainly through the Calvin cycle, and secondary leaves through the C_4 pathway, it would not be surprising that there would be a difference in the response of CO_2 fixation by the two kinds of chloroplasts to temperature. In the following experiments two sets of plants were used. One set was grown under normal temperature condition with 25°C and 20°C as day and night temperatures respectively, the other set was grown at both decreased day and night temperatures of 15°C and 10°C respectively. The cold treated plants showed some necrosis at the tip of the leaves and these necrotic spots were cut away before the leaves were lacerated.

1. Isolated Chloroplasts from Primary Leaves

a) Normal temperature treatment:- ($25^{o}C$ day/ $20^{o}C$ night)

Figure 26a summarizes the results of $^{14}CO_2$ fixation into isolated primary leaf chloroplasts under different fixation temperatures. The optimal temperature for this process was found to be $30^{o}C$. Further increase in temperature resulted in lower rate of CO_2 fixation. In two other experiments the optimum was found at $35^{o}C$

b) Cold treatment:- ($15^{o}C$ day/$10^{o}C$ night)

When isolated chloroplasts from primary leaves of cold treated plants were used the optimal temperature for CO_2 fixation was found to be $25^{o}C$ as can be seen from Figure 27a. Though the fixation rate from this figure seemed to be higher than that of normal temperature treatment plants, it must be remembered that the rate varied with each preparation and therefore may not be of too much significance.

2. Supernatant from Primary Leaves

a) Normal temperature treatment:- ($25^{o}C$ day/ $20^{o}C$ night)

The optimal temperature of $^{14}CO_2$ incorporation into supernatant of primary maize leaves was found to be higher than that found with isolated chloroplasts. Figure 26b shows that the optimal temperature for the process was $40^{o}C$

and was consistent with other experiments.

b) Cold treatment:- (15°C day/10°C night)

Temperature response of $^{14}CO_2$ fixation in primary leaf supernatant fraction was essentially the same whether the plants were grown at normal or subnormal temperatures. Figure 27b shows that the optimal temperature for CO_2 fixation by chloroplasts isolated from primary leaves of chilled plants was still 40°C.

3. Isolated Chloroplasts from Secondary Leaves

a) Normal temperature treatment:- (25°C day/20°C night)

Figure 28a summarizes the results of the effect of temperature on $^{14}CO_2$ fixation in secondary leaf chloroplasts of maize grown under normal temperature treatment. The rate of fixation rose sharply from 15°C to 40°C, further increase in temperature resulted in decreased rate of incorporation.

b) Cold treatment:- (15°C day/10° night)

Cold temperature treatment had a drastic effect on the $^{14}CO_2$ fixation capacity of isolated chloroplasts from secondary leaves of maize. As can be seen from Figure 29a, the rate of CO_2 fixation was extremely low. The highest fixation rate was obtained at 20°C instead of 40°C with

a value of only 1 μm CO_2/mg protein/hr as compared with well over 10 μm CO_2/mg protein/hr usually observed at this temperature for plants grown under normal temperature treatment. These results agree with those of Williams (1974).

4. Supernatant from Secondary Leaves

a) Normal temperature treatment:- (25°C day/20°C night)

The effect of temperature on $^{14}CO_2$ incorporation into supernatant of secondary maize leaves was summarized in Figure 28b. It was more or less the same as that found with isolated chloroplasts of the same preparation, with the optimal temperature at 40°C, and was also similar to the supernatant fraction from primary leaves of maize.

b) Cold temperature treatment:- (15°C day/10°C night)

Cold temperature pretreatment did not seem to have altered the pattern of response to temperature by secondary maize leaf supernatant. As can be seen from Figure 29b, the optimal temperature was still at 40°C, which was the same found in plants grown under normal temperature.

V. STUDIES OF PHOSPHOENOLPYRUVATE AND RIBULOSE DIPHOSPHATE CARBOXYLASES IN THE CHLOROPLAST AND SUPERNATANT OF MAIZE LEAVES

As reported previously in Section IV, the chloroplast and supernatant fractions from primary and secondary leaves of maize were not able to fix CO_2 without the addition of PEP. However, their CO_2 fixation capacity in response to different concentrations of PEP, HCO_3^- and Mg^{+2} were different from each other. It has been reported by Ting and Osmond (1973a) that multiple forms of PEP carboxylase are present in maize. It is the purpose of this section to report on the properties of PEP carboxylase isolated from each cell fraction and to determine whether the differences in their CO_2 fixation capacity are directly related to the properties of this enzyme.

In experiments investigating the effect of temperature on PEP carboxylase the activity of RuDP carboxylase was also assayed to determine whether this key carboxylase in the bundle sheath chloroplasts (Hatch and Kagawa, 1973) was also affected in the same way as PEP carboxylase in mesophyll cells.

Enzyme preparations and assay systems were the same as described in Methods and Materials except where stated otherwise. In studying chloroplastic enzymes most investigators ruptured the chloroplasts either by sonication or mechanical force (Pan, 1974). It has been noticed that too much physical agitation will lower the activity of PEP carboxylase from the chloroplast

preparation so a modified method of Bahr and Jensen (1974) was employed to prepare ruptured chloroplasts in hypotonic medium.

In both chloroplast and supernatant fractions of either primary or secondary leaves the progress curves of the PEP carboxylase reaction showed a linear relationship with time up to about 6 minutes. In addition the activity was linear with protein concentration up to 0.6 mg in chloroplasts and 0.4 mg in the supernatant.

RuDP carboxylase activity was also linear with time and protein concentration up to 15 minutes and 1.0 mg protein respectively in all fractions.

A. Determination of Apparent Km Values for PEP, HCO_3^- and Mg^{+2} of Phosphoenolpyruvate Carboxylase

Method of determining apparent Km values for reaction components of PEP carboxylase was as described in Section IV.B.

1. Chloroplastic PEP Carboxylase from Primary Leaves

The apparent Km values for PEP, HCO_3^- and Mg^{+2} for PEP carboxylase in this fraction were calculated to be 10.00, 0.43 and 4.00 mM respectively as shown in Figures 30 to 32. Mg^{+2} concentration of 10mM had not yet attained the saturation level for this enzyme. Moreover the initial velocity exhibited a linear relationship with Mg^{+2} from 0 to 10mM. Bicarbonate inhibited enzyme activity above 10 mM.

2. Supernatant PEP Carboxylase from Primary Leaves

The apparent Km values for PEP, HCO_3^- and Mg^{+2} for PEP carboxylase in this fraction were calculated to be 10.00, 0.44 and 1.14 mM respectively as shown in Figures 33 - 35 inclusive. Again Mg^{+2} at a concentration of 10mM failed to attain the saturation point, and the initial velocity of the enzyme exhibited a linear relationship with Mg^{+2} concentration from 0 to 10 mM.

3. Chloroplastic PEP Carboxylase from Secondary Leaves

The apparent Km values for PEP, HCO_3^- and Mg^{+2} for PEP carboxylase of this fraction were calculated to be 1.40, 0.27 and 5.71 mM respectively as shown in Figures 36 to 38 inclusive. Differing from primary leaf PEP carboxylases Mg^{+2} at a concentration ca. 3 mM was inhibitory. There was no inhibition by HCO_3^- above 10 mM as observed in primary leaf chloroplast enzyme.

4. Supernatant PEP Carboxylase from Secondary Leaves

The apparent Km values for the two substrates and cofactor for PEP carboxylase were calculated to be 3.85 mM for PEP, 1.33 mM for bicarbonate and 0.21 mM for magnesium ion as shown in Figures 39 to 41 inclusive. Above ca. 3mM Mg^{+2} inhibited the reaction.

B. Effect of Temperature on the Activities of Phosphoenolpyruvate and Ribulose Diphosphate Carboxylase in Maize Leaves

It has been shown that the rate of CO_2 fixation by isolated chloroplasts and the supernatant from both primary and secondary leaves of maize is affected by incubation temperature. In addition, a drop in growth temperature of maize plant caused almost complete inhibition of the CO_2 fixation capacity of isolated chloroplasts from secondary maize leaves, but not chloroplasts from primary maize leaves. In this section the effect of both assay and growth temperature on PEP carboxylase and RuDP carboxylase in maize leaves is reported. The enzyme preparations and assay systems were the same as described in Methods and Materials.

1. Chloroplastic PEP and RuDP Carboxylases from Primary Leaves

a) Normal temperature treatment:- ($25^{o}C$ day/$20^{o}C$ night)

In plants grown under normal temperature conditions PEP carboxylase from isolated chloroplasts showed increased activity with an increase in temperature attaining a maximum between 30 and $35^{o}C$. RuDP carboxylase activity increased with increasing incubation temperature from 15^{o} to $45^{o}C$. However, the specific activity of the latter enzyme was much lower than that of the PEP carboxylase (Figure 42a).

b) Cold treatment:- (15°C day/10°C night)

In plants grown under low temperature condition the activity of PEP carboxylase isolated from the same cell fraction decreased in specific activity to about half of the value of the enzyme from plants grown under normal temperature condition. There was also a shift in optimum temperature from between 30 and 35°C to 25°C. However the response of RuDP carboxylase activity to incubation temperature remained the same as that grown under normal temperature, though again there was a decrease in specific activity (Figure 43a).

2. Supernatant PEP and RuDP Carboxylases from Primary Leaves

a) Normal temperature treatment: (25°C day/20°C night)

Figure 42b summarizes the results of the carboxylases response to incubation temperature. PEP carboxylase activity was considerably higher than that in the chloroplast fraction and it attained a maximum rate at 35°C whereas RuDP carboxylase activity continued to rise from 15° to 45°C. However, the supernatant fraction was very low in RuDP carboxylase activity in comparison with that of PEP carboxylase, and it was of the same order of magnitude as that from the chloroplast fraction (Fig.42a).

b) Cold treatment:- (15°C day/10°C night)

Figure 43b shows that neither the activity of PEP carboxylase nor that of RuDP carboxylase from the supernatant fraction was affected by cold treatment during growth. PEP carboxylase still reached a maximum in activity between 35° to 40°C while the activity of RuDP carboxylase likewise increased from 15° to 45°C. However the specific activity was less than half the value of the enzyme from plants grown under normal temperature condition.

3. Chloroplastic PEP and RuDP Carboxylases from Secondary Leaves

a) Normal temperature treatment:- (25°C day/ 20°C night)

Figure 44a shows the incubation temperature response curve of the two carboxylases from isolated chloroplasts from secondary leaves grown under normal temperature. The response was essentially the same as that found in primary leaf chloroplasts (Fig. 42a). The optimum temperature for PEP carboxylase was 35°C. RuDP carboxylase activity again continued to increase with the increase of incubation temperature from 15° to 45°C.

b) Cold treatment:- (15°C day/10°C night)

Figure 45a shows that cold treatment during the growth of plants markedly influenced the PEP carboxylase activity from secondary leaf chloroplasts. There was an almost complete inhibition of this enzyme from 15° to 40°C. Some activity of the enzyme was observed at 45°C. However, within the same chloroplast fraction RuDP carboxylase showed the same temperature - activity relationship as that in chloroplasts of primary leaves grown at normal and subnormal temperatures (Figs. 42a, 43a).

The absence of PEP carboxylase activity in secondary leaf chloroplasts from plants grown at subnormal temperatures may explain the failure to fix CO_2 by isolated chloroplasts of similarly grown plants (Fig. 29a).

4. Supernatant PEP and RuDP Carboxylases from Secondary Leaves

a) Normal temperature treatment:- (25°C day/20°C night)

Figure 44b shows that the response of the carboxylases to incubation temperature was essentially similar to that observed in the supernatant fraction of primary leaves.

b) Cold treatment:- (15°C day/10°C night)

Figure 45b shows that the response of the two

carboxylases to incubation temperature was again essentially similar to that observed in all other supernatant fractions.

From these results it is obvious that cold temperature treatment during growth did not affect the activity of RuDP carboxylase. It is interesting to note that the enzyme activity continued to increase above 40°C. Cold temperature treatment during growth did not influence the activity of the cytoplasmic PEP carboxylases from both primary and secondary leaves. But on the other hand, chloroplasts isolated from secondary leaves grown at subnormal temperatures showed no PEP carboxylase activity except at 45°C in the assay system. However, the activity of PEP carboxylase in chloroplasts from primary leaves grown under the same condition was only slightly affected in comparison with that from chloroplasts in primary leaves grown at normal temperature.

VI. EVALUATION OF THE 'LACERATION TECHNIQUE'

Due to the fibrous nature of maize leaf tissue it is difficult to discrupt the tissue without damaging the chloroplasts. Most studies with isolated chloroplasts were based on methods of blending or grinding (Woo et al., 1970; Bishop et al., 1972; Andersen et al., 1972; Bazzaz and Govindjee, 1973). The 'laceration technique' was first developed by Mache and Waygood (1969), and later on modified by Waygood et al. (1971). It is obvious that each method has its own advantages and limitations. It is the purpose of this section to report on some comparative studies of the commonly employed methods of isolation with particular reference to the 'laceration technique' and to evaluate the validity of this technique and its application to maize leaves.

A. Physical Integrity of Isolated Chloroplasts as Observed with Light Microscope

Three isolation methods were compared, namely (a) 'laceration technique', (b) gentle grinding in a chilled mortar, and (c) blending with waring blender. The method of isolation and chloroplast count were as described in Methods and Materials. Chloroplasts were isolated from 3 g of leaves in 15 ml of Solution H and resuspended in 2 ml of Solution A. In cases where the media were heavily laden with broken chloroplast fragments and starch granules the chloroplast suspension was diluted again with Solution A in a 1:1 ratio before counting.

The amount of foaming observed during the process of isolation and the amount of chloroplast fragments in suspension media observed under the microscope are indicated with the "+" sign (Table XIV). Intact chloroplasts were those observed with complete unbroken membrane. Broken chloroplasts referred to those plastids with broken membrane but retaining at least three quarters of the chloroplast body. Fragments usually appeared in the form of broken membranes, starch granules and other particles of smaller sizes. Table XIV summarizes the results of this comparative studies. It can be seen that the 'laceration technique' gives the highest percentage of intact chloroplasts recovered, and the least amount of broken fragments and there was no foaming during isolation of the plastids from both primary and secondary leaves. Grinding and blending were lower by 5 to 10% as far as the percentage of intact chloroplasts were concerned. The method of grinding broke many of the chloroplasts and released numerous starch granules and broken fragments into the media. Blending was a much gentler technique, but it produced a lot of foaming which renders it undesirable for enzyme studies. It can also be seen that with all the advantages afforded by the 'laceration technique' it gives the lowest yield of chloroplast number per gram of leaf tissue.

Table XV shows the distribution of intact chloroplasts between mesophyll and bundle sheath chloroplasts. The latter were identified by their positive reaction with I-KI. It can be seen that the chloroplast pellet obtained with the 'laceration technique' is predominantly mesophyll. Bundle sheath chloroplasts only account

for 10 to 12% of the total number of chloroplasts. However, among the broken chloroplasts, between one-third to two-thirds of the plastids were bundle sheath in origin. So the 'laceration technique' should not be recommended for bundle sheath chloroplasts.

B. Recovery of Phosphoenolpyruvate and Ribulose Diphosphate Carboxylases by Different Techniques

It has been found by Kanai and Edwards (1973b) that sequential release of mesophyll protoplasts and bundle sheath cell contents by respective enzymatic maceration and grinding would give a higher total recovery of PEP and RuDP carboxylase activities as compared with the values obtained from whole leaf preparation. Accordingly, an experiment was undertaken with the view of comparing PEP and RuDP carboxylases recovery by different methods, namely (a) grinding with acid washed sand, (b) blending with waring blender and (c) 'laceration technique' plus grinding. Enzyme preparations were made from 5 g of leaf tissue and 15 ml of buffer each. Blending was carried out for 5 minutes at full speed in the waring blender and at 15 second pulses. Grinding was carried out with acid washed sand to ensure breakage of the fibrous bundle sheath tissue. The leaf tissue after laceration was further ground gently in a chilled mortar to release the contents of bundle sheath cells. The chloroplast and supernatant fractions obtained by the laceration technique was designated Chl_L and $supt_L$ respectively, while those obtained from subsequent gentle grinding were designated as Chl_G

and $Supt_G$ respectively. From Table XVI the total units of PEP and RuDP carboxylases recovered by grinding were 442 and 158 units respectively (1 unit being 1 μm CO_2/mg protein/hr) for primary leaves and 479 and 174 units respectively for secondary leaves.

As compared with grinding, blending appeared to be more efficient in PEP carboxylase recovery with 625 and 596 units for primary and secondary leaves respectively. But it only recovered 148 units for RuDP carboxylase for primary leaves and 159 units for secondary leaves. The 'laceration technique' recovered a total 1,146 units of PEP carboxylase and 198 units of RuDP carboxylase from primary leaves, and 928 units of PEP carboxylase and 163 units of RuDP carboxylase for secondary leaves. The figure for RuDP carboxylase was comparable with that of Kanai and Edwards (1973b)while that of PEP carboxylase was higher than that reported by these workers.

The PEP carboxylase: RuDP carboxylase ratio was also calculated for each technique and for each leaf type. It can be seen that the 'laceration technique' is the best for PEP carboxylase activity recovery for both leaf types.

C. Comparison of CO_2 Fixation Capcity of Secondary Leaf Chloroplasts Isolated by Different Techniques

It has been shown in the previous section that 'laceration technique' coupled with grinding produced the best results as far as enzyme recovery is concerned. It was thought that chloroplasts obtained

by different techniques may demonstrate differences in CO_2 fixation capacity. Accordingly, an experiment was undertaken to compare the CO_2 fixation capacity of chloroplasts isolated by the 'laceration technique' and grinding. Table XVII shows that the chloroplasts isolated by both techniques demonstrated a slight increase in CO_2 fixation with increase of temperature from 20^o to 40^oC in the absence of substrate. However, at 4mM PEP, chloroplasts isolated by laceration technique showed a steady increase in this capacity from 20^o to 40^oC, which was also observed previously (Fig. 28a) where 40^oC was the optimum temperature for CO_2 fixation by chloroplasts isolated by this method. But, the chloroplasts isolated by grinding showed a higher rate of fixation at 20^oC in comparison with the chloroplasts isolated by the 'laceration technique'. The maximum rate was attained at 30^oC and the rate at 40^oC dropped to half of the maximum value. Chloroplast preparation from both techniques demonstrated enhanced CO_2 fixation in the presence of RuDP (4 mM) and both attained the highest rate at 40^oC. The chloroplasts obtained by 'laceration technique' showed a steeper increase between 30 to 40^oC. However, in the presence of both PEP and RuDP, only chloroplasts isolated by the 'laceration technique' demonstrated the synergistic effect of the two compounds on CO_2 fixation. The chloroplasts isolated by grinding did not demonstrate such a phenomenon.

D. Separation of Bundle Sheath and Mesophyll Chloroplasts by Differential Centrifugation

Arya and Waygood (unpublished) developed the technique of separation of bundle sheath and mesophyll chloroplasts on the basis of weight difference between the starch loaded bundle sheath chloroplasts and the lighter mesophyll chloroplasts. The chloroplast suspension after laceration was filtered through 2 layers of Kleenex tissue and then centrifuged successively for 10 seconds at 750 g, 20 seconds at 1,000 g and 50 seconds at 2,000 g. Table XVIII summarizes the results for biochemical differnces among these pellets which may be used as an indication for the degree of separation.

It was observed that the 750 g pellet, containing the heaviest chloroplasts, was highest in RuDP carboxylase activity, while the 2,000 g pellet, containing the lightest chloroplasts, was highest in PEP carboxylase activity. This indicates that the 750 g pellet was enriched by bundle sheath chloroplasts whereas the 2,000 g pellet by mesophyll chloroplasts. The capacity for CO_2 fixation in the absence or presence of either PEP or RuDP was highest in the 1,000 g pellet presummably enriched by both types of chloroplasts.

DISCUSSION

The $^{14}CO_2$ fixation experiments with primary and secondary leaf discs in Sections I and II were designed to answer the question whether malate in maize leaves serves as a means of storage of CO_2 as suggested by O'Neal et al. (1972) or as a necessary carrier of CO_2 as suggested by others (Slack et al., 1969; Farineau, 1971; Samejima and Miyachi; 1971) and whether primary leaves and secondary leaves of maize differ in their photosynthetic carbon metabolism. If malate were merely a CO_2 storage agent and the Calvin cycle were the dominant photosynthetic carbon pathway it would be expected that in kinetic experiments the percentage of radioactivity recovered from malate would increase while that from 3PGA would remain relatively low having a rapid turnover. In pulse-chase experiments, other photosynthetic intermediates would gain radiocarbon at the expense of 3PGA, but not malate or aspartate. On the other hand, if malate were a necessary CO_2 carrier, then it would be expected that in kinetic experiments the levels of radioactivity of both malate and 3PGA would remain low being immediately metabolised. And in pulse-chase experiments, 3PGA and other photosynthetic intermediates would gain radioactivity with a concomitant decrease of radiocarbon in malate.

From the results, it can be seen that in kinetic experiments of primary leaf discs, the percentage of radiocarbon in malate remained relatively high for the 20 minutes duration of feeding time while that of 3PGA decreased with time indicating a slow metabolism of malate

and a rapid turnover of 3PGA. In pulse-chase experiments with primary leaf tissue, at the end of the 15 second pulse, both malate and 3PGA were heavily labelled, then both decreased in radiocarbon during the chase period with 3PGA exhibiting a more rapid loss. This pattern was consistent with other experiments (not reported in this thesis) with 2 second pulse. Malate was heavily labelled at the end of the 15 second pulse (also at the end of 2 second pulse in other experiments), and never appeared to gain radiocarbon at the expense of 3PGA, indicating an independent β-carboxylation reaction. If this interpretation is correct, then multiple carboxylation reactions do take place in these green tissues as suggested by Zelitch (1971). In primary leaf experiments the low ^{14}C content of 3PGA suggests a heavy demand for this compound and consequently rapid protein synthesis (Bassham, 1971). This was also observed in $^{14}CO_2$ fixation experiments with isolated chloroplasts from primary leaves. The concomitant rise in radiocarbon of sugar phosphates with the fall of that in 3PGA implies an actively operating Calvin cycle in vivo. Isocitrate lactone was significantly labelled at the end of the 15 second pulse period (also at the end of 2 second pulse in other experiments). Concomitant with its subsequent fall in radioactivity was the rise of glycine and serine in pulse-chase experiments. This compound, isocitrate lactone, has not been reported previously as a product of photosynthesis in maize. However, the results agree

with the work of Mahon et al. (1974) who reported that glycine and serine in maize leaves were found to be more heavily labelled in experiments conducted in 21% oxygen than those in 1% oxygen, the reverse was found true for alanine and 3PGA. It is possible that low oxygen tension inhibits the incorporation of ^{14}C into isocitrate lactone, or that isocitrate lactone is a product of steady state photosynthesis only. This may explain why it was not reported by investigators who conducted their experiments in controlled atmosphere and dark and light transients (Latzko et al., 1971; Farineau, 1971; Samejima and Miyachi, 1971). The early appearance of this compound (even at the end of 2 second pulse) suggests that it could have acquired its radioactivity from a C_4-pool as proposed by Tan (1974). This compound then in turn passes on its radioactivity to glycine and serine possibly via glyoxylate and isocitrate lyase. Alanine was heavily labelled at the end of 1 minute fixation in kinetic experiments. But in pulse-chase experiments it was not so highly labelled at the end of the pulse period. Rather it gained radioactivity at the expense of 3PGA, which might be its precursor. Formation of aspartate presumably via oxaloacetate was a slow process in these experiments with primary leaves.

The labelling pattern of $^{14}CO_2$ fixation experiments with secondary maize leaf discs was somewhat different. The low level of radiocarbon in both malate and 3PGA in kinetic experiments indicates a rapid turnover rate for both of these compounds. The increased radiocarbon level in 3PGA presumably at the expense of malate, with

the subsequent gain of radioactivity by sugar phosphates and sugars at the expense of 3PGA indicates that malate is perhaps a carrier of CO_2 in these tissues. This carboxyl group is in turn passed onto RuDP to form 3PGA for the Calvin cycle. This labelling pattern agrees with what was reported by Johnson and Hatch (1969). Differing from primary leaf discs is the more rapid formation of aspartate in secondary leaf tissues and its rapid turnover as indicated in pulse-chase experiments, suggesting rapid C_4 acid synthesis and their demand by the cell. Isocitrate lactone bore a similar relationship with glycine and serine as that observed with primary leaf tissues. The carbon flow into sugar from sugar phosphates also followed that of the Calvin cycle pattern.

It can thus be concluded that in young developing maize primary leaves, C_4 acids may not be the sole nor the dominant route of entry of carbon into cellular metabolism, but rather, they act as an accessory route to the Calvin cycle which is the dominant photosynthetic pathway. However, this acid is the necessary CO_2 carrier in mature fully differentiated secondary leaf tissues. The labelling pattern as seen in secondary leaves does not preclude the possibility that CO_2 can enter bundle sheath cells directly via the Calvin cycle without going through the process of β-carboxylation. However, it probably proceeds at a very slow rate because of the difficulty to gain access into the deep lying bundle sheath tissues in mature leaves. Supporting evidence for this process is that isolated bundle sheath strands in C_4 plants including maize have been shown to be able to fix CO_2 without

the addition of substrates (Edwards and Black, 1971; Edwards et al., 1970; Chollet and Ogren, 1972, 1973). In any case β-carboxylation is the dominant route for mature secondary leaf tissue as compared to the Calvin cycle in young primary leaves.

Electron microscopy (not reported in this thesis) showed that the chloroplasts in primary leaf tissue were largely undifferentiated, while those from secondary leaf tissues were differentiated. Therefore the data obtained in this section are not sufficient to provide evidence as to whether the different role of C_4 acids observed in primary and secondary leaves is a result of development alone or that of a basic difference in metabolism between primary and secondary leaves as well. To further clarify this point it would be necessary to undertake experiments with both leaf types and at various stages of development of each. This study also showed that the choice of plant material of comparable stages of development is essential for C_4 plant research workers.

Experiments on $^{14}CO_2$ fixation by cellular fractions of primary and secondary leaves and their activities of PEP and RuDP carboxylases were designed to answer the following questions: (a) is there a basic difference in carbon metabolism of the chloroplasts from primary and secondary leaves of maize, (b) if there is, would this explain the difference observed with leaf tissues in Sections I and II. (c) would these differences be attributed to the differences in the key carboxylase --- phosphoenolpyruvate carboxylase, and (d) does the possibility exist that the activity observed in chloroplasts is but contamination by the cytoplasmic (supernatant) content? Since

it is difficult to discuss $^{14}CO_2$ fixation of chloroplasts without referring to the enzyme activity, Sections III, IV and V will be discussed together.

The primary leaf chloroplasts isolated in experiments in Section III were not capable of fixing CO_2 without the addition of substrate, with an exception on one occasion. Even then, the rate was very low in comparison with what was reported by other investigators (O'Neal et al., 1972; Chollet and Ogren, 1972). It is very likely that the chloroplasts isolated from expanded primary leaves were much more advanced in their differentiation than those from unrolled primary leaves (2 to 4 days after planting) employed by these workers. The significant experiment as shown in Fig.11 might have included in the preparation the odd chloroplasts which were less differentiated. This also implies that the younger the primary leaf tissue the better the chance of isolating chloroplasts which can fix CO_2 without the addition of substrate. In these cases the 'laceration technique' cannot be employed since the laceration blades are designed for expanded leaves which can lie flat in the glass trough during isolation.

Isolated chloroplasts from secondary leaves were unable to fix $^{14}CO_2$ at significant rates. However, this should not be surprising, since the generation of the CO_2 acceptor, PEP, in mature mesophyll cells is dependent on the availability of pyruvate, derived from malate and transported to the mesophyll cells by the bundle sheath cells (Slack et al., 1969). It was also found that even isolated mesophyll protoplasts from mature secondary leaves were unable to fix CO_2

without the addition of pyruvate or phosphopyruvate (Edwards and Black, 1971; Kanai and Edwards,1973a). The ability to fix CO_2 without the addition of exogenous substrate may not therefore be an appropriate criterion for photosynthetic activity for C_4 mesophyll chloroplasts. In its place, the capacity of light dependent conversion of pyruvate into PEP can be used. So the failure of endogenous $^{14}CO_2$ fixation by secondary leaf chloroplasts does not necessarily imply that they were photosynthetically inactive. Again there is always the possibility that from very young unrolled secondary leaves of maize, one could isolate young undifferentiated chloroplasts which can fix CO_2 endogenously.

Isolated chloroplasts from primary and secondary leaves of maize were found to possess the capacity for $^{14}CO_2$ fixation in the presence of PEP, which is in agreement with the reports of Baldry et al. (1969) for sugar cane and Waygood et al. (1971) for maize. It was also found that isolated chloroplasts from primary and secondary leaves of maize could fix CO_2 at comparable rates on a per milligram protein basis. In addition, it has been the author's experience that supernatant fractions always fixed $^{14}CO_2$ at rates at least three fold higher than that of the chloroplast fractions.

Table XIX summarizes the apparent Km values of PEP, HCO_3^- and Mg^{+2} for the CO_2 fixation capacity and PEP carboxylase activities of the chloroplast and cytoplasm (supernatant) fractions of primary and secondary leaves of maize. The Km values reported for the substrates are considerably higher than those reported in the

literature (Waygood et al., 1969; Walker, 1957; Ting and Osmond, 1973a; 1973b), but these should be evaluated in the view of the preparations used, which may have contained membranes and substrates which directly or indirectly inhibited the reaction pathways. There was no attempt made to remove any potential inhibitory effectors. Nevertheless, there was a degree of correlation between the CO_2 fixation capacity and PEP carboxylase activity to suggest that the carboxylase participated in a regulatory role for CO_2 fixation and this is supported by experiments to be discussed later.

It is important to note that the primary leaf PEP carboxylase under these experimental conditions had a much lower affinity for its substrate, PEP, than the secondary leaf enzyme. This may be partly responsible for the predominance of the Calvin cycle in primary leaves in comparison to the predominance of the C_4-dicarboxylic acid cycle in secondary leaves. There were also other differences which were not evident in the apparent Km values. For example there was a linear relationship between Mg^{+2} concentration and initial velocity of PEP carboxylase up to 10 mM Mg^{+2} in both the chloroplast and supernatant enzyme fractions of primary leaves and a V_{max} could not be calculated. On the other hand the curves for the secondary leaf enzymes showed a V_{max} from 1 to 4 mM Mg^{+2} above which concentration it was inhibitory.

From all of these results the author suggests that, under these experimental conditions, the basic metabolism of primary and secondary leaves are different in the predominance of one or the other of the

two generally held pathways of photosynthetic carbon metabolism. The difference between the primary and secondary leaf PEP carboxylases may also point to the probability of multiple forms (isoenzymes) of this enzyme in the developmental phases of leaf tissue (Ting and Osmond, 1973a, 1973b). The respective apparent Km values do not allow us to draw any conclusive evidence that the chloroplast and cytoplasmic PEP carboxylases are different either within or between the primary and secondary leaves, but other evidence focuses on this point as follows.

In later experiments, the response to temperature of PEP carboxylase and also RuDP carboxylase was followed in relation to normal (20^{o}-25^{o}C) and subnormal (15^{o}-20^{o}C) temperature treatment during growth, 4 to 5 days for primary leaves and 2 to 3 weeks for secondary leaves depending on the temperature range. CO_2 fixation experiments were always fortified with PEP, but not RuDP, although both substrates were tested in the enzyme preparation. The results from 8 cellular fractions from each set of the plants given in Figures 26 - 29 and 42 - 45 are summarized in tabular form below:

	Primary<------LEAVES------>Secondary							
	10 - 15°C		20 - 25°C		10 - 15°C		20 - 25°C	
	Chl	Cyt	Chl	Cyt	Chl	Cyt	Chl	Cyt
CO_2 Fixation	+	+	+	+	-	+	+	+
PEP Carboxylase	+	+	+	+	-	+	+	+
RuDP Carboxylase	+	+	+	+	+	+	+	+

+ : Positive temperature response up to 40^{o}C (Figs. 26-29, 42-45)
- : Negative temperature response up to 40^{o}C (Figs. 29a, 45a)

The significant finding is the absence of CO_2 fixation and PEP carboxylase in the chloroplast fraction of secondary leaves grown at subnormal temperature. However, there was some activity of PEP carboxylase, but not CO_2 fixation at an assay temperature of 45^oC.

The present investigation shows that both the CO_2 fixation capacity and the activities of PEP and RuDP carboxylases in maize grown at normal temperatures acquired optimum activity from 30 to 40^oC. This is in agreement with the report of Treharne and Cooper (1969).

Chilling temperatures have been shown to modify both the in vivo and in vitro activity of photosynthetic enzymes such as NADP-malate dehydrogenase and pyruvate Pi dikinase (Taylor et al., 1974) from maize and Sorghum. In this study the loss of CO_2 fixation capacity by secondary leaf chloroplasts was probably a result of the loss of PEP carboxylase activity when plants were subjected to subnormal temperature condition. If this is the case, the results imply that PEP carboxylase in this fraction may be associated with chloroplasts and not merely a chance contamination from the supernatant, and also, the enzyme must be participating in a regulatory role governing CO_2 fixation. Supporting evidence for this concept includes the report by Phillips and McWilliam (1971) that at reduced temperatures (below 12^oC), the PEP carboxylase of maize showed a sudden increase in activation energy, suggestive of a conformational change in a membrane-bound enzyme due to a phase change occurring in membrane lipids. Moreover, Taylor and Craig (1971) also observed extensive ultrastructural changes in chloroplasts of the C_4 plant Sorghum after subjecting it

to subnormal temperatures for three days. Although maize is not as sensitive to chilling as Sorghum, it is not surprising that similar changes would be observed when it has been subjected to 3 weeks of subnormal temperatures. Treatment of maize chloroplast extracts with Triton-X eliminated the sudden increase in activation energy as reported by Phillips and McWilliam (1971), but resulted in a higher but constant activation energy over a wide temperature range. This provided evidence for a loose association between PEP carboxylase and a lipid component of a membrane as was also suggested by Slack et al. (1969) and Baldry et al. (1969). Many phenomena observed by workers in this field also support a loose association of PEP carboxylase with chloroplasts. Slack et al. (1969) obtained occasional association of the enzyme with chloroplasts using non-aqueous method of isolation although other workers found its association with the cytoplasmic fraction when using aqueous method of isolation (Hatch and Kagawa, 1973; Lyttleton, 1971). Washed chloroplasts lost their ability to fix CO_2, but when heated to 45°C they regained their CO_2 fixation capacity (Arya and Waygood, personal communication). The present author has observed activity of PEP carboxylase at 45°C in chloroplasts from chill treated secondary leaves, which were completely inactive below this temperature (Fig. 45a). PEP carboxylase activity was found to be totally lost with one wash after isolation (Lyttleton, 1971), which was also the experience of the author in this investigation, and sonicated chloroplasts were unable to fix CO_2 even in the presence of PEP. Positive evidence for a chloroplastic PEP carboxylase was obtained by Pan and Waygood

(1971) which was thermostable, had an optimum pH of 5.4, but still had considerable activity at pH 7.0 in chloroplast preparations (Pan, 1974). It is uncertain whether the enzyme observed in this study is the same as the thermostable one. If these enzymes have a similar origin, then the heat activation phenomena in the author's experiments and those of Arya and Waygood may be explained by a reassociation of the enzyme with a membrane from which it may have dissociated during the cold treatment or in washing the preparation. Further evidence for the presence of more than one PEP carboxylase isoenzymes in C_4 tissues has been provided by Ting and Osmond (1973a, 1973b) who observed that there were shoulders in the elution peaks of PEP carboxylase purified with DEAE-cellulose. The isoenzymes from these shoulders were remarkably constant in their kinetic parameters.

Some investigators (Taylor et al., 1974) have cast doubt on the regulatory role of PEP carboxylase on photosynthetic rates because of its high level even in chilling conditions (1 to 3 days of chilling) as compared with other C_4 pathway enzymes. However, the data in this thesis indicates that this high level of PEP carboxylase can be attributed to the cytoplasmic enzyme which may or may not be a regulatory photosynthetic carboxylase, and its activity was not severely affected by lowering the growth temperature as is the enzyme associated with the chloroplasts from secondary leaves. Furthermore, 1 to 3 days of chilling in their experiments might not have provided enough time for the change in enzyme level to take place.

The kinetic data in this thesis neither supports nor precludes the possibility of isoenzymes in the chloroplast and supernatant fractions or that the chloroplast PEP carboxylase is cytoplasmic in origin. But chill treatment experiments show that (at least for secondary leaves) if it is cytoplasmic in origin it may become associated with chloroplast membranes under condition of plant growth at 20 - 25°C or at temperatures above 40°C in chloroplast enzyme preparation from chilled leaves. Under these circumstance, PEP produced by pyruvate Pi dikinase (a mesophyll chloroplast enzyme) would be available as substrate for PEP carboxylase in vivo.

It has also been observed (Brooking and Taylor, 1973; Hofstra and Nelson, 1969) that low temperature treatment during plant growth slowed down the release of photosynthetically incorporated radiocarbon from aspartate and malate into the C_3 cycle in Sorghum and maize respectively. In the light of the present study another way to interprete this phenomenon is that cytoplasmic PEP carboxylase is still active and C_4 acids are being produced even under chilling temepratures. However, further metabolism of C_4 acids through a possible transcarboxylation reaction necessitates their intimate contact with mesophyll chloroplast membranes from which the cytoplasmic malate and aspartate would not have ready access because of their spatial separation.

Further evidence for differences between supernatant and chloroplast CO_2 fixation in the presence of PEP is shown in the distribution of ^{14}C in products of CO_2 fixation. It was observed that the

supernatant fractions had a considerable higher percentage of radioactivity in sugar phosphates whereas the chloroplasts fractions were more heavily labelled in amino acids and sugars. The high percentage of label in sugar phosphates in the supernatant fraction may reflect the possibility that some of the bundle sheath chloroplasts were broken during isolation and the enzymes therefore escaped into the laceration medium (supernatant). The high activity of RuDP carboxylase in the supernatant fraction as compared to the chloroplast fraction supports this.

A high percentage of labelling was located in the 3C (three carbon) compounds namely, glyceraldehyde, dihydroxy acetone and glycerate. The chloroplast fractions, which were predominantly mesophyllic (see Section VI) were heavily labelled in all 3C compounds, whereas in the supernatant, only glycerate was heavily labelled, but the other two were not. It is well known that glycerate kinase, NADP glyceraldehyde dehydrogenase and triose phosphate isomerase are located in both bundle sheath and mesophyll cells (Slack et al., 1969; Hatch and Kagawa, 1973). These enzymes lead to the formation of 3PGA, dihydroxyacetone phosphate and glyceraldehyde phosphate. However in these experiments, the phosphorylated forms of the 3C compounds were not found probably due to phosphatase activity. If this interpretation is correct and the 3C compounds were phosphorylated reaction products, they could have arisen from a transcarboxylation reaction first postulated by Hatch and Slack (1966). A positive evidence for a transcarboxylation reaction in maize extract was

obtained by Pan (1974) who obtained malate and 3PGA when the C_4 phosphorylated product of his thermostable acid PEP carboxylase reaction reacted with a 5C (five carbon) phosphorylated sugar.

Further evidence in support of some type of transcarboxylation reaction in C_4 plants has been provided by Whelan et al.(1972) while studying the enzymatic fractionation of carbon isotopes by PEP and RuDP carboxylases. They argue that if the route of carbon in C_4 plants is $CO_2 \xrightarrow{PEP}$ malate $\dashrightarrow C_3$ $\dashrightarrow CO_2 \xrightarrow{RuDP}$ PGA then RuDP carboxylase would be the step in this sequence which determines the isotopic composition of the intermediates. The δ^{13} values for the carbon intermediates of C_4 plants would then resemble that of C_3 plants which they do not. They also argue that only if all of the CO_2 fixed into malate is transferred (or decarboxylated to CO_2 and refixed) to RuDP carboxylase would the δ^{13} C content of the carbon intermediates be determined at the PEP carboxylase step. All the evidence indicates that these two enzymes respectively determine the isotopic composition of C_3 and C_4 plants. The enzymatic mechanism which could satisfy the isotopic discrimination data would therefore be a transcarboxylation in C_4 plants. Finally, Kortschak (1971) found that in mesophyll chloroplasts of sugarcane leaves, fructose was labelled more rapidly from 3-^{14}C labelled aspartate and malate than from those labelled in the β-carboxyl. This is further evidence supporting a transcarboxylation reaction.

As previously mentioned, malate was not found as a product of CO_2 fixation in these cellular fractions at a concentration of

3.2 mM PEP whereas it was the principal product at a concentration of 16 mM PEP (Arya and Waygood, 1971), and this has also been the author's experience. The absence of malate may be due to a very rapid decarboxylation in which the $^{14}CO_2$ would either enter the CO_2 pool or be transferred directly to RuDP forming 3PGA by the very low amount of RuDP carboxylase found in the chloroplast fraction. It is doubtful, however, that malate could have been formed and decarboxylated. In experiments not reported in this thesis, secondary leaf chloroplasts were fed with U-^{14}C-malate (s.a. 20 µCi/4mM), and it was found that there was no decarboxylation of malate and over 90% of radioactivity was recovered in malate after column and thin layer chromatography of the chloroplast extract, radioactivity was not recovered in any other compound.

There are two other possible ways to interprete the absence of malate as a product of the β-carboxylation reaction at a concentration of 3.2 mM PEP and its presence at 16 mM PEP. Phosphoenolpyruvate could play a regulatory role in the process of transfer of carbon obtained by β-carboxylation from mesophyll cell to bundle sheath cells. For example, at high levels of PEP, the transfer of carbon may be predominantly by the decarboxylation of malate, an hypothesis which is favoured by most investigators (Huber et al., 1973). Perhaps at low level of PEP a transcarboxylation reaction is the preferred pathway. Another explanation could be that at 16 mM PEP β-carboxylation resulted in a large pool of oxaloacetate or the initial C_4 product and a transcarboxylation reaction may not be efficient enough to deplete the pool before it was converted into malate.

PEP carboxylase activity in chloroplasts from primary leaves was not influenced by low temperature treatment to the extent of that in chloroplasts from secondary leaves. The primary leaves were harvested 4 to 5 days after being transferred into a low temperature growth chamber, and there may not have been sufficient time for the chilling effect to produce any significant influence on the PEP carboxylase of the chloroplast enzyme of these leaves. The secondary leaves were processed in the laboratory after two to three weeks exposure to chilling temperature. There is also a possibility that the properties of the chloroplast PEP carboxylase from primary leaves were totally different from that of the secondary leaf, for example, the linear relationship between Mg^{+2} and the initial velocity of PEP carboxylase as mentioned (Section V).

β-carboxylation appears to be very active in primary leaf cell fractions when fortified with 3.2 mM PEP. The carbon flow data for these leaves indicate the predominance of the Calvin cycle (Section I). This may be due to a lower level of pyruvate Pi dikinase in the relatively undifferentiated mesophyll chloroplasts, or if pyruvate arises from 3PGA, and 3PGA is drawn into extensive amino acid synthesis, (e.g. alanine, Fig.5) then there would be a scarcity of PEP for β-carboxylation. Also the data indicate that PEP carboxylase in primary leaf fractions has a relatively lower affinity for PEP and higher requirement for Mg^{+2} than that in secondary leaves in which the carbon flow data indicate predominance of the Hatch and Slack cycle (Section II). It must be remembered that with the laceration

technique' the chloroplast pellet was predominantly mesophyll in origin. Though the C_4 enzymes appear very active we are not able to assess their activity relative to those of the Calvin cycle in the bundle sheath chloroplasts.

To summarize: the study in Sections III, IV and V has produced evidence that the CO_2 fixation capacity of secondary leaf chloroplasts is not due to contamination by cytoplasm, and is regulated by a chloroplastic PEP carboxylase since it cannot be demonstrated in chloroplasts from secondary leaves grown at subnormal temperatures (10 - 15°C) unless the assay medium is held at a temperature of 45°C. If the C_4 primary product of β-carboxylation undergoes a transcarboxylation rather than the formation of malate and its subsequent decarboxylation in the bundle sheath cells, then the transfer of photosynthetically incorporated carbon from mesophyll to bundle sheath cells would more likely be in the form of dihydroxy acetone phosphate or a related compound.

The fact that pyruvate Pi dikinase is located in mesophyll chloroplasts (Slack et al. 1969); the biochemical enzymatic evidence for a transcarboxylation reaction produced by Pan (1974); the evidence produced in this investigation of the presence of a chloroplastic PEP carboxylase, and the formation of 3PGA, dihydroxyacetone phosphate, glyceraldehyde phosphate in mesophyll chloroplasts; the absence of malate as a product of CO_2 fixation by chloroplasts at 3.2 mM PEP; the failure of the chloroplast system to decarboxylate malate make it very tempting to suggest that the role of the mesophyll chloroplasts

in maize plant is (a) to produce the CO_2 acceptor, PEP, (b) to mediate the β-carboxylation of CO_2 resulting in the production of a C_4 initial product, and (c) to allow this product to undergo a transcarboxylation reaction to form 3C photosynthetic intermediates which are then transported to the bundle sheath chloroplasts. If this is the case, then the C_4 mechanism of maize under certain experimental conditions may not be that of a CO_2 pump as suggested by Slack et al.(1969), but that of a CO_2 trap as suggested by Laetsch (1974) with the exception that CO_2 is not decarboxylated in the mesophyll chlorplasts but transcarboxylated in the mesophyll chloroplasts (see Literature Review).

The last section of this thesis (Section VI) concerns the evaluation of the 'laceration technique' in comparison to other techniques of isolating chloroplasts.

The results in this section reveal that the 'laceration technique' is the most efficient method in obtaining physically intact mesophyll chloroplasts, but not bundle sheath chloroplasts. Moreover, the isolation medium did not foam and the suspension medium was almost free from broken chloroplasts fragments. It is suspected that the foaming produced by blending implies certain oxidation reaction which might be undesirable for enzyme studies. Broken fragments and starch granules indicate the breakage of chloroplasts and organelles. Accordingly, under these conditions there would be many compounds released from the organelles, which otherwise would not have come into contact with chloroplasts. The effect of such fragments on the

biochemical behaviour of chloroplasts or cytoplasmic fractions have yet to be studied.

The chloroplasts isolated by the 'laceration technique' and those by grinding were affected differently by temperature with respect to their CO_2 fixation capacity. Chloroplasts isolated by the 'laceration technique' attained the highest rate of CO_2 fixation at 40^oC and exhibited a synergistic effect of PEP and RuDP on this process. But chloroplasts isolated by the method of grinding attained highest fixation rates of CO_2 at 30^oC and did not exhibit a synergistic effect in the presence of PEP and RuDP. This indicates differences in the biochemical integrity of the two types of chloroplast preparations. The synergistic effect of PEP and RuDP on the CO_2 fixation capacity of the chloroplasts isolated by the 'laceration technique' could be taken as further evidence for a transcarboxylation reaction. If experiments were undertaken to determine the presence of a transcarboxylation reaction, a procedure should be used which provides minimal agitation of the chloroplasts as exemplified by the laceration technique.

The data also revealed that the 'laceration technique' is the most efficient in recovery of PEP carboxylase activity as compared with blending and grinding. The supernatant is considerably higher in total RuDP carboxylase activity from both primary and secondary leaves as compared with chloroplasts. It is possible that RuDP carboxylase leached out of some of the bundle sheath chloroplasts during the process of isolation, or that the RuDP carboxylase is

loosely bound to chloroplast membranes and is readily dissociated into the suspension medium. Supporting this loose association of RuDP carboxylase with the chloroplast membrane is the report by Lyttleton (1971) that washed chloroplasts lost both PEP and RuDP carboxylase activities in maize. Or as proposed by David and Merrett (1975) that some of the RuDP carboxylase subunits are synthesized in the cytoplasm and are assembled at the chloroplasts, the activities of RuDP carboxylase observed in the cytoplasmic fraction could be attributed to these preassembled subunits which could somehow show a certain amount of enzyme activity.

The major limitation of the 'laceration technique' is the inability for complete separation of the two kinds of chloroplasts. This, perhaps, is encountered in both grinding and blending and possibly other techniques as well. However, partial separation of bundle sheath and mesophyll chloroplasts can be achieved by the method of differential centrifugation developed by Arya and Waygood (unpublished). The results show that there was considerable differences in enzyme activity of the carboxylases between the 750 g and the 2,000 g pellets. The best fixation rate for CO_2 fixation came from the intermediate pellet, possibly enriched by both mesophyll and bundle sheath chloroplasts. It is suggested that CO_2 fixation is optimized when both types of chloroplasts are present. Isolated chloroplasts by the 'laceration technique' were unable to fix CO_2 endogenously, but would do so only in the presence of PEP or RuDP or both together.

It is concluded that the 'laceration technique' is suitable for the study of mesophyll chloroplasts, but not bundle sheath chloroplasts, of mature monocotyledonous plants including maize and wheat (Arya and Waygood, 1973) and is more desirable than the commonly employed methods of grinding and blending.

SUMMARY

On the basis of the present study with leaf tissue, isolated chloroplasts and cytoplasm of primary and secondary leaves of Zea mays L., the following conclusions can be drawn.

1. There is a difference in the basic photosynthetic carbon metabolism between primary leaves of 6 to 8 day old seedlings and secondary leaves of 16 to 18 day old seedlings of maize. The Calvin cycle is the predominant photosynthetic cycle in the primary leaves whereas the C_4 pathway predominates in the secondary leaves. The enzyme, PEP carboxylase, from the two types of leaves also differs in certain properties. The primary leaf enzymes never attained saturation at the concentrations of magnesium ion tested (0 - 10 mM), whereas the secondary leaf enzymes were saturated at the concentration of 1 to 4 mM. Enzymes from primary leaves also have a higher apparent Km value for PEP as compared with the values obtained with secondary leaf enzymes.
2. The products of CO_2 fixation by the cellular fractions of both leaf types indicate the possibility of a transcarboxylation reaction subsequent to the formation of the initial product of β-carboxylation. The major products of such a transcarboxylation reaction have been found to be the three carbon compounds, glycerate, dihydroxyacetone and glycerabldehyde. The site of this reaction is most probably located in the mesophyll chloroplasts for both primary and secondary leaves.

3. The CO_2 fixation capacity of secondary leaf chloroplasts in the presence of PEP is not an artifact due to contamination by the cytoplasmic PEP carboxylase. Evidence shows that it is mediated by a chloroplast-associated PEP carboxylase which can be inhibited by cold treatment during growth. This property of cold sensitivity is not shared by the cytoplasmic PEP carboxylase. The chloroplastic PEP carboxylase also plays a role in regulating incorporation of CO_2 into isolated chloroplasts.
4. The findings in the present investigation indirectly point out that the role of mesophyll chloroplasts in mature secondary leaf tissue may include the following:
   (a) to mediate the β-carboxylation of CO_2 resulting in the production of a C_4 initial product,
   (b) to allow this product to undergo a transcarboxylation reaction to form 3C photosynthetic intermediates.
5. The 'laceration technique' is recommended for isolation of mesophyll chloroplasts, but not bundle sheath chloroplasts, from monocotyledonous leaf tissue for studies on CO_2 fixation and enzyme activities. The chloroplast preparation is predominantly mesophyll in origin. The low amount of bundle sheath chloroplasts can be partially eliminated by differential centrifugation. This technique excels other commonly employed methods in maintaining <u>in vivo</u> physical and biochemical integrity of the chloroplasts. However, it does not give as high a yield of chloroplasts on a per gram fresh weight basis as compared with the others.

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APPENDIX

Table I. Distribution of radioactivity in extracts from maize primary leaf discs. 5 μCi $NaH^{14}CO_3$ (59 μCi/mM) were fed. Calculations were based on the specific activity of $NaH^{14}CO_3$. Results are expressed as μg CO_2 dm^{-2}.

$^{14}CO_2$ Fixation Time (min.)	Fraction	μg CO_2 dm^{-2}	%
1	Methanol-water	0.81	94.19
	Residue + Chloroform	0.05	5.81
	Total	0.86	100.00
2	Methanol-water	1.69	91.85
	Residue + Chloroform	0.15	8.15
	Total	1.84	100.00
5	Methanol-water	3.41	89.50
	Residue + Chloroform	0.40	10.50
	Total	3.81	100.00
10	Methanol-water	7.32	85.61
	Residue + Chloroform	1.23	14.39
	Total	8.55	100.00
20	Methanol-water	14.62	82.32
	Residue + Chloroform	3.14	17.68
	Total	17.76	100.00

Table II. Distribution of radioactivity in the various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs as a function of time. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

$^{14}CO_2$ Fixation Time (min.)	Fraction	dpm	%
1	Organic acid	12,523	79.70
	AminO-acid	3,190	20.30
	Sugar	---	0.00
	Total	15,713	100.00
2	Organic acid	26,499	79.92
	Amino-acid	6,656	20.08
	Sugar	---	0.00
	Total	33,155	100.00
5	Organic acid	49,777	68.54
	Amino-acid	15,468	21.30
	Sugar	7,377	10.16
	Total	72,622	100.00
10	Organic acid	81,314	55.50
	Amino-acid	27,523	18.78
	Sugar	37,687	25.72
	Total	146,524	100.00
20	Organic acid	153,672	49.93
	Amino-acid	38,709	12.58
	Sugar	115,402	37.49
	Total	307,783	100.00

Table III. Distribution of radioactivity in the various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs as a function time. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

Time (min.)	1		2		5		10		20	
	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
Organic acids										
Glycerate	---	0.00	---	0.00	2,444	3.37	6,002	4.10	10,368	3.37
Glycolate	---	0.00	---	0.00	---	0.00	---	0.00	5,391	1.75
Isocitrate lactone	1,396	8.88	818	2.47	1,890	2.60	3,361	2.29	5,184	1.68
Malate	3,129	19.91	7,841	23.65	17,386	23.94	26,527	18.10	48,520	15.76
3-Phosphoglycerate	2,419	15.39	6,548	19.75	6,966	9.59	10,443	7.13	10,160	3.30
Sugar monophosphates	2,648	16.85	6,707	20.23	10,238	14.10	16,925	11.55	23,846	7.75
Sugar diphosphates	---	0.00	---	0.00	3,029	4.17	4,305	2.94	2,488	0.81
Amino acids										
Alanine	1,736	11.05	1,630	4.92	2,510	3.46	3,975	2.71	3,134	1.02
Aspartate	---	0.00	1,175	3.54	3,128	4.31	4,287	2.93	5,044	1.64
Glutamate	---	0.00	626	1.89	1,651	2.27	4,521	3.09	7,148	2.32
Glycine + Serine	---	0.00	684	2.06	1,548	2.13	5,534	3.78	11,720	3.81
Sugar										
Fructose	---	0.00	---	0.00	3,620	4.98	14,967	10.21	38,483	12.50
Glucose	---	0.00	---	0.00	2,927	4.03	12,611	8.61	27,960	9.08
Sucrose	---	0.00	---	0.00	735	1.01	7,745	5.29	46,300	15.04

Table IV. Distribution of radioactivity in various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs in pulse chase experiment. Pulse: 30 µl $NaH^{14}CO_3$ (60.3 µCi/mM) for 15 seconds. Results are expressed in dpm and percentage of total radioactivity recovered from fractions.

$^{14}CO_2$ Fixation Chase period (min.)	Fraction	dpm	%
0	Organic acid	79,415	88.30
	Amino-acid	10,522	11.70
	Sugar	---	0.00
	Total	89,937	100.00
0.5	Organic acid	73,035	84.70
	Amino-acid	13,192	15.30
	Sugar	---	0.00
	Total	86,227	100.00
1.0	Organic acid	77,189	80.60
	Amino-acid	18,578	19.40
	Sugar	---	0.00
	Total	95,767	100.00
1.5	Organic acid	64,371	70.80
	Amino-acid	21,548	23.70
	Sugar	5,001	5.50
	Total	90,920	100.00
2.5	Organic acid	63,308	67.40
	Amino-acid	21,604	23.00
	Sugar	9,017	9.60
	Total	93,929	100.00
6.0	Organic acid	54,982	59.20
	Amino-acid	21,175	22.80
	Sugar	16,718	18.00
	Total	92,875	100.00

Table V. Distribution of radioactivity in the various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs in pulse chase experiment. Pulse: 30 µl $NaH^{14}CO_3$ (60.3 µCi/mM) for 15 seconds. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

Chase periods (min.)	0		0.5		1.0		1.5		2.5		6.0	
	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
Organic acids												
Glycerate	---	0.00	---	0.00	---	0.00	---	0.00	---	0.00	1,689	1.82
Glycolate	---	0.00	---	0.00	---	0.00	---	0.00	---	0.00	1,102	1.19
Isocitrate Lactone	12,118	13.47	8,307	9.63	8,583	8.96	5,173	5.69	5,857	6.24	2,571	2.77
Malate	20,672	22.98	12,838	14.89	14,903	15.56	13,105	14.41	14,905	15.87	13,515	14.55
3-phosphoglycerate	27,106	30.14	15,607	18.10	14,747	15.40	9,932	10.92	10,355	11.02	5,802	6.25
Sugar monophosphates	3,316	3.69	13,593	15.76	16,308	17.03	17,520	19.27	19,454	20.71	17,040	18.35
Sugar diphosphates	---	0.00	2,832	3.28	3,667	3.83	3,104	3.41	2,275	2.42	1,763	1.90
Amino acids												
Alanine	1,378	1.53	2,229	2.59	2,692	2.81	3,194	3.51	3,351	3.57	2,456	2.64
Aspartate	1,428	1.59	1,505	1.75	995	1.04	1,952	2.15	1,341	1.43	1,551	1.67
Glutamate	541	0.60	984	1.14	1,463	1.53	1,833	2.02	2,146	2.29	2,068	2.23
Glycine + serine	2,412	2.68	4,052	4.70	6,202	6.48	7,038	7.74	8,580	9.13	8,661	9.33
Sugar												
Fructose	---	0.00	---	0.00	---	0.00	2,540	2.79	3,469	3.69	5,256	5.66
Glucose	---	0.00	---	0.00	---	0.00	2,117	2.33	2,579	2.75	4,178	4.50
Sucrose	---	0.00	---	0.00	---	0.00	---	0.00	2,772	2.95	6,466	6.96

Table VI. Distribution of radioactivity in extracts from maize secondary leaf discs. 5 μCi $NaH^{14}CO_3$ (59 μCi/mM) were fed. Calculations were based on specific activity of $NaH^{14}CO_3$. Results are expressed as μg CO_2 dm^{-2}.

$^{14}CO_2$ Fixation Time (min.)	Fraction	μg CO_2 dm^{-2}	%
1	Methanol-water	1.01	96.19
	Residue + Chloroform	0.04	3.81
	Total	1.05	100.00
2	Methanol-water	1.88	92.16
	Residue + Chloroform	0.16	7.84
	Total	2.04	100.00
5	Methanol-water	4.69	90.19
	Residue + Chloroform	0.51	9.81
	Total	5.20	100.00
10	Methanol-water	8.99	86.28
	Residue + Chloroform	1.43	13.72
	Total	10.42	100.00
20	Methanol-water	17.18	81.62
	Residue + Chloroform	3.87	18.38
	Total	21.05	100.00

Table VII. Distribution of radioactivity in the various fractions of the methanol-water soluble fraction of the plant extracts from maize secondary leaf discs as function of time. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

$^{14}CO_2$ Fixation Time (min.)	Fraction	dpm	%
1	Organic acid	13,049	62.27
	Amino-acid	3,328	15.88
	Sugar	4,580	21.85
	Total	20,957	100.00
2	Organic acid	21,768	56.10
	Amino-acid	5,123	13.20
	Sugar	11,909	30.69
	Total	38,800	99.99
5	Organic acid	40,158	44.30
	Amino-acid	17,016	18.77
	Sugar	33,484	36.93
	Total	90,658	100.00
10	Organic acid	60,268	33.58
	Amino-acid	25,549	14.23
	Sugar	93,679	52.19
	Total	179,496	100.00
20	Organic acid	87,604	24.72
	Amino-acid	45,011	12.70
	Sugar	221,709	62.57
	Total	354,324	99.99

Table VIII. Distribution of radioactivity in the various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs as a function of time. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

Time (min.)	1		2		5		10		20	
	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
Organic acids										
Glycerate	---	0.00	1,762	4.54	1,483	1.64	1,936	1.08	3,059	0.86
Isocitrate Lactone	1,471	7.02	1,664	4.29	1,918	2.12	3,574	1.99	4,235	1.20
Malate	5,150	24.57	4,991	12.86	6,076	6.70	6,701	3.73	7,959	2.24
3-phosphoglycerate	2,638	12.59	4,078	10.51	7,488	8.26	10,275	5.72	8,236	2.32
Sugar monophosphates	1,920	9.16	6,460	16.65	11,444	12.62	20,848	11.61	24,924	7.03
Sugar diphosphates	---	0.00	---	0.00	5,369	5.92	8,637	4.81	13,648	3.85
Amino acids										
Alanine	---	0.00	---	0.00	1,455	1.60	1,666	0.93	2,593	0.73
Aspartate	1,885	8.99	2,115	5.45	2,840	3.13	2,435	1.36	4,007	1.13
Glutamate	---	0.00	---	0.00	1,533	1.69	1,538	0.86	3,065	0.87
Glycine + serine	537	2.56	1,374	3.54	7,205	7.95	12,558	7.00	20,273	5.72
Sugar										
Fructose	451	2.15	3,017	7.78	10,460	11.54	24,898	13.87	55,540	15.67
Glucose	353	1.68	2,086	5.38	9,279	10.24	20,856	11.62	39,446	11.13
Sucrose	510	2.43	3,017	7.78	11,051	12.19	38,640	21.53	102,560	28.95
Unidentified	2,346	11.19	1,733	4.47	---	0.00	---	0.00	---	0.00

Table IX. Distribution of radioactivity in various fractions of the methanol-water soluble fraction of the plant extracts from maize secondary leaf discs in pulse chase experiment. Pulse: 30 µl $NaH^{14}CO_3$ (60.3 µCi/mM) for 15 seconds. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

$^{14}CO_2$ Fixation Chase period (min.)	Fraction	dpm	%
0	Organic acid	61,095	77.10
	Amino-acid	18,146	22.90
	Sugar	----	0.00
	Total	79,241	100.00
0.5	Organic acid	71,269	86.50
	Amino-acid	11,123	13.50
	Sugar	----	0.00
	Total	82,392	100.00
1.0	Organic acid	64,543	81.30
	Amino-acid	14,846	18.70
	Sugar	----	0.00
	Total	79,389	100.00
1.5	Organic acid	60,111	77.90
	Amino-acid	17,053	22.10
	Sugar	----	0.00
	Total	77,164	100.00
2.5	Organic acid	57,306	68.70
	Amino-acid	19,769	23.70
	Sugar	6,340	7.60
	Total	83,415	100.00
6.0	Organic acid	44,138	56.90
	Amino-acid	20,711	26.70
	Sugar	12,722	16.40
	Total	77,571	100.00

Table X. Distribution of radioactivity in the various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs in pulse chase experiment. Pulse: 30 µl $NaH^{14}CO_3$ (60.3 µCi/mM) for 15 seconds. Results are expressed as dpm and percentage of total radioactivity recovered from fractions.

Chase periods (min.)	0		0.5		1.0		1.5		2.5		6.0	
	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
Organic acids												
Glycerate	---	0.00	---	0.00	---	0.00	666	0.86	444	0.53	551	0.71
Isocitrate Lactone	12,441	15.70	8,214	9.97	5,884	7.41	3,754	4.86	3,558	4.27	2,039	2.63
Malate	29,256	36.92	17,908	21.74	12,091	15.23	9,869	12.79	9,466	11.35	7,110	9.17
3-phosphoglycerate	11,005	13.89	22,495	27.30	15,648	19.71	12,533	16.24	11,118	13.33	6,062	7.81
Sugar monophosphates	---	0.00	15,392	18.68	18,945	23.86	17,921	23.22	19,503	23.38	16,205	20.89
Sugar diphosphates	---	0.00	---	0.00	---	0.00	3,088	4.00	1,335	1.60	1,268	1.63
Amino acids												
Alanine	1,537	1.94	2,009	2.44	1,682	2.12	1,584	2.05	1,485	1.78	2,028	2.61
Aspartate	8,936	11.28	1,039	1.26	1,009	1.27	1,278	1.66	1,102	1.32	1,612	2.08
Glutamate	---	0.00	1,385	1.68	1,442	1.82	1,942	2.52	2,155	2.58	2,496	3.22
Glycine + serine	2,561	3.23	4,918	5.97	4,854	6.11	6,491	8.41	6,610	7.92	7.747	9.99
Sugars												
Fructose	---	0.00	---	0.00	---	0.00	---	0.00	3,263	3.91	6,637	8.56
Glucose	---	0.00	---	0.00	---	0.00	---	0.00	2,641	3.17	5,068	6.53

Table XI. Distribution of radioactivity in various fractions of maize leaf chloroplast and supernatant extracts after 2 minutes of $NaH^{14}CO_3$ fixation in the presence of 3.2 mM phosphoenolpyruvate. Specific activity of $NaH^{14}CO_3$: 200 μCi/7.5mM. Results are expresse in dpm, percentage of total fixation and μm CO_2/mg protein.

	Primary Leaf				Secondary Leaf			
	Chloroplast		Supernatant		Chloroplast		Supernatant	
Fraction	dpm	%	dpm	%	dpm	%	dpm	%
Methanol-water	3,158,240	99.65	9,860,900	100.00	1,107,600	99.77	2,476,400	100.00
Chloroform	11,085	0.35	---	---	2,540	0.23	---	---
Total	3,169,325	100.00	9,860,900	100.00	1,110,140	100.00	2,476,400	100.00
μm CO_2/mg protein	0.28		2.04		0.15		1.02	

Table XII. Distribution of radioactivity in organic acid, amino acid and sugar fractions of maize leaf chloroplast and supernatant extracts after 2 minutes of $NaH^{14}CO_3$ fixation in the presence of 3.2 mM phosphoenolpyruvate. Specific activity of $NaH^{14}CO_3$: 200 µCi/7.5 mM. Results are expressed in dpm and percentage of total radioactivity recovered from columns.

	Primary Leaf				Secondary Leaf			
	Chloroplast		Supernatant		Chloroplast		Supernatant	
Fraction	dpm	%	dpm	%	dpm	%	dpm	%
Organic acid	480,496	40.27	1,810,408	51.92	183,882	40.10	329,820	54.62
Amino acid	72,511	6.08	102,099	2.93	16,005	3.49	14,927	2.47
Sugar	640,134	53.65	1,574,238	45.15	258,690	56.41	259,116	42.91
Total	1,193,141	100.00	3,486,745	100.00	458,577	100.00	603,863	100.00

Table XIII. Distribution of radioactivity in products of maize leaf chloroplast and supernatant extracts after 2 minutes of $NaH^{14}CO_3$ fixation in the presence of 3.2 mM phosphoenolpyruvate. Specific activity of $NaH^{14}CO_3$: 200 µCi/7.5 mM. Results are expressed in dpm and percentage of total radioactivity recovered from thin layer plates.

	Primary Leaf				Secondary Leaf			
	Chloroplast		Supernatant		Chloroplast		Supernatant	
	dpm	%	dpm	%	dpm	%	dpm	%
Amino acid								
Aspartate	42,528	6.68	65,241	1.90	8,307	3.01	5,807	1.42
Organic acids								
Glycerate	68,270	10.72	560,149	16.34	26,256	9.51	41,190	10.05
Glycollate	106,710	16.76	248,997	7.26	23,621	8.56	40,886	9.98
Sugar phosphates	35,236	5.54	799,729	23.33	30,344	10.99	79,187	19.33
Unknown	35,036	5.50	201,533	5.88	21,532	7.80	48,181	11.76
Sugars								
Dihydroxyacetone	56,512	8.88	128,519	3.75	31,362	11.36	18,808	4.59
Glyceraldehyde	99,271	15.59	111,017	3.24	31,762	11.51	23,410	5.71
Pentoses	76,766	12.06	164,025	4.78	18,977	6.87	20,409	4.98
Hexoses	39,008	6.13	250,038	7.29	23,172	8.39	30,214	7.37
Sucrose	22,755	3.57	335,551	9.79	23,172	8.39	50,022	12.21
Polysaccharide	54,511	8.56	563,585	16.44	37,555	13.60	51,623	12.60
Total	636,603	99.99	3,428,384	100.00	276,060	99.99	409,737	100.00
Total C_3 products	224,053	35.20	799,685	23.33	89,380	32.38	83,408	20.36

Table XIV. Comparison of Methods of Isolation for Chloroplasts from Primary and Secondary Leaves.

Isolation Method	Leaf Source	Intact Chloroplasts		Broken Chloroplasts		Fragments	Foaming
		Number	%	Number	%		
Laceration	Primary C_1	242	90.64	25	9.36	+	-
	C_2	344	90.77	35	9.23	+	-
	Secondary C_1	564	92.61	45	7.39	+	-
	C_2	674	95.87	29	4.13	+	-
Grinding	Primary C_1	221	85.00	39	15.00	+++++++++	+++
	C_2	498*	86.91	75	13.09	+++++++++	+++
	Secondary C_1	146	83.91	28	16.09	+++++++++	+++
	C_2	337*	83.42	67	16.58	+++++++++	+++
Blending	Primary C_1	225	84.59	41	15.41	+++++	+++++++++
	C_2	205*	83.33	41	16.67	+++++	++++
	Secondary C_1	295	85.51	50	14.49	+++++	+++++++++
	C_2	455*	87.33	66	12.67	+++++	++++

C_1: Chloroplast count before centrifugation

C_2: Chloroplast count after centrifugation

* : Chloroplast count after 1:1 dilution with solution A

+ : Presence -: Absence

Table XV. Distribution of Intact Chloroplasts between Mesophyll and Bundle Sheath Chloroplasts by the 'Laceration Technique'.

	Primary Leaf Chloroplasts		Secondary Leaf Chloroplasts	
	Mesophyll	Bundle Sheath	Mesophyll	Bundle Sheath
Number Intact	606	104	694	84
% Intact	81.0	13.9	84.3	10.2
Number Broken	28	10	27	18
% Broken	3.7	1.3	3.3	2.2

Table XVI. Levels of Phosphoenolpyruvate and Ribulose Diphosphate Carboxylases by Different Extraction Methods from Primary and Secondary Leaves.

Extraction Method	Leaf Source	PEP Carboxylase	RuDP Carboxylase	PEP C./RuDP C.
			μm CO_2/mg protein/hr	
Grinding with sand (whole leaf)	Primary	442	158	2.80
	Secondary	479	174	2.75
Blending (whole leaf)	Primary	625	148	4.22
	Secondary	596	159	3.75
$Laceration_L$ + $Grinding_G$	Primary, Chl_L	16	5	14.04
	$Supt_L$	363 → 379	22 → 27	
	Chl_G	82	21	5.79
	$Supt_G$	685 → 767 → 1,146	150 → 171 → 198	4.49
	Secondary, Chl_L	30	7	8.16
	$Supt_L$	337 → 367	38 → 45	
	Chl_G	46	21	5.69
	$Supt_G$	515 → 561 → 928	97 → 118 → 163	4.75

PEP C./RuDP C.: Phosphoenolpyruvate carboxylase activity to ribulose diphosphate carboxylase activity ratio

Table XVII. Comparison of CO_2 Fixation Capacity of Chloroplasts Isolated by Different Techniques from Secondary Leaves.

	CO_2 Fixation, μm CO_2/mg protein/hr					
	Laceration			Grinding		
Addition	20°C	30°C	40°C	20°C	30°C	40°C
Nil	0.04	0.04	0.09	0.02	0.02	0.04
PEP (4mM)	0.35	0.73	3.69	1.18	2.23	1.01
RuDP (4mM)	0.12	0.59	1.17	0.16	0.28	0.53
PEP (4mM), RuDP (4mM)	1.24	1.23	7.15	1.30	2.33	1.74

Table XVIII. Separation of Bundle Sheath and Mesophyll Chloroplasts by Differential Centrifugation

	Chloroplast Fraction		
	750 g	1,000 g	2,000 g
Enzyme	Enzyme activity, μm CO_2/mg protein/hr		
PEP carboxylase	1.35	3.14	3.25
RuDP carboxylase	0.95	0.66	0.49
CO_2 Fixation Capacity	CO_2 fixation, μm CO_2/mg protein/hr		
With no addition	0.03	0.05	0.04
PEP (4mM)	0.31	2.71	2.22
RuDP (4mM)	0.17	0.20	0.17

Table XIX. Summary of apparent Km values for PEP, HCO_3^- and Mg^{+2} for the CO_2 fixation capacity and PEP carboxylase activity of maize leaf cellular fractions. Data obtained from Figures 14 - 25 and 30 - 41.

	Primary Leaf		Secondary Leaf	
	Chloroplast	Supernatant	Chloroplast	Supernatant
	Km, mM	Km, mM	Km, mM	Km, mM
PEP				
CO_2 Fixation	4.00	7.40	2.04	2.44
PEP Carboxylase	10.00	10.00	1.40	3.85
HCO_3^-				
CO_2 Fixation	0.56	0.89	0.67	0.40
PEP Carboxylase	0.43	0.44	0.27	1.33
Mg^{+2}				
CO_2 Fixation	2.00	0.30	2.22	0.35
PEP Carboxylase	4.00	1.14	5.71	0.21

Figure 1. Incorporation of $^{14}CO_2$ into the various fractions of plant extracts from maize primary leaf discs in kinetic experiments. Data obtained from Table I.

T = Total fixation
M = Methanol-water soluble fraction
R = Residue and chloroform soluble fraction

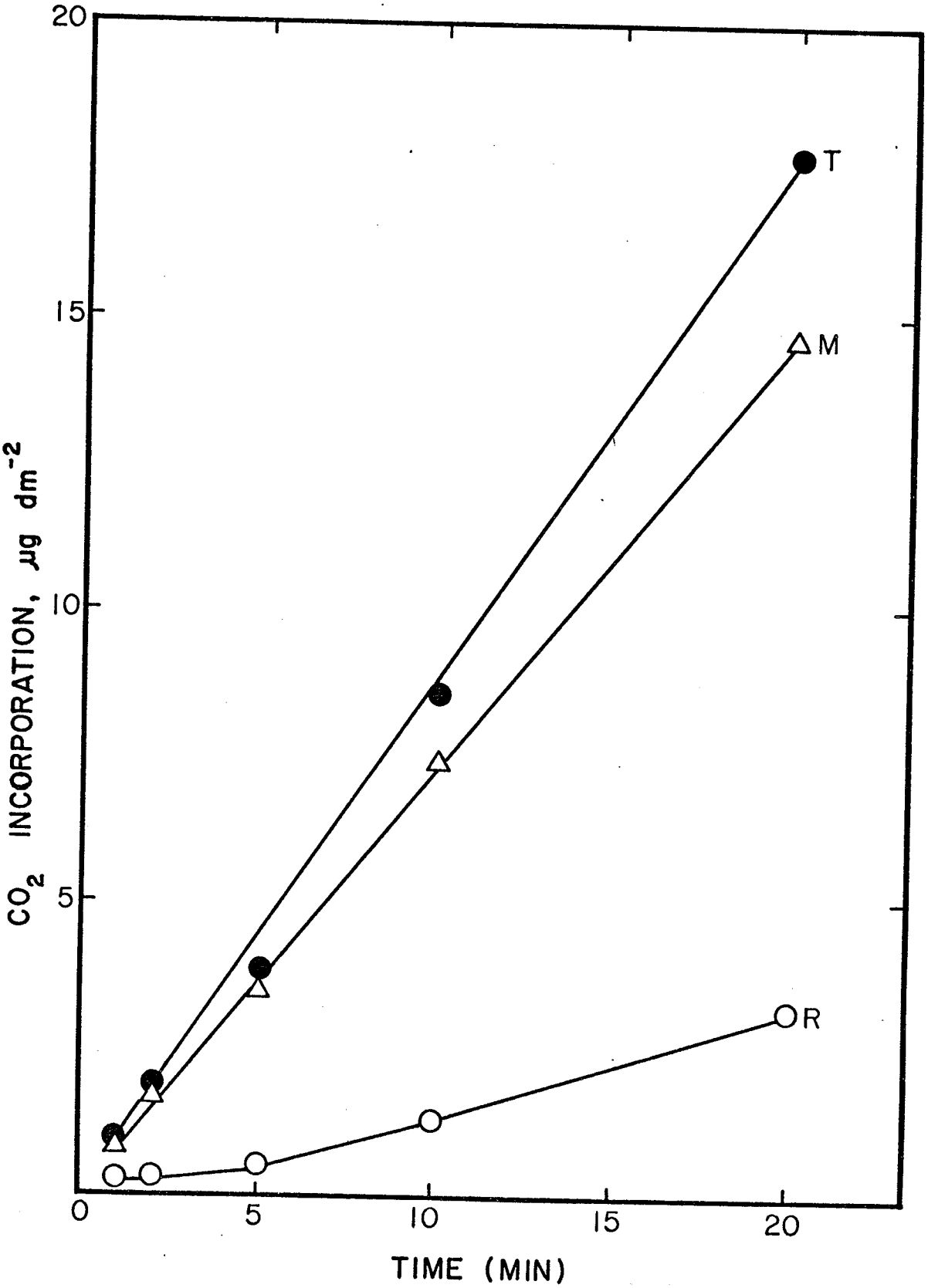

20
15
10
5
0
5
10
15
20
T
M
R
CO_2 INCORPORATION, µg dm^{-2}
TIME (MIN)

Figure 2. Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of plant extracts from maize primary leaf discs in kinetic experiments. Data obtained from Table II.

OA = Organic acid fraction
AA = Amino acid fraction
S = Sugar fraction

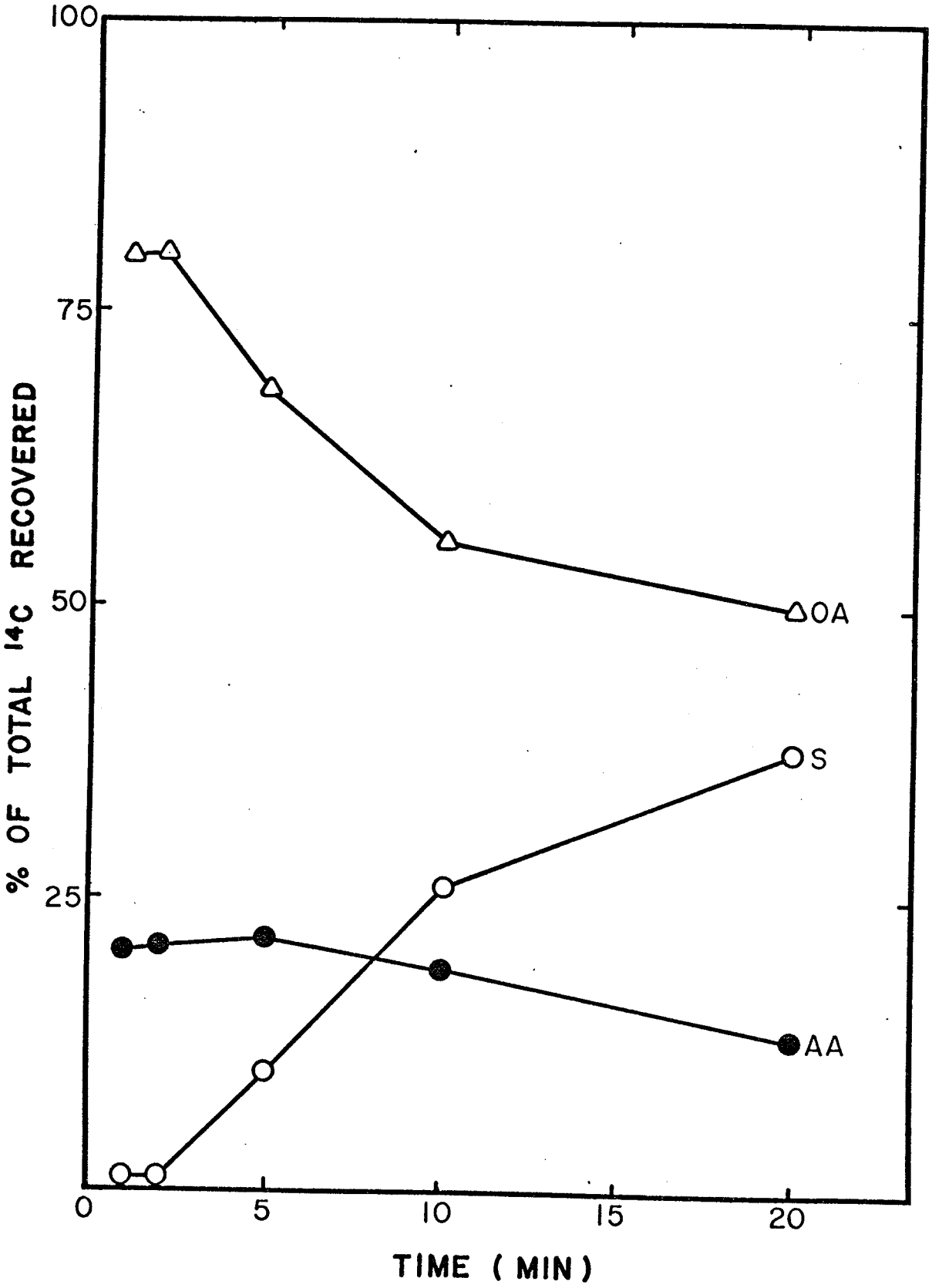

100
75
50
25
0
5
10
15
20
% OF TOTAL 14C RECOVERED
TIME (MIN)
OA
S
AA

Figure 3. Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs in kinetic experiments. Data obtained from Table III.

Organic Acid Fraction

ISL = Isocitrate lactone
MAL = Malate
PGA = 3-phosphoglyceric acid
SMP = Sugar monophosphates
SDP = Sugar diphosphates

Amino Acid Fraction

ALA = Alanine
ASP = Aspartate
GLU = Glutamate
G+S = Glycine + Serine

Sugar Fraction

FRU = Fructose
GLU = Glucose
SUC = Sucrose

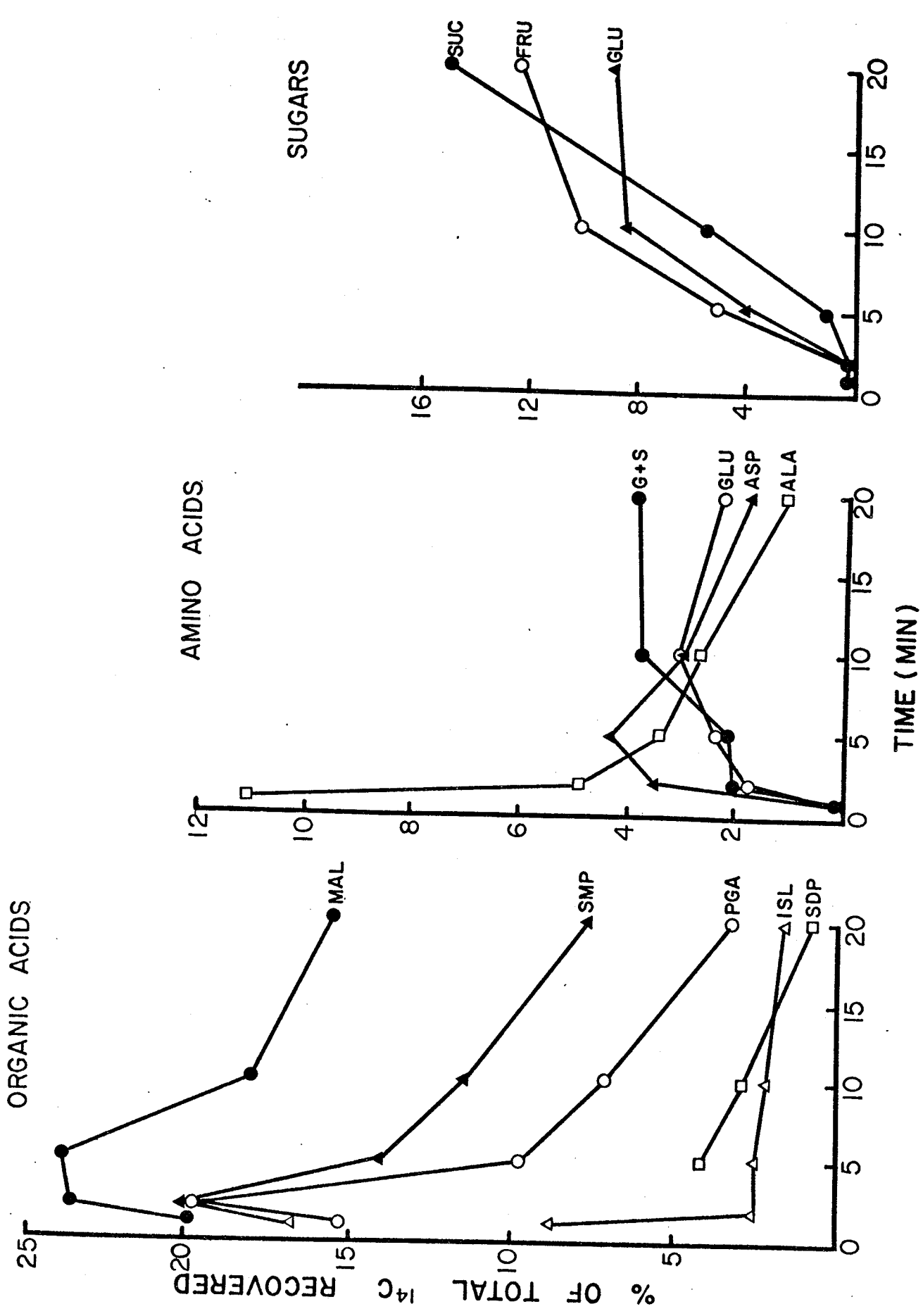

ORGANIC ACIDS
AMINO ACIDS
SUGARS
% OF TOTAL 14C RECOVERED
TIME (MIN)
MAL
SMP
PGA
ISL
SDP
G+S
GLU
ASP
ALA
SUC
FRU
GLU

Figure 4. Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of the plant extracts from maize primary leaf discs in pulse-chase experiments. Data obtained from Table IV.

OA = Organic acid fraction
AA = Amino acid fraction
S = Sugar fraction

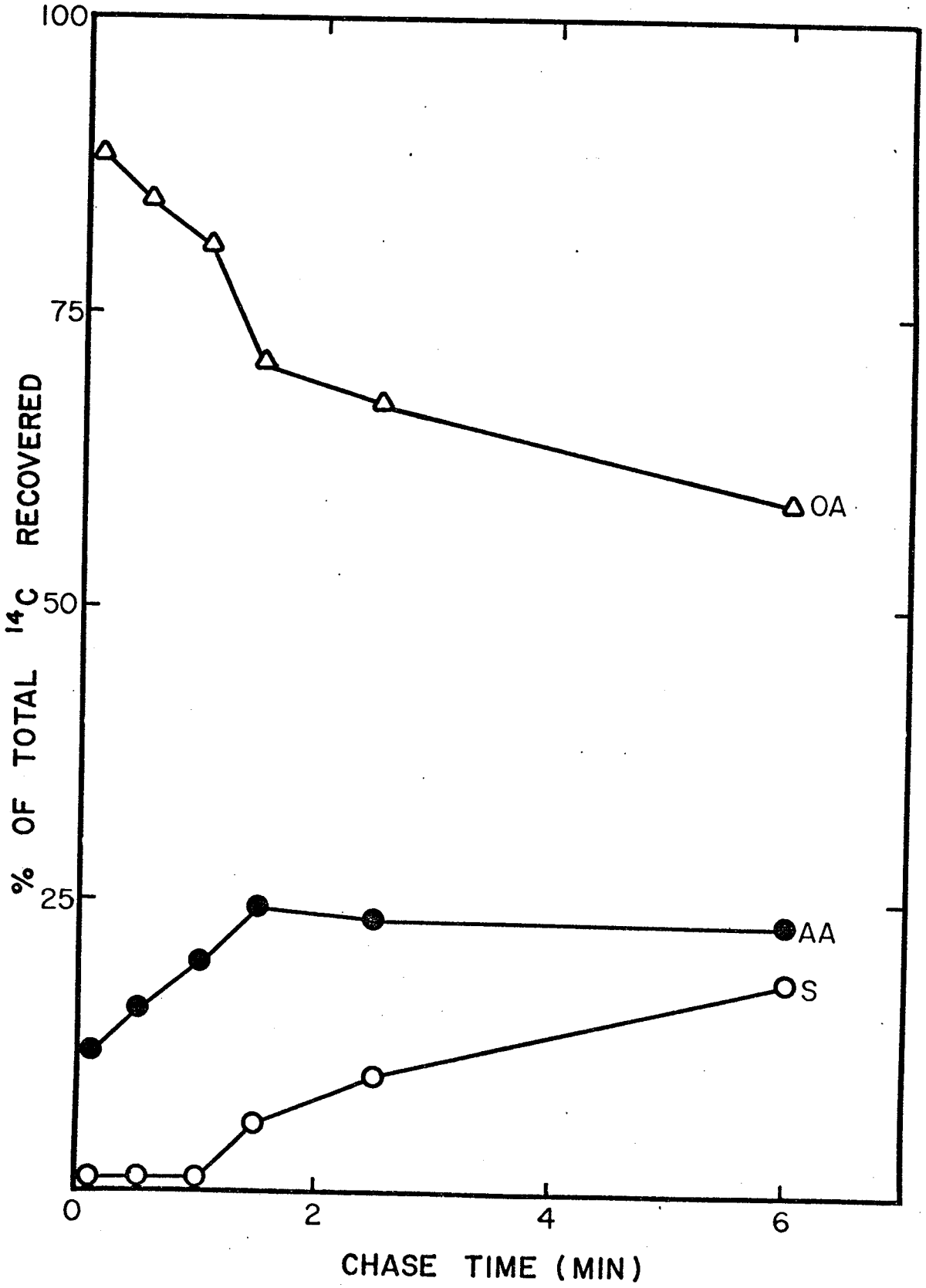

100
75
50
25
0
2
4
6
% OF TOTAL ^{14}C RECOVERED
CHASE TIME (MIN)
OA
AA
S

Figure 5. Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize primary leaf discs in pulse-chase experiments. Data obtained from Table V.

Organic Acid Fraction

ISL= Isocitrate lactone
MAL= Malate
PGA= 3-phosphoglyceric acid
SMP= Sugar monophosphates
SDP= Sugar diphosphates

Amino Acid Fraction

ALA= Alanine
ASP= Aspartate
GLU= Glutamate
G+S= Glycine + Serine

Sugar Fraction

FRU= Fructose
GLU= Glucose
SUC= Sucrose

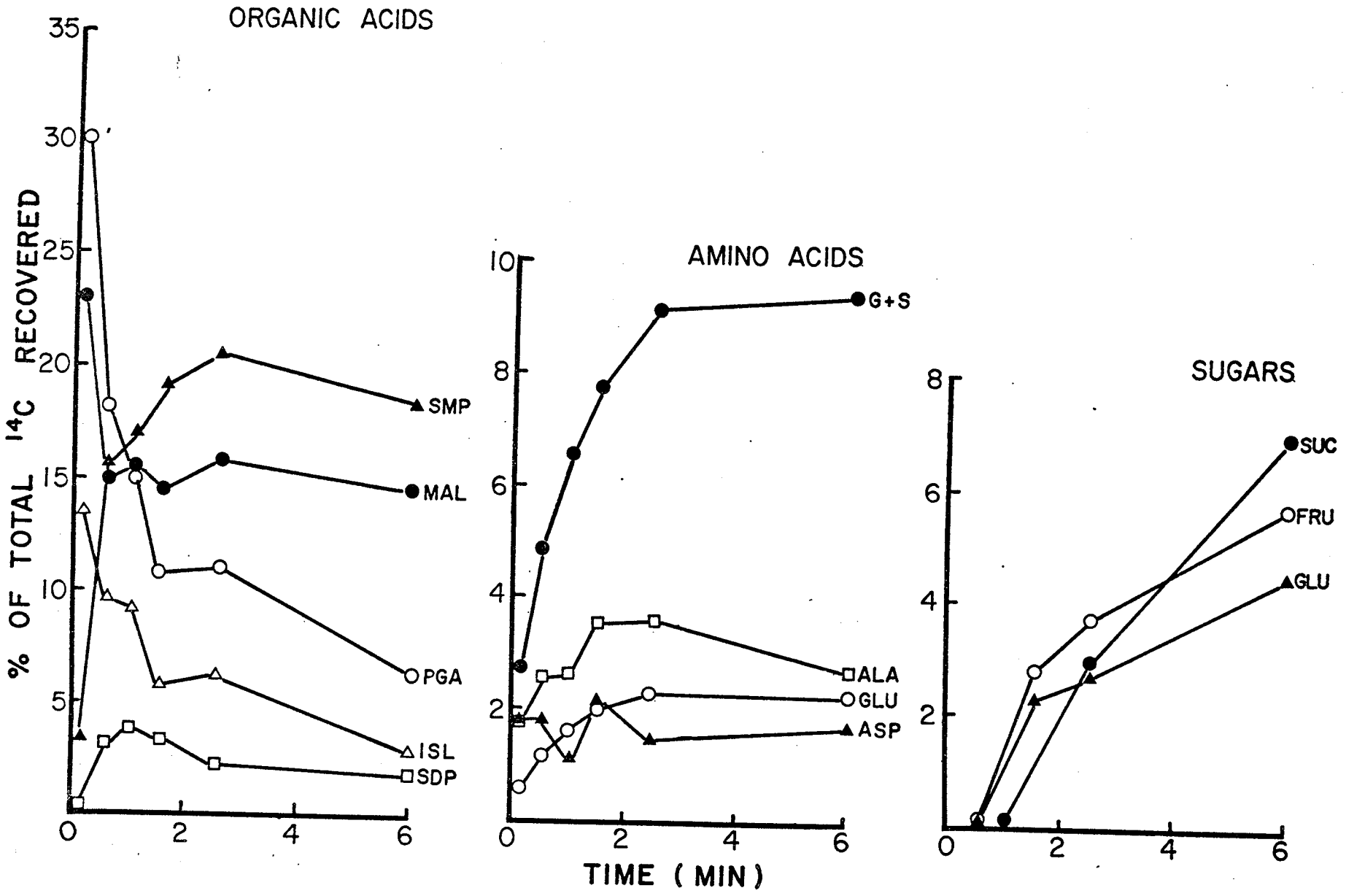

ORGANIC ACIDS
% OF TOTAL ^{14}C RECOVERED
35
30
25
20
15
10
5
0
2
4
6
SMP
MAL
PGA
ISL
SDP
AMINO ACIDS
10
8
6
4
2
G+S
ALA
GLU
ASP
SUGARS
SUC
FRU
GLU
TIME (MIN)

Figure 6. Incorporation of $^{14}CO_2$ into the various fractions of plant extracts from maize secondary leaf discs in kinetic experiments. Data obtained from Table VI.

T = Total fixation
M = Methanol-water soluble fraction
R = Residue and chloroform soluble fraction

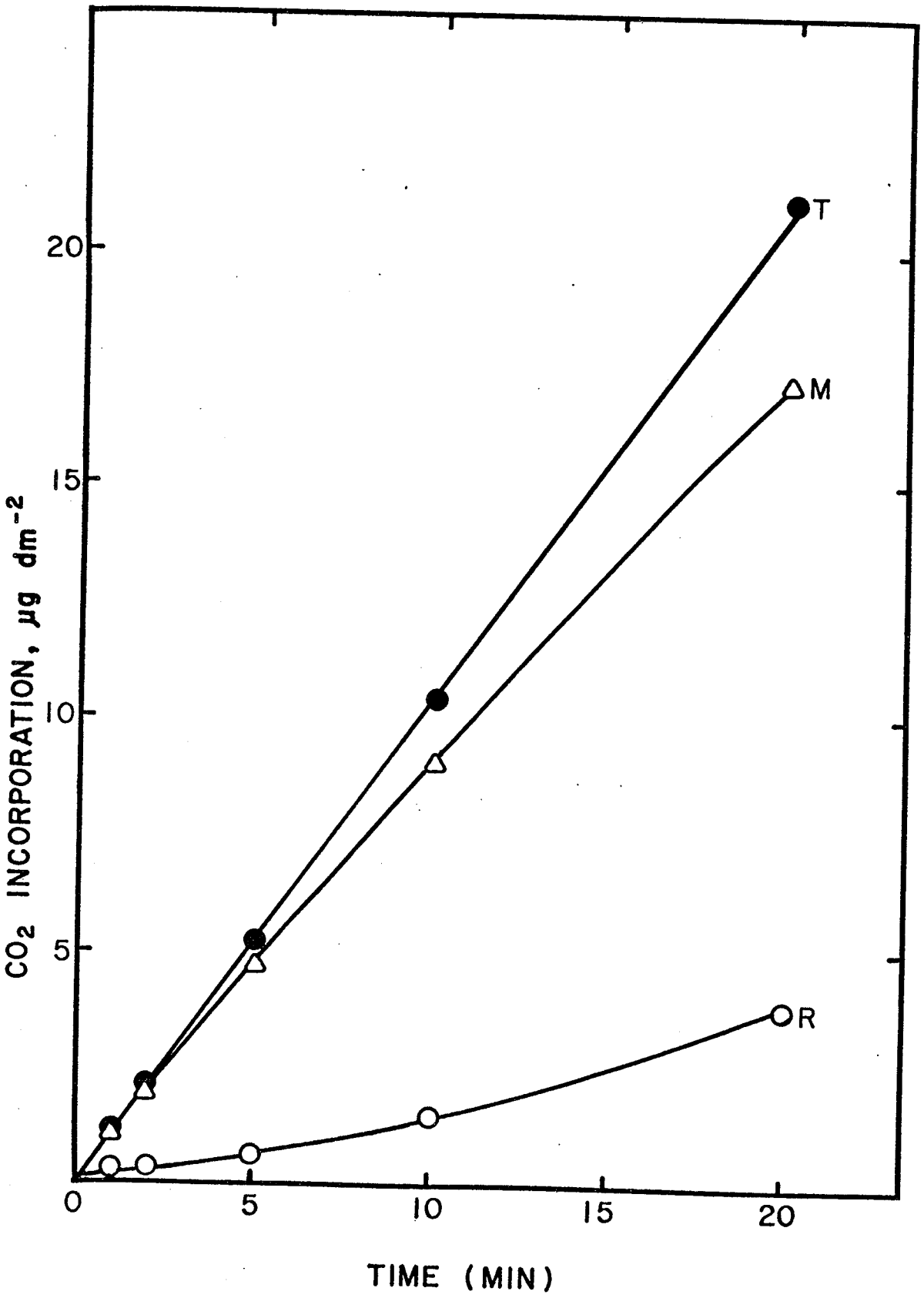

T
M
R
20
15
10
5
0
5
10
15
20
CO_2 INCORPORATION, µg dm^{-2}
TIME (MIN)

Figure 7. Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of plant extracts from maize secondary leaf discs in kinetic experiments. Data obtained from Table VII.

OA = Organic acid fraction
AA = Amino acid fraction
S = Sugar fraction

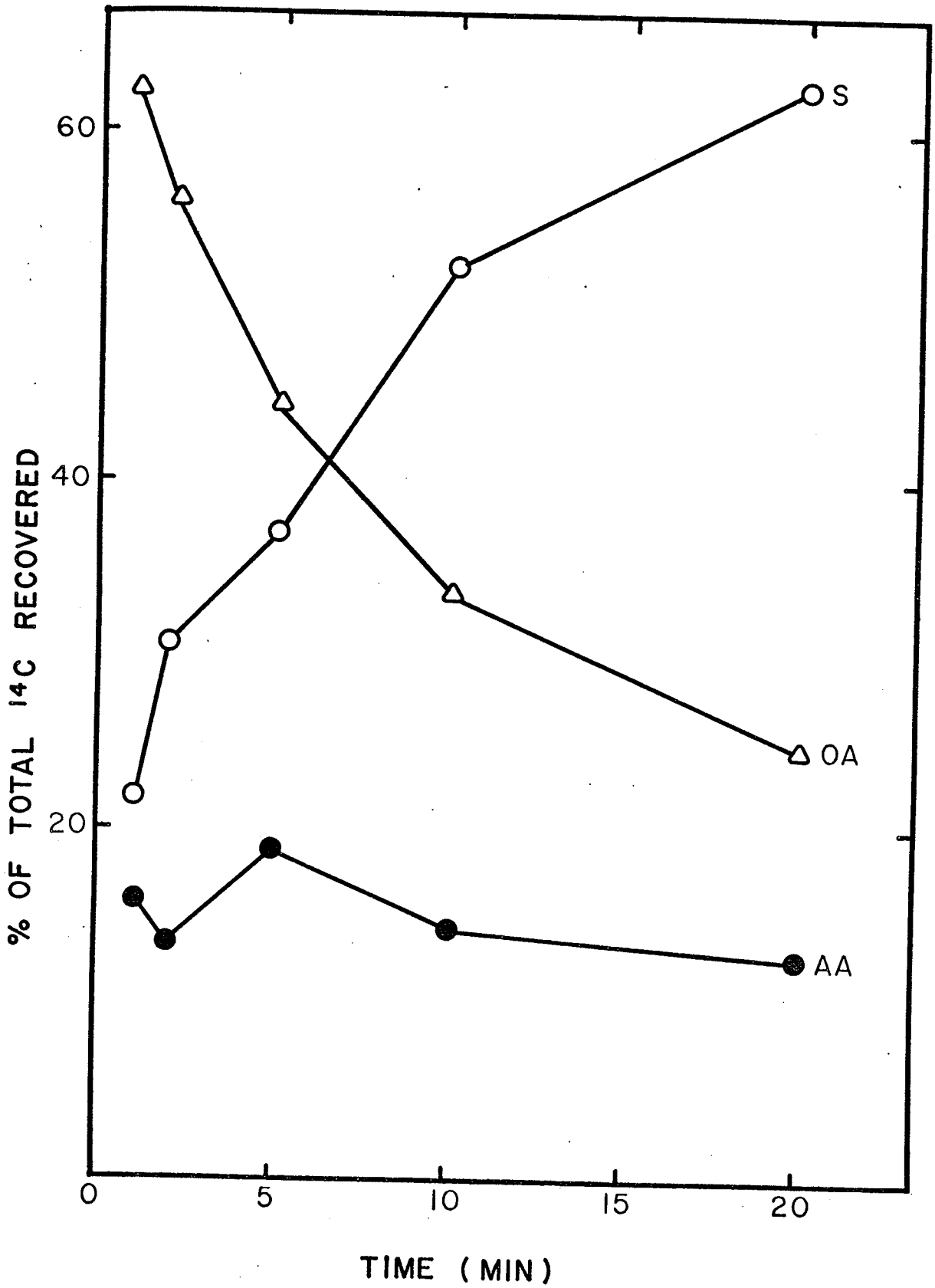

60
40
20
0
5
10
15
20
S
OA
AA
% OF TOTAL 14C RECOVERED
TIME (MIN)

Figure 8. Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs in kinetic experiments. Data obtained from Table VIII.

Organic Acid Fraction

ISL = Isocitrate lactone
MAL = Malate
PGA = 3-phosphoglyceric acid
SMP = Sugar monophosphates
SDP = Sugar diphosphates

Amino Acid Fraction

ALA = Alanine
ASP = Aspartate
GLU = Glutamate
G+S = Glycine + Serine

Sugar Fraction

FRU = Fructose
GLU = Glucose
SUC = Sucrose

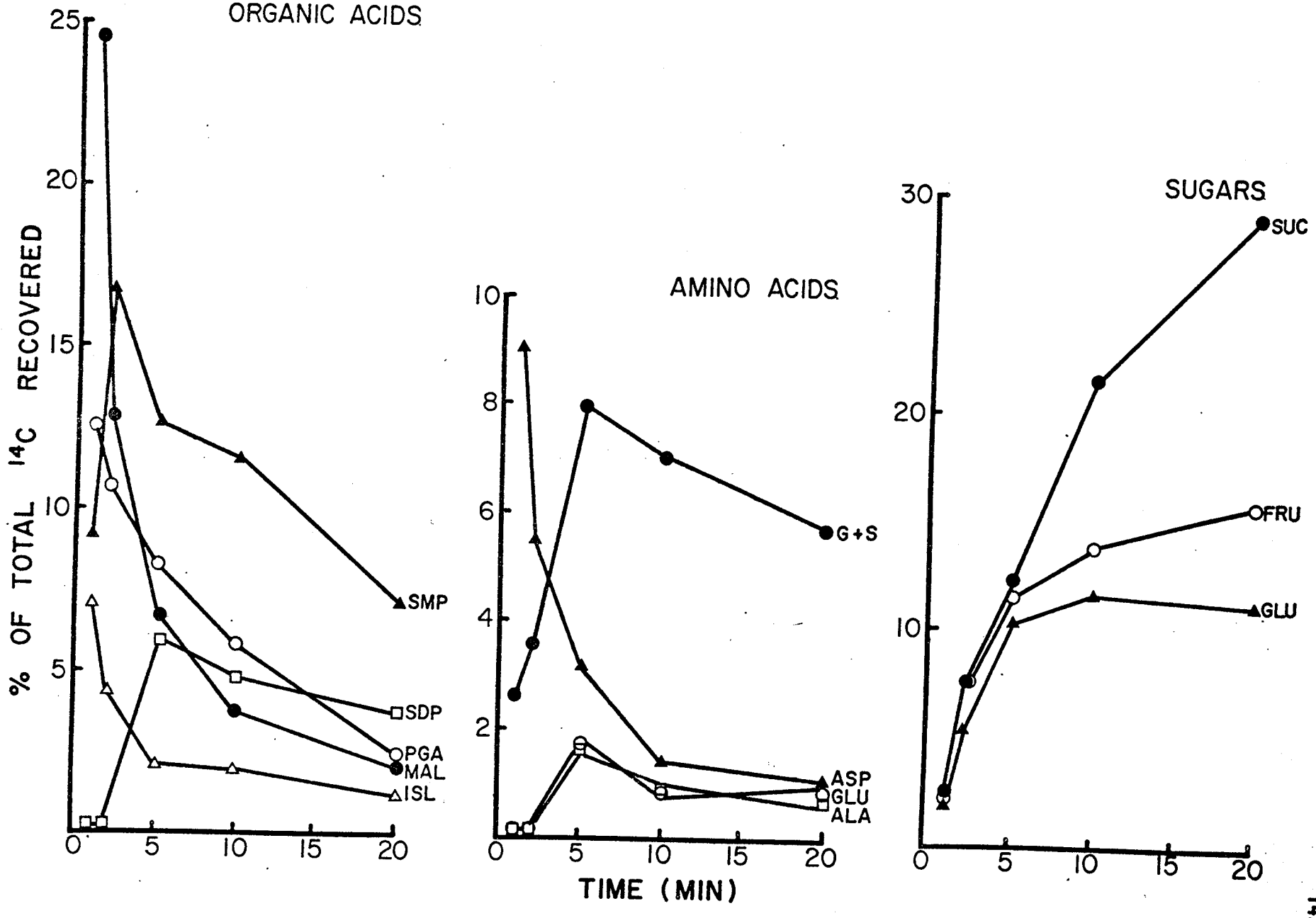

ORGANIC ACIDS
% OF TOTAL ^{14}C RECOVERED
SMP
SDP
PGA
MAL
ISL
AMINO ACIDS
G+S
ASP
GLU
ALA
SUGARS
SUC
FRU
GLU
TIME (MIN)

Figure 9. Distribution of ^{14}C in various fractions of the methanol-water soluble fraction of plant extracts from maize secondary leaf discs in pulse-chase experiments. Data obtained from Table IX.

OA = Organic acid fraction
AA = Amino acid fraction
S = Sugar fraction

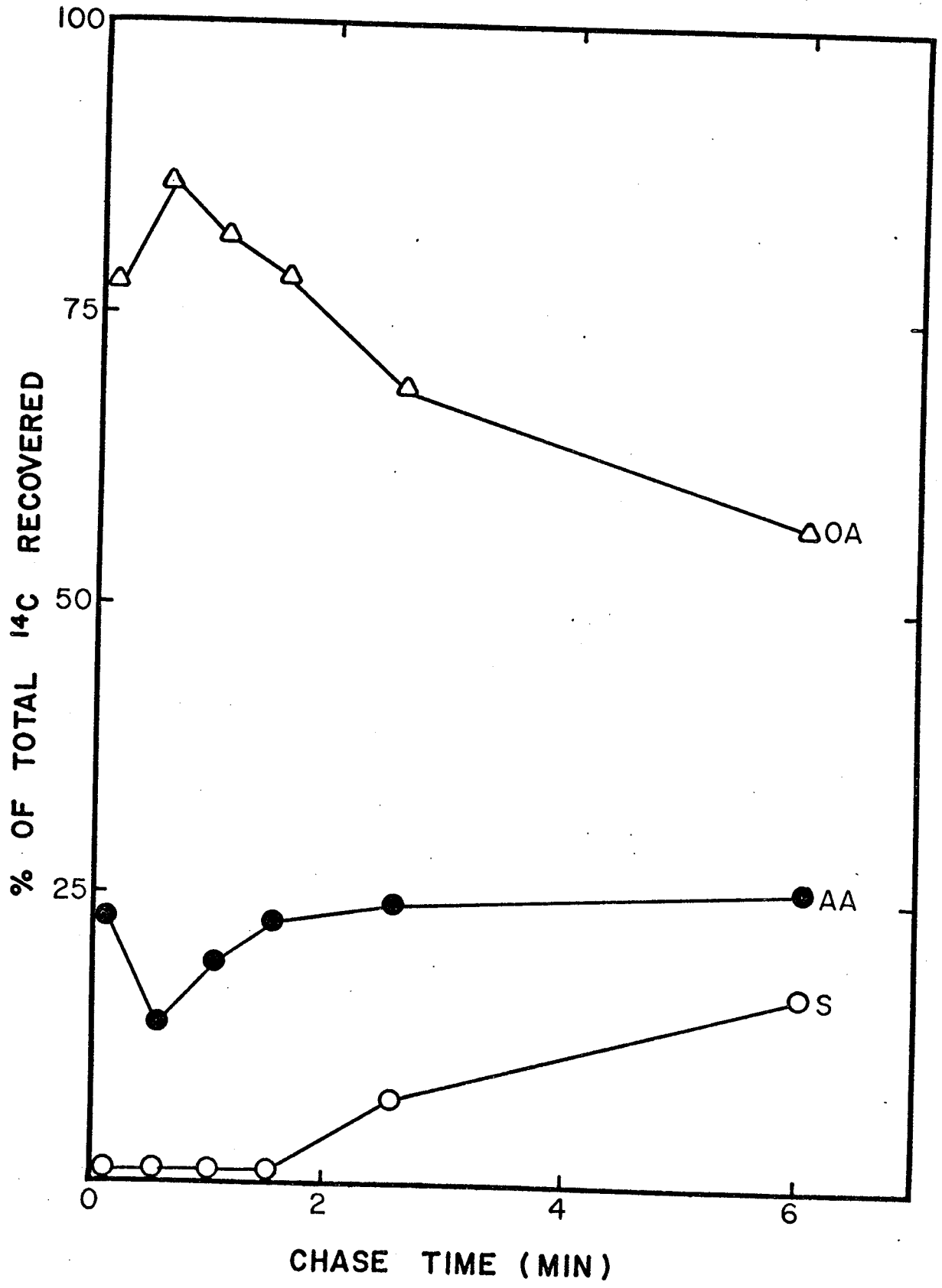

100
75
50
25
0
2
4
6
% OF TOTAL 14C RECOVERED
CHASE TIME (MIN)
OA
AA
S

Figure 10. Distribution of ^{14}C in various products of the organic acid, amino acid and sugar fractions of the plant extracts from maize secondary leaf discs in pulse-chase experiments. Data obtained from Table X.

Organic Acid Fraction

ISL = Isocitrate lactone
MAL = Malate
PGA = 3-phosphoglyceric acid
SMP = Sugar monophosphates
SDP = Sugar diphosphates

Amino Acid Fraction

ALA = Alanine
ASP = Aspartate
GLU = Glutamate
G+S = Glycine + Serine

Sugar Fraction

FRU = Fructose
GLU = Glucose

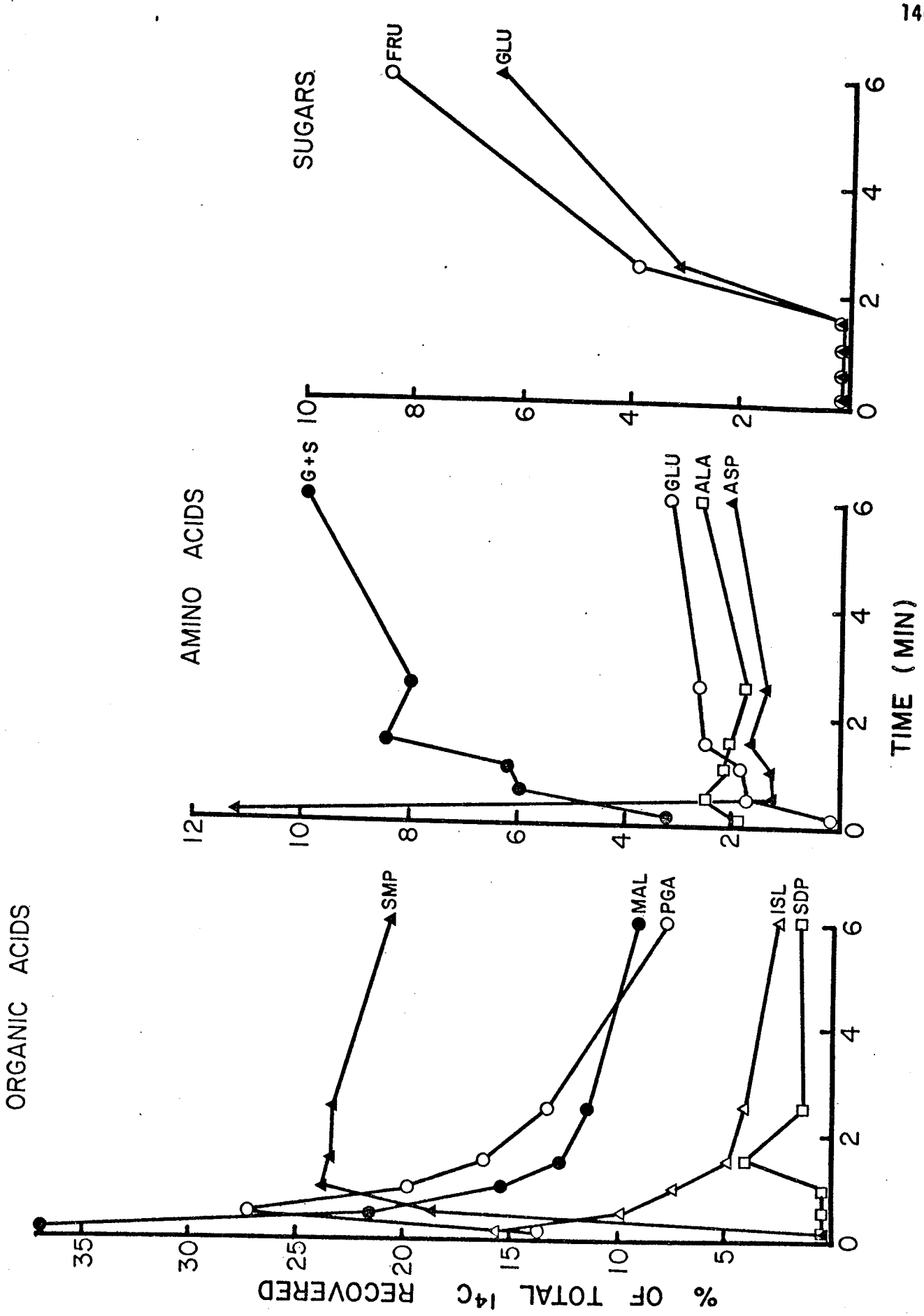

ORGANIC ACIDS
AMINO ACIDS
SUGARS
% OF TOTAL 14C RECOVERED
TIME (MIN)
SMP
MAL
PGA
ISL
SDP
G+S
GLU
ALA
ASP
FRU
GLU

Figure 11. Incorporation of $^{14}CO_2$ into isolated chloroplasts of maize primary leaves in the absence of PEP

● = Light

⬢ = Darkness

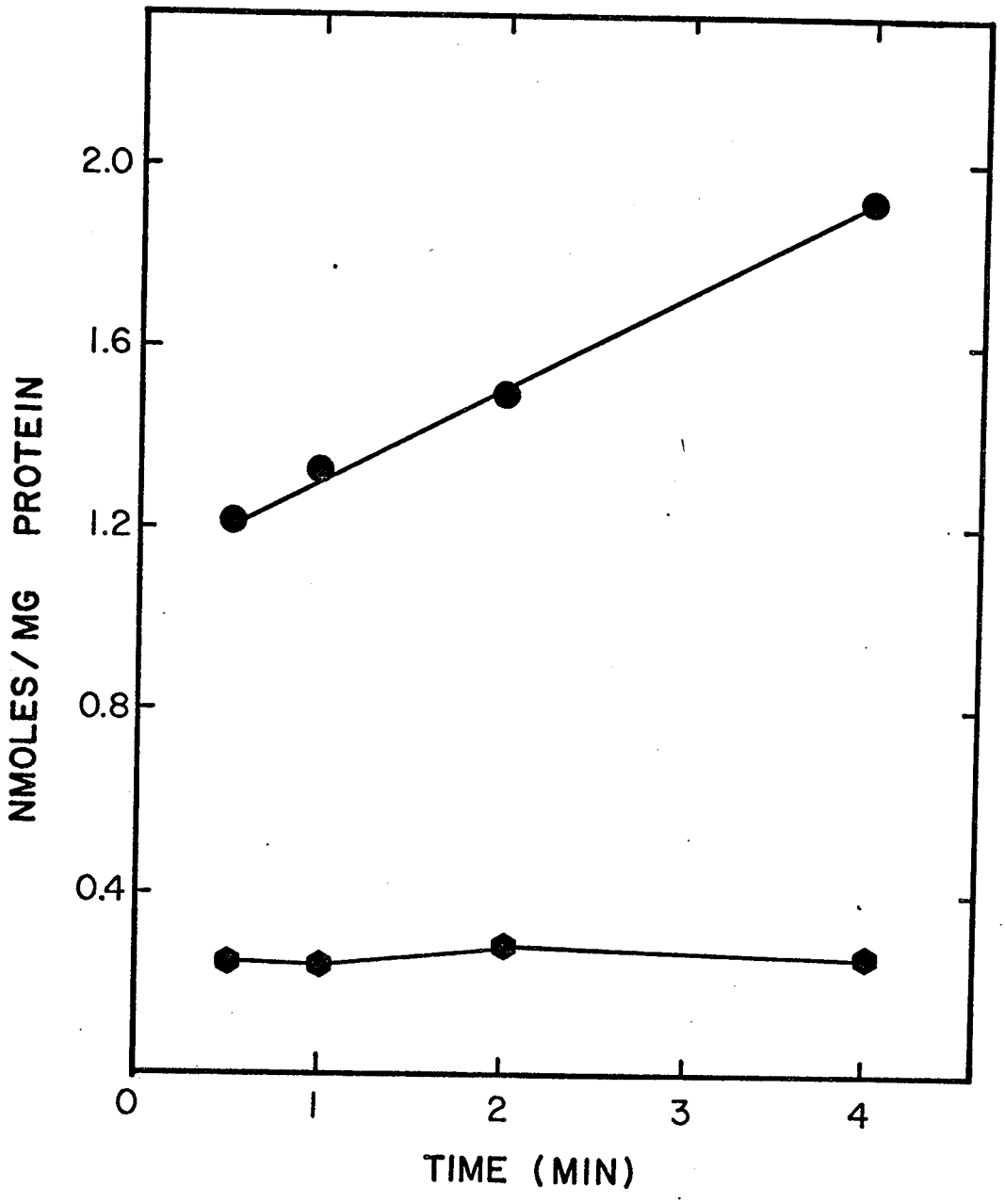

2.0
1.6
1.2
0.8
0.4
NMOLES/MG PROTEIN
0
1
2
3
4
TIME (MIN)

Figure 12. Incorporation of $^{14}CO_2$ into cellular fractions of maize primary leaves as a function of time.

a. Isolated Chloroplasts

● = Methanol-water soluble fraction, 3.2mM PEP

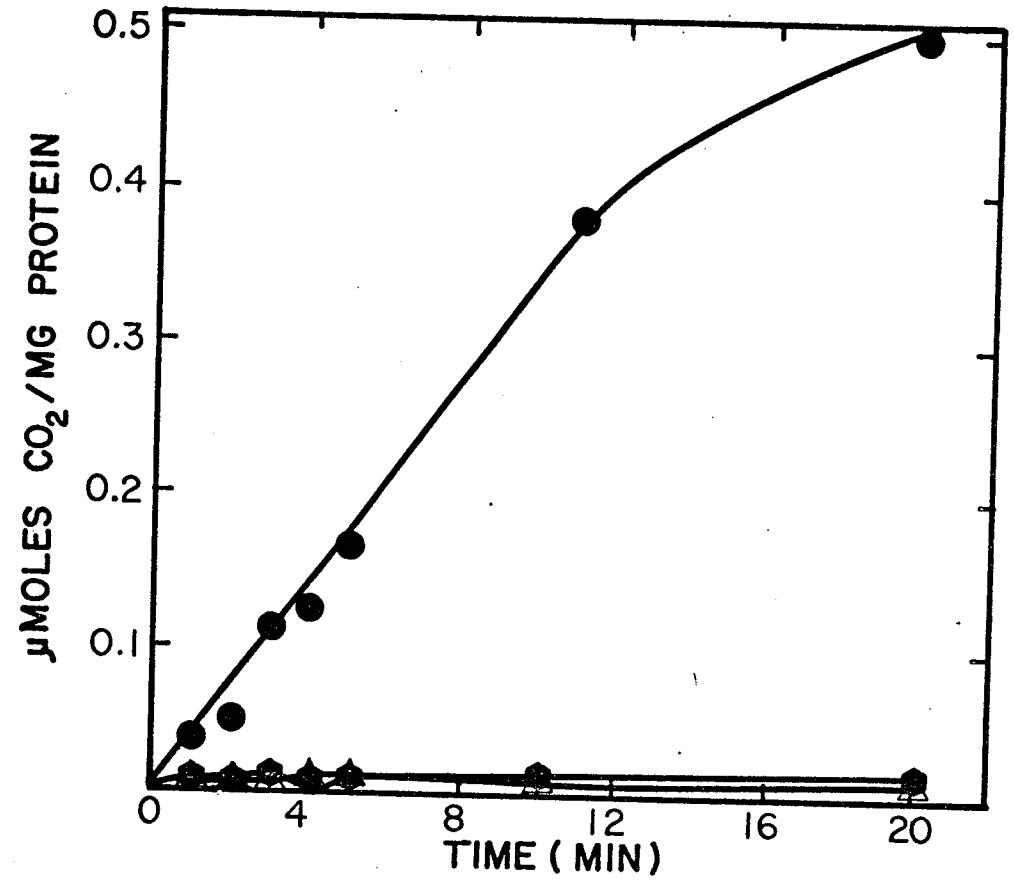
● = Chloroform soluble fraction, 3.2mM PEP

△ = Methanol-water soluble fraction, 0 mM PEP

b. Supernatant

■ = Total fixation, 3.2 mM PEP

▲ = Total fixation, 0 mM PEP


0.5
0.4
0.3
0.2
0.1
µMOLES CO_2/MG PROTEIN
0
4
8
12
16
20
TIME (MIN)

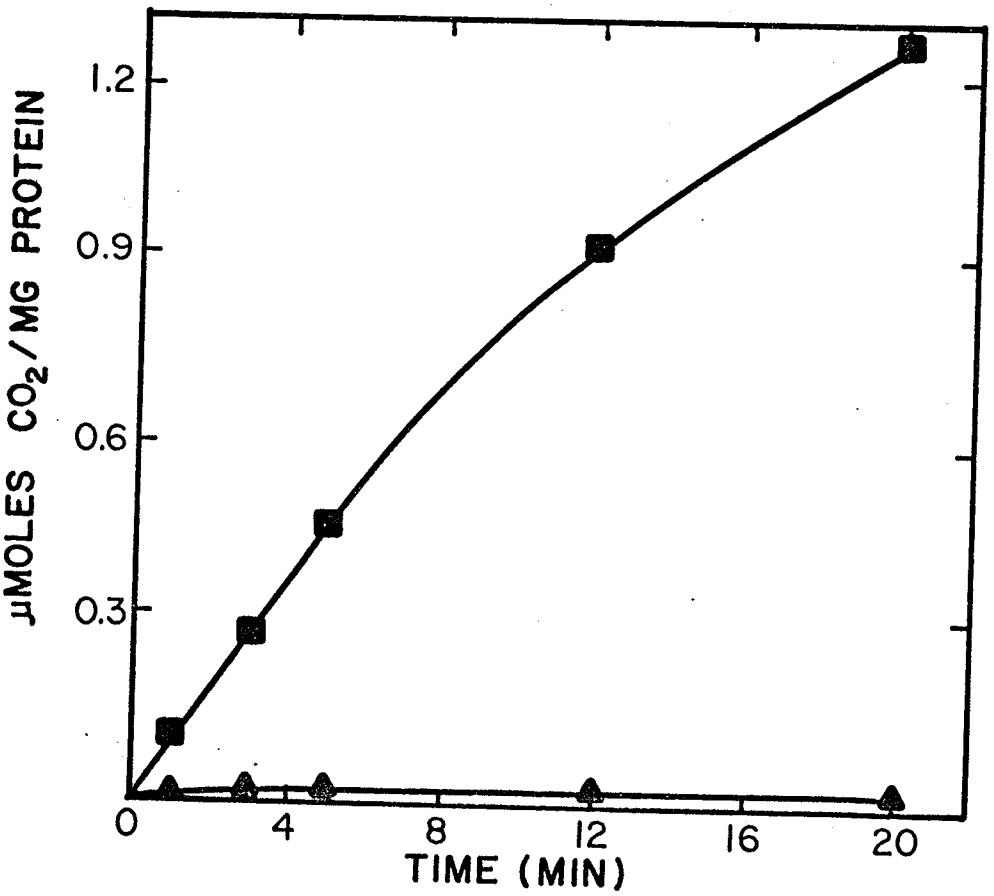

1.2
0.9
0.6
0.3
µMOLES CO_2/MG PROTEIN
0
4
8
12
16
20
TIME (MIN)

Figure 13. Incorporation of $^{14}CO_2$ into cellular fractions of maize secondary leaves as a function of time.

a. Isolated Chloroplasts

● = Methanol-water soluble fraction, 3.2mM PEP

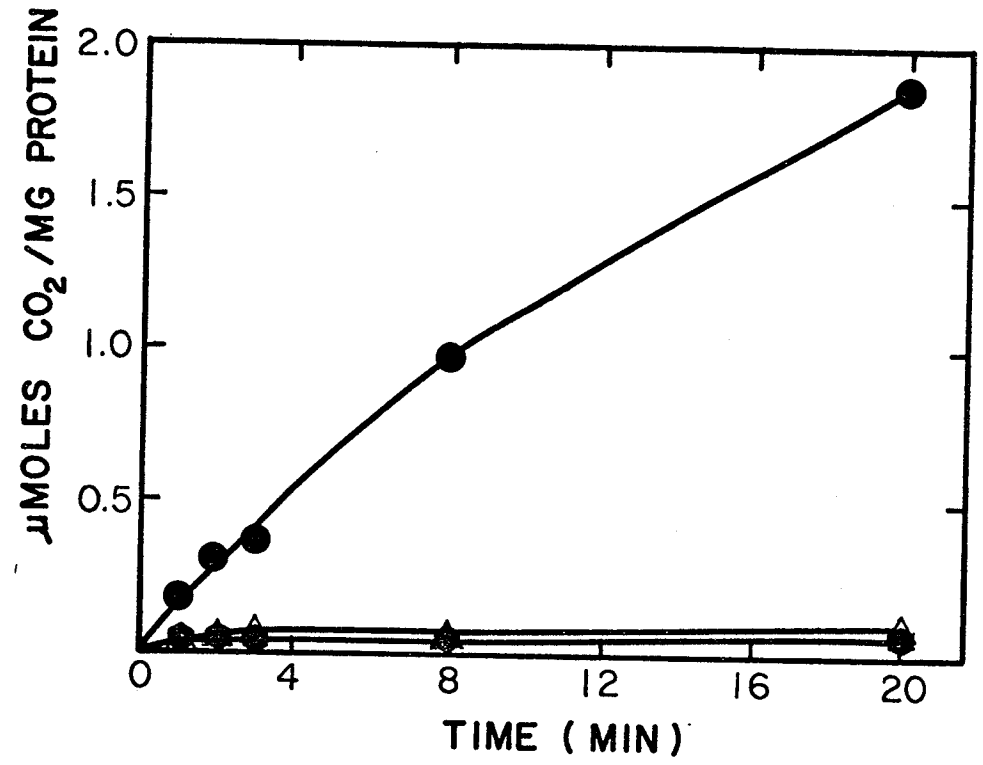
⬢ = Chloroform soluble fraction, 3.2mM PEP

△ = Methanol-water soluble fraction, 0 mM PEP

b. Supernatant

■ = Total fixation, 3.2 mM PEP

▲ = Total fixation, 0 mM PEP


µMOLES CO_2 /MG PROTEIN
2.0
1.5
1.0
0.5
0
4
8
12
16
20
TIME (MIN)

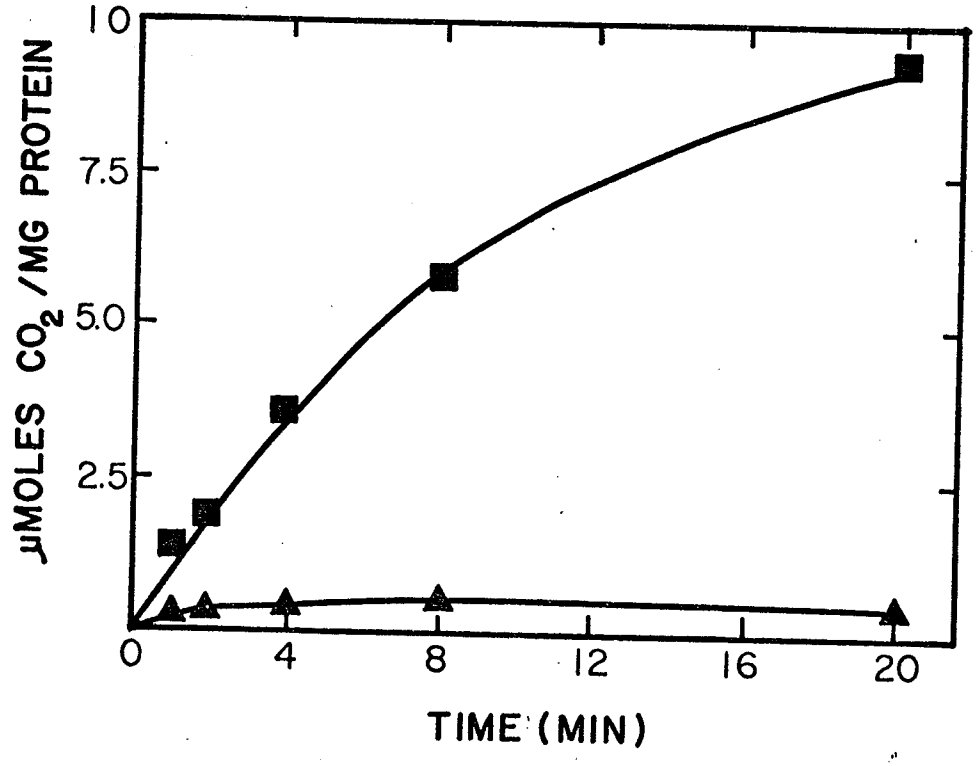

µMOLES CO_2 /MG PROTEIN
10
7.5
5.0
2.5
0
4
8
12
16
20
TIME (MIN)

Figure 14. Effect of PEP concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize primary leaves.
Reaction systems (0.5 ml) contained in μmoles the following: $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); $MgCl_2$, 5; Solution A, enough to make up 0.5 ml; 0.6 mg chloroplast protein and varying amounts of PEP as indicated. v is in μm CO_2/mg protein/hr. S is in mM of PEP.

Km = 4.00 mM

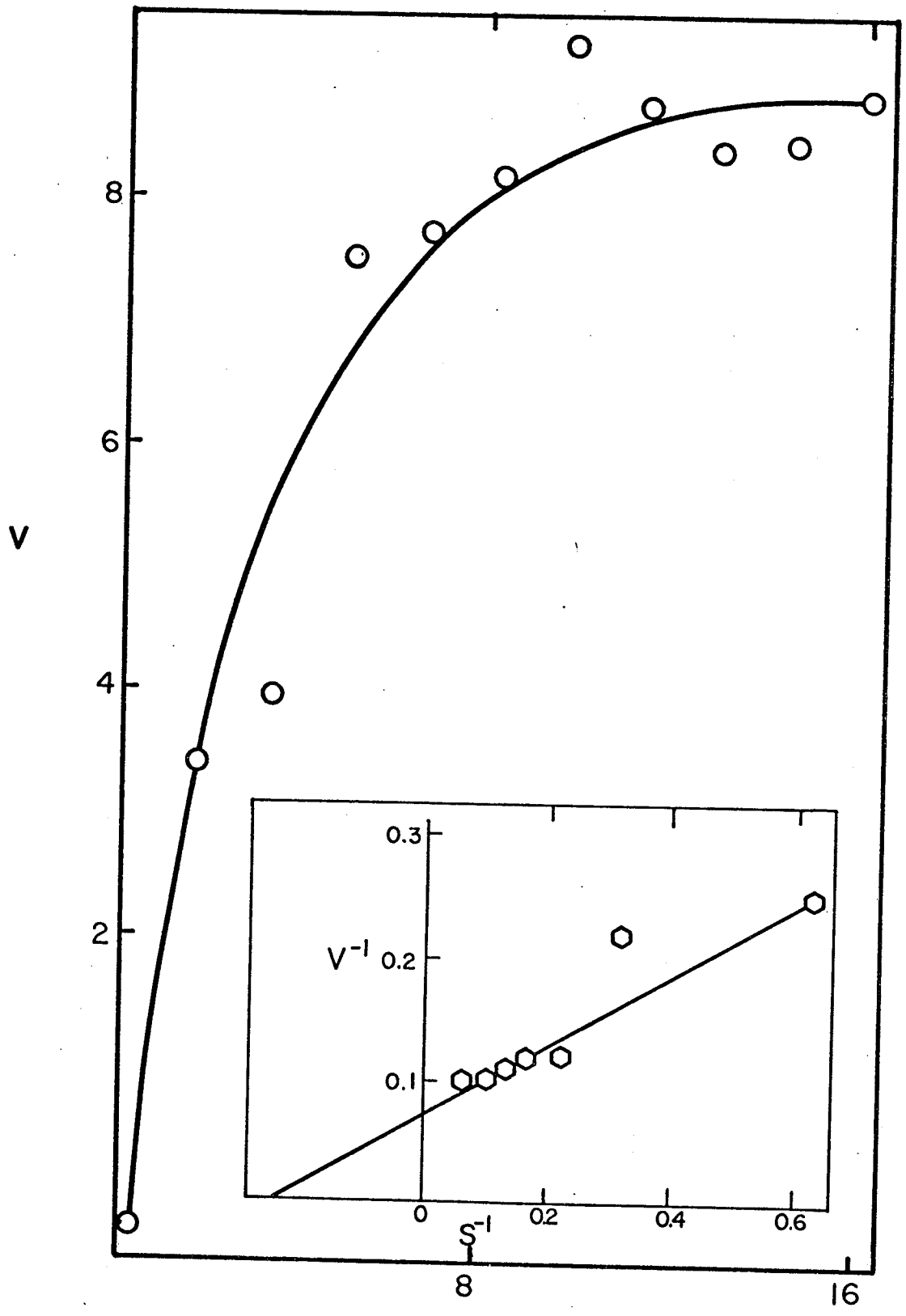

V
8
6
4
2
8
16
PEP (mM)
0.3
V^{-1} 0.2
0.1
0
S^{-1}
0.2
0.4
0.6

Figure 15. Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize primary leaves. Reaction systems (0.5 ml) contained in μmoles the following: PEP, 8; $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume; 0.6 mg of crude chloroplast protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$.

Km = 0.56 mM

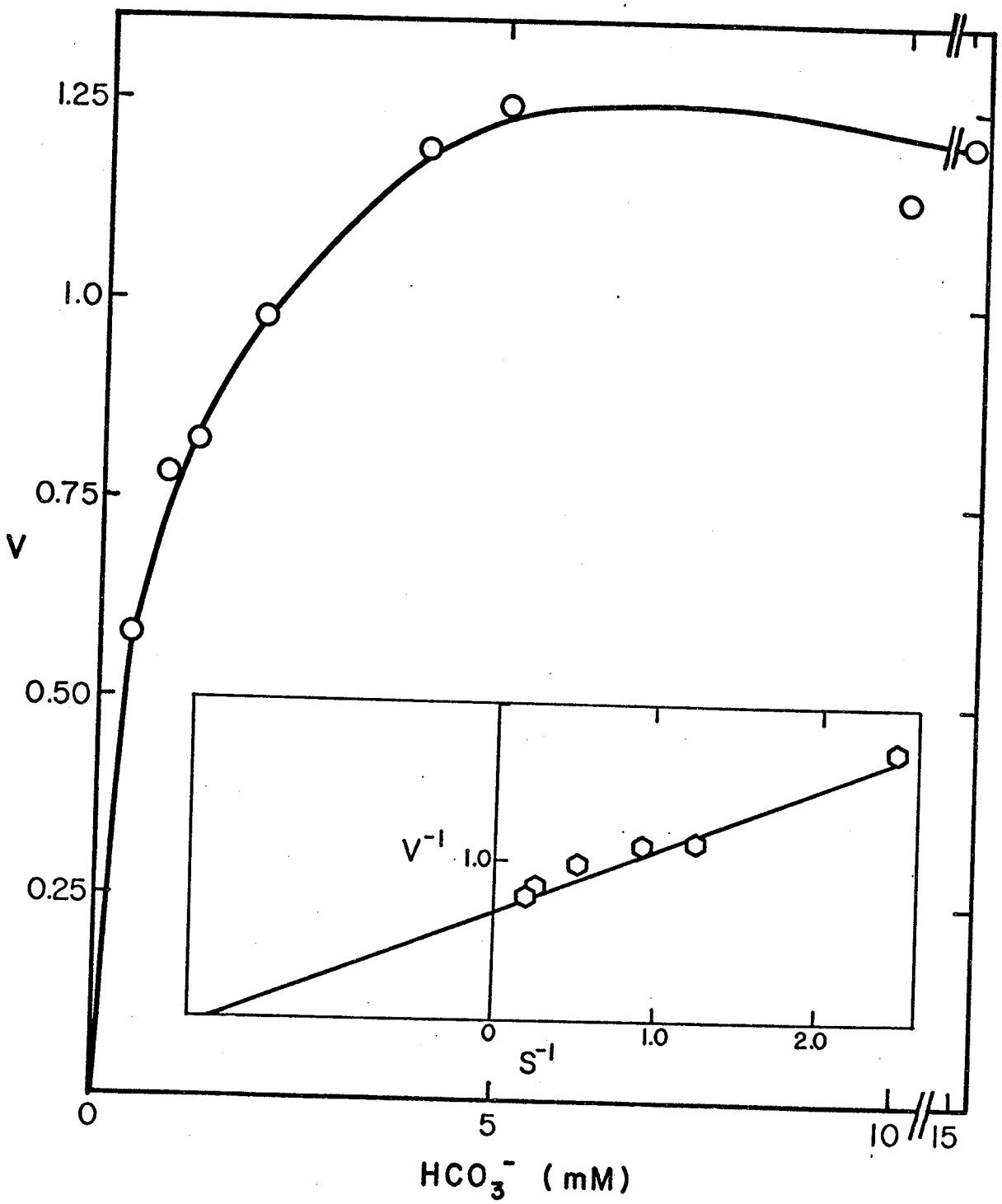

1.25
1.0
0.75
V
0.50
0.25
0
5
10
15
HCO_3^- (mM)
V^{-1}
1.0
0
1.0
2.0
S^{-1}

Figure 16. Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize primary leaves. Reaction systems (0.5 ml) contained in µmoles the following: PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); Solution A, enough to make up 0.5 ml final volume; 0.6 mg of chloroplast protein and varying amounts of $MgCl_2$ as indicated. v is in µm CO_2/mg protein/hr. S in in mM of $MgCl_2$.

Km = 2.00 mM

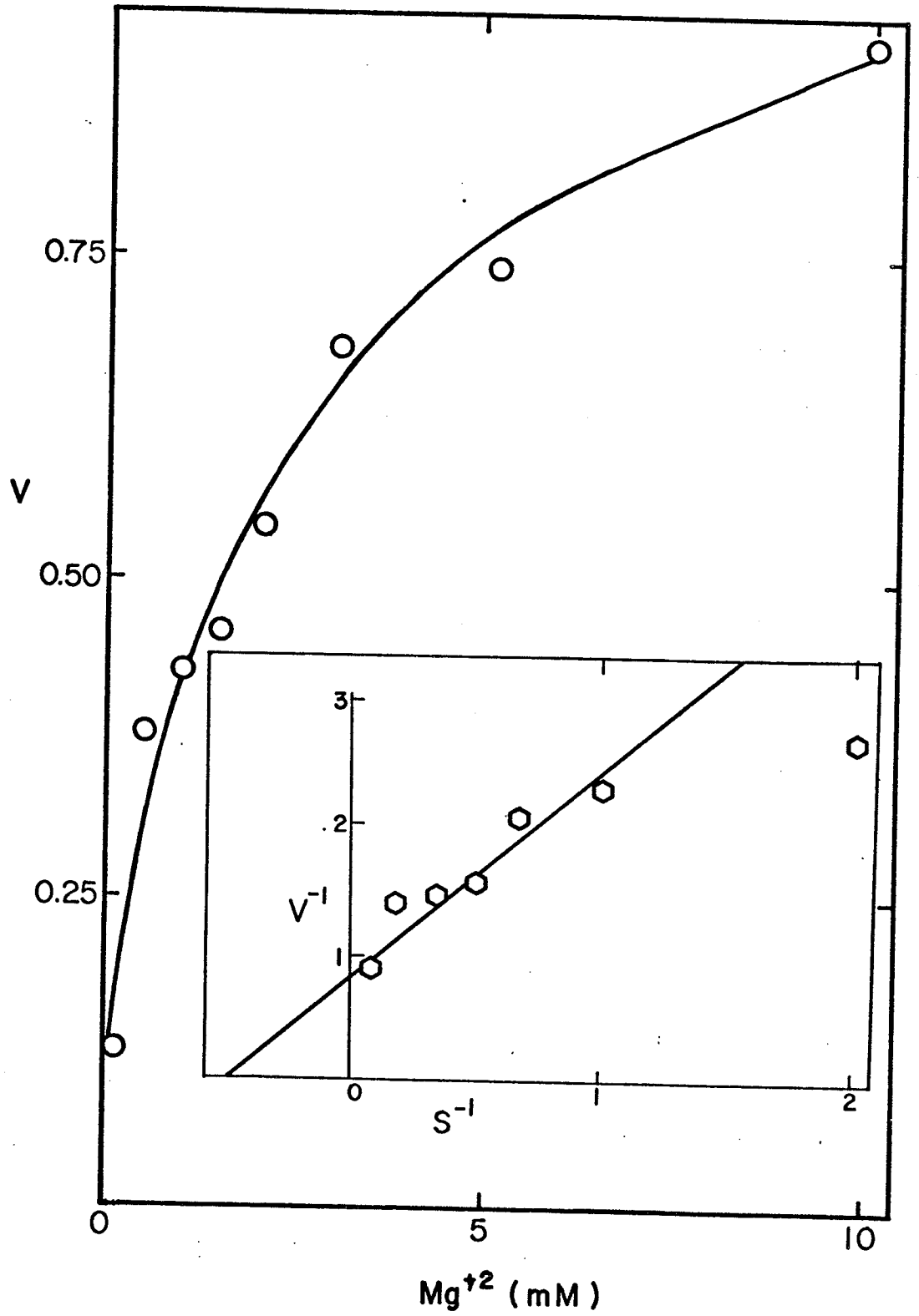

0.75
0.50
0.25
V
0
5
10
Mg+2 (mM)
3
2
1
V-1
0
1
2
S-1

Figure 17. Effect of PEP concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize primary leaves.
Reaction systems (0.5 ml) contained in μmoles the following: $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume; 0.4 mg crude supernatant protein and varying amounts of PEP as indicated. v is in μm CO_2/mg protein/hr. S is in mM of PEP.

Km = 7.40 mM

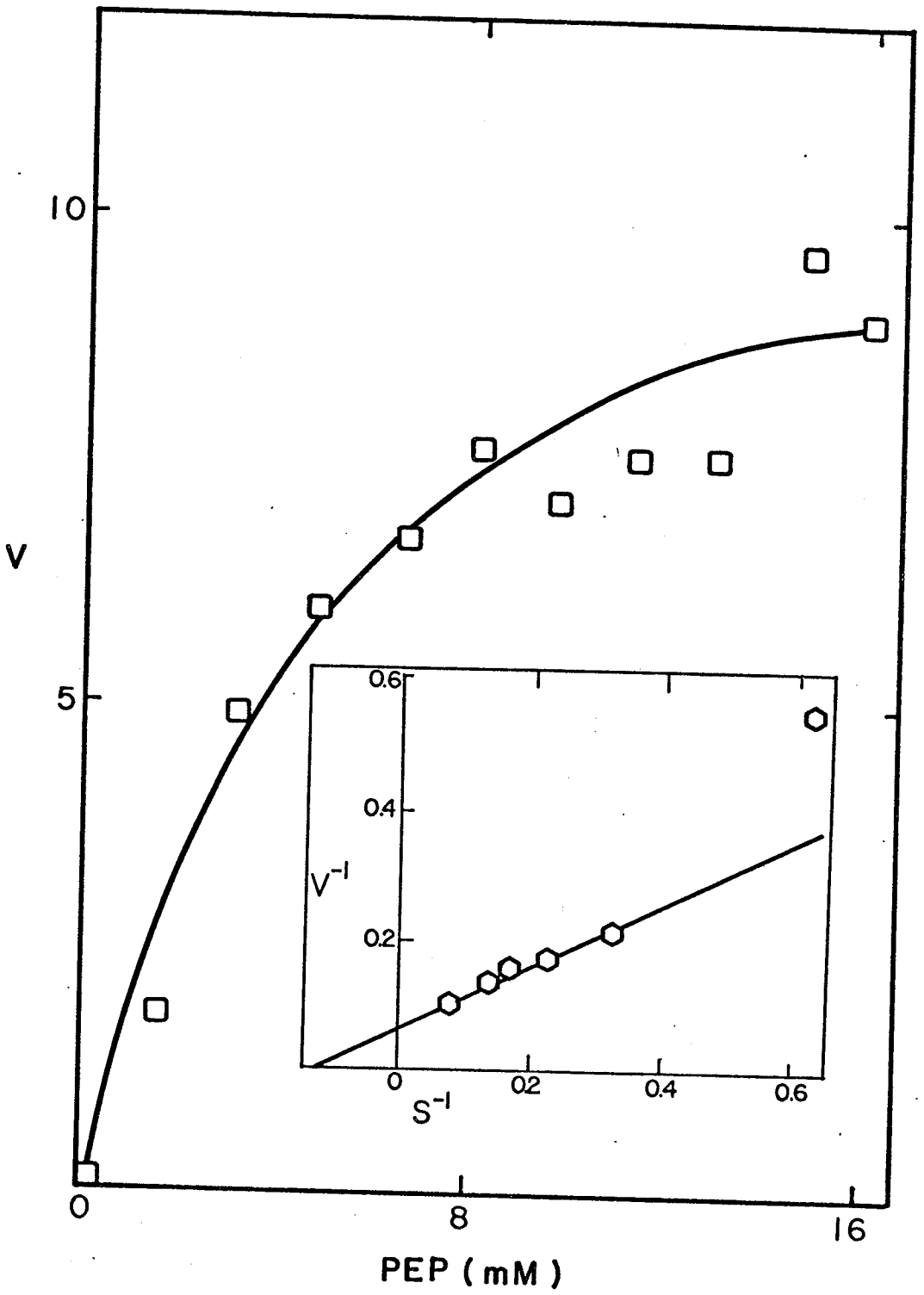

10
5
V
0
8
16
PEP (mM)
0.6
0.4
0.2
V^{-1}
0
0.2
0.4
0.6
S^{-1}

Figure 18. Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize primary leaves.
Reaction systems (0.5 ml) contained in μmoles the following: PEP, 8; $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume; 0.4 mg of crude supernatant protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$

Km = 0.89 mM

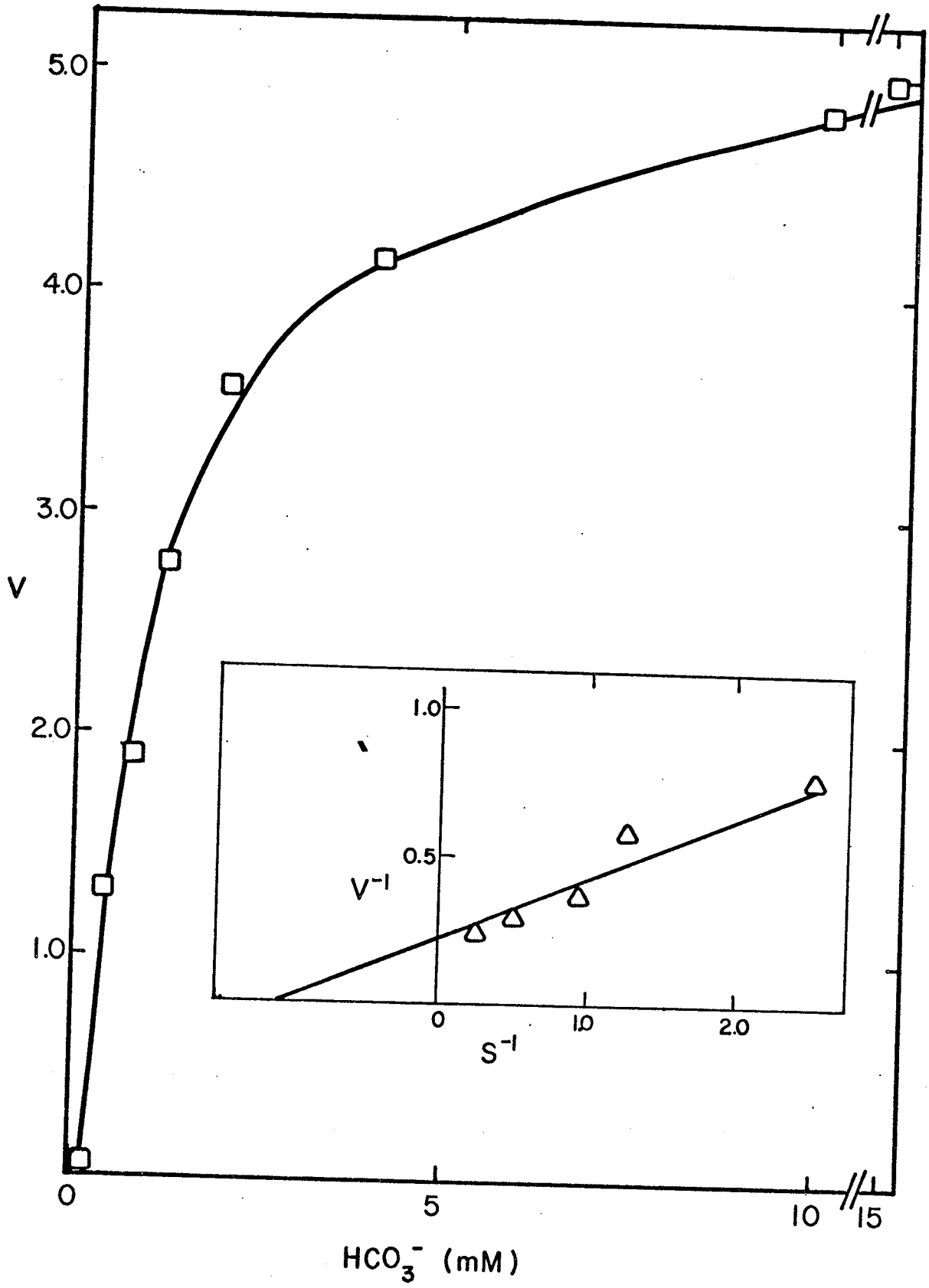

5.0
4.0
3.0
2.0
1.0
V
0
5
10
15
HCO3- (mM)
1.0
0.5
V-1
0
1.0
2.0
S-1

Figure 19. Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize primary leaves.
Reaction systems (0.5 ml) contained in µmoles the following: PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); Solution A, enough to make up 0.5 ml final volume; 0.4 mg of crude supernatant protein and varying amounts of $MgCl_2$ as indicated. v is in µm CO_2/mg protein/hr. S is in mM of $MgCl_2$.

Km = 0.30 mM

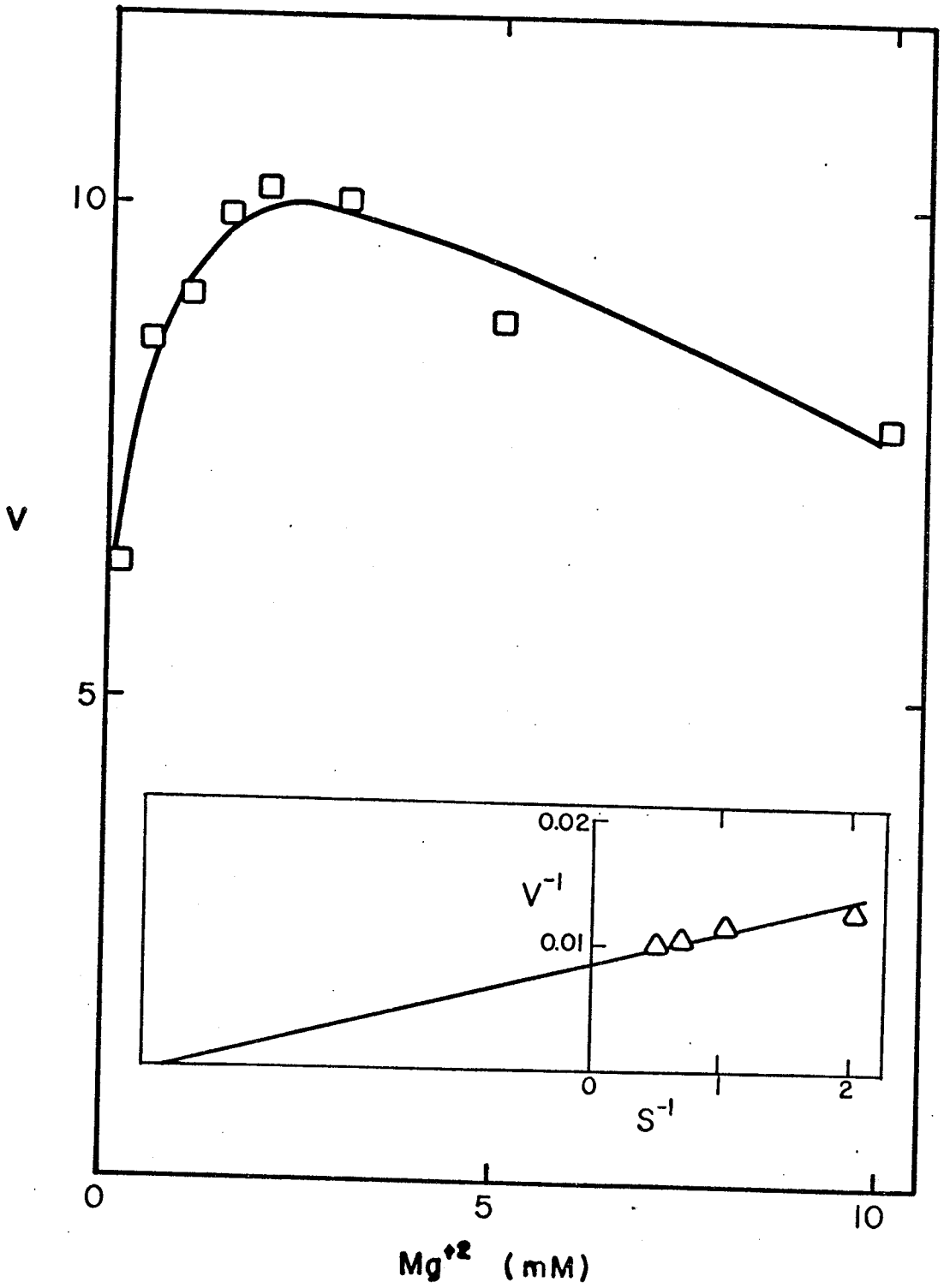

10
5
V
0
5
10
Mg+2 (mM)
0.02
V-1
0.01
0
2
S-1

Figure 20. Effect of PEP concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize secondary leaves.
Reaction systems (0.5 ml) contained in μmoles the following: $NaH^{14}CO_3$, 25 (>6 x 10^6); $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume, 0.6 mg of crude chloroplast protein and varying amounts of PEP as indicated. v is in μm CO_2/mg protein/hr. S is in mM of PEP.

Km = 2.04 mM

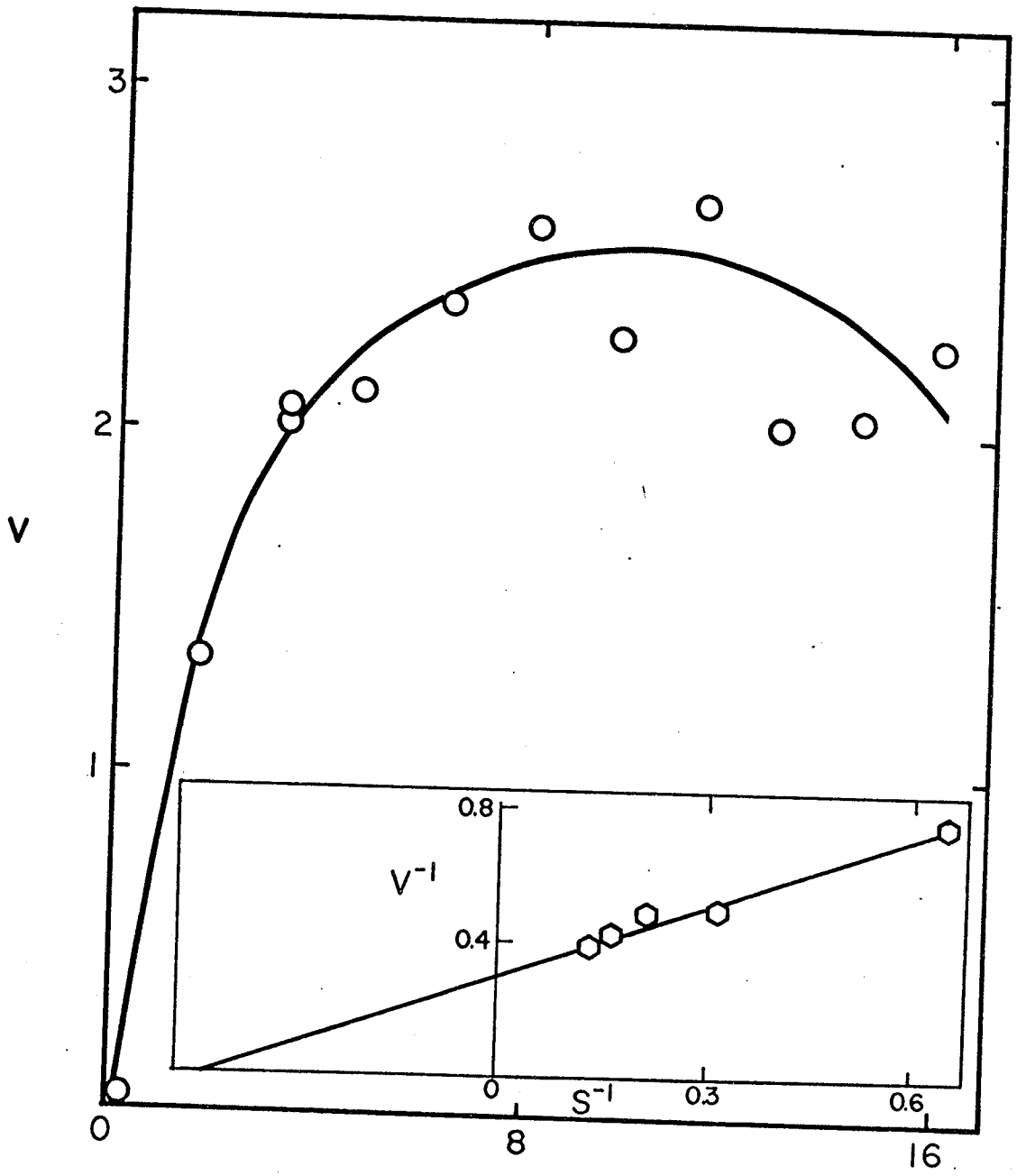

V
3
2
1
0
8
16
PEP (mM)
0.8
V^{-1}
0.4
0
S^{-1}
0.3
0.6

Figure 21. Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize secondary leaves.
Reaction systems (0.5 ml) contained in µmoles the following: PEP, 8; $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume; 0.6mg of crude chloroplast protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in µm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$.

Km = 0.67 mM

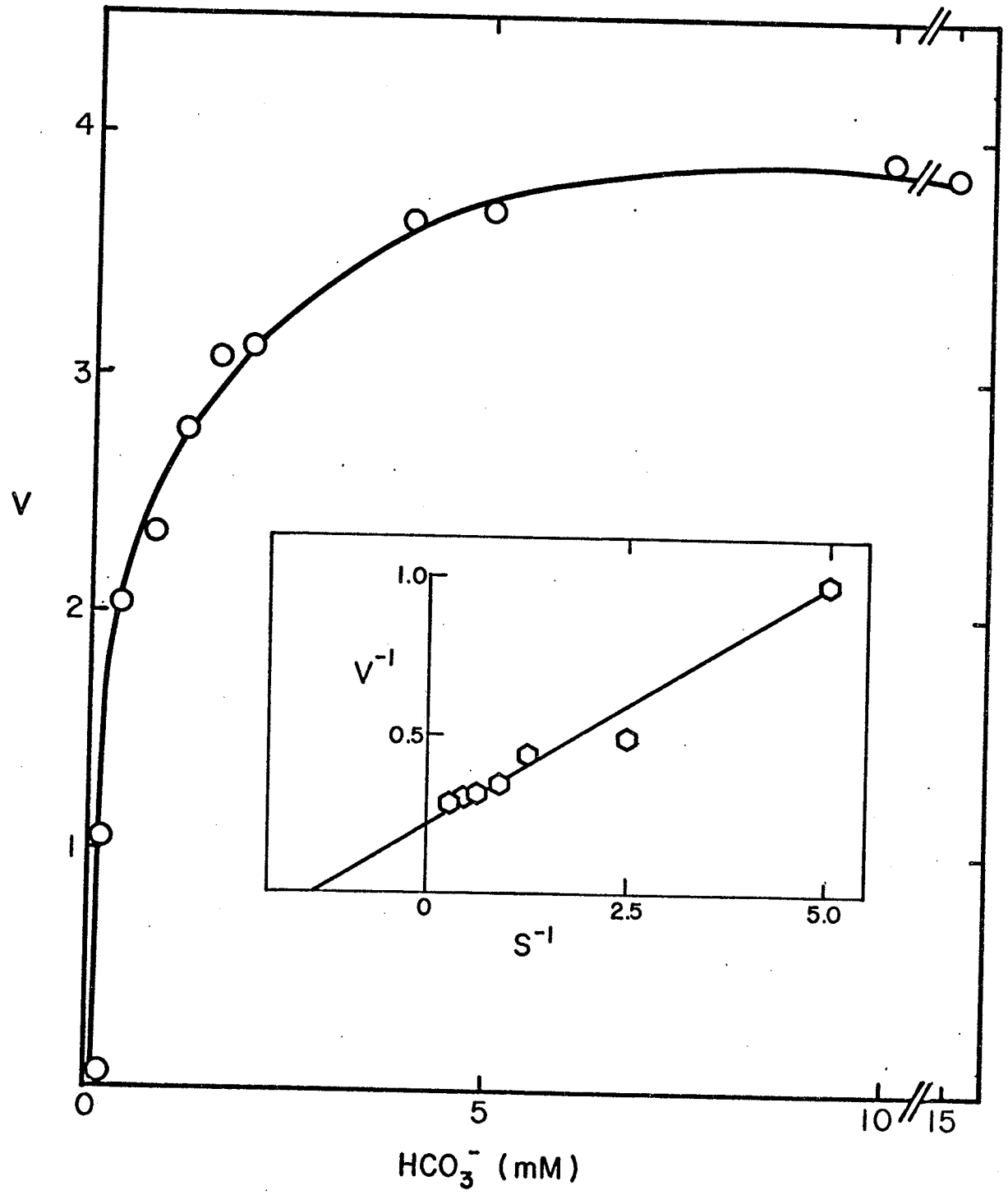

V
4
3
2
1
0
5
10
15
HCO_3^- (mM)
1.0
V^{-1}
0.5
0
2.5
5.0
S^{-1}

Figure 22. Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by isolated chloroplasts of maize secondary leaves. Reaction systems (0.5 ml) contained in μmoles the following: PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); Solution A, enough to make up 0.5 ml final volume; 0.6 mg of crude chloroplast protein and varying amounts of $MgCl_2$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $MgCl_2$.

Km = 2.22 mM

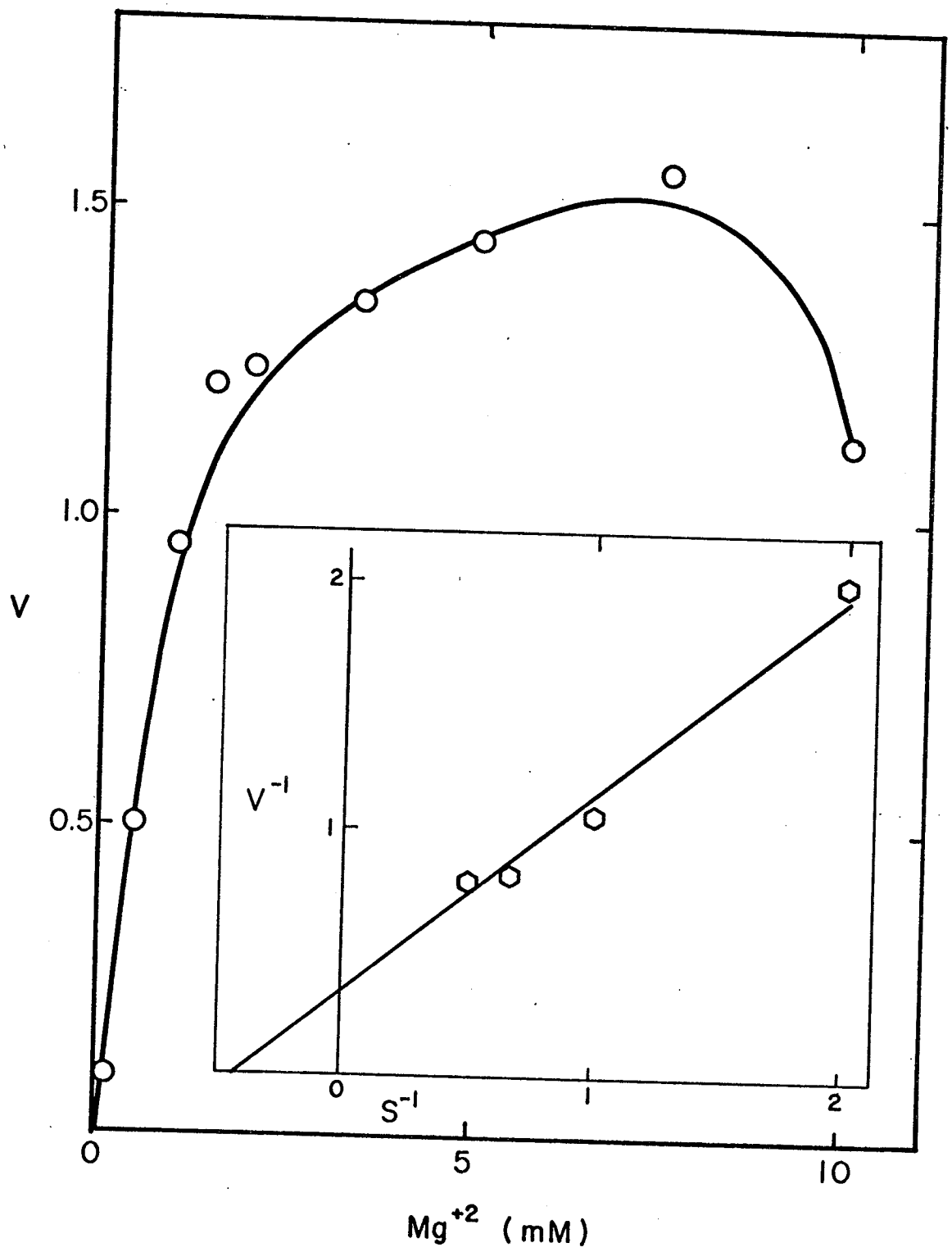

1.5
1.0
0.5
V
0
5
10
Mg+2 (mM)
2
V-1
1
0
S-1
1
2

Figure 23. Effect of PEP concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize secondary leaves. Reaction systems (0.5 ml) contained in µmoles the following: $NaH^{14}CO_3$, 25 (> 6 x 10^6 dpm); $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume; 0.4 mg of crude supernatant protein and varying amounts of PEP as indicated. v is in µm CO_2/mg protein/hr. S is in mM of PEP.

Km = 2.44 mM

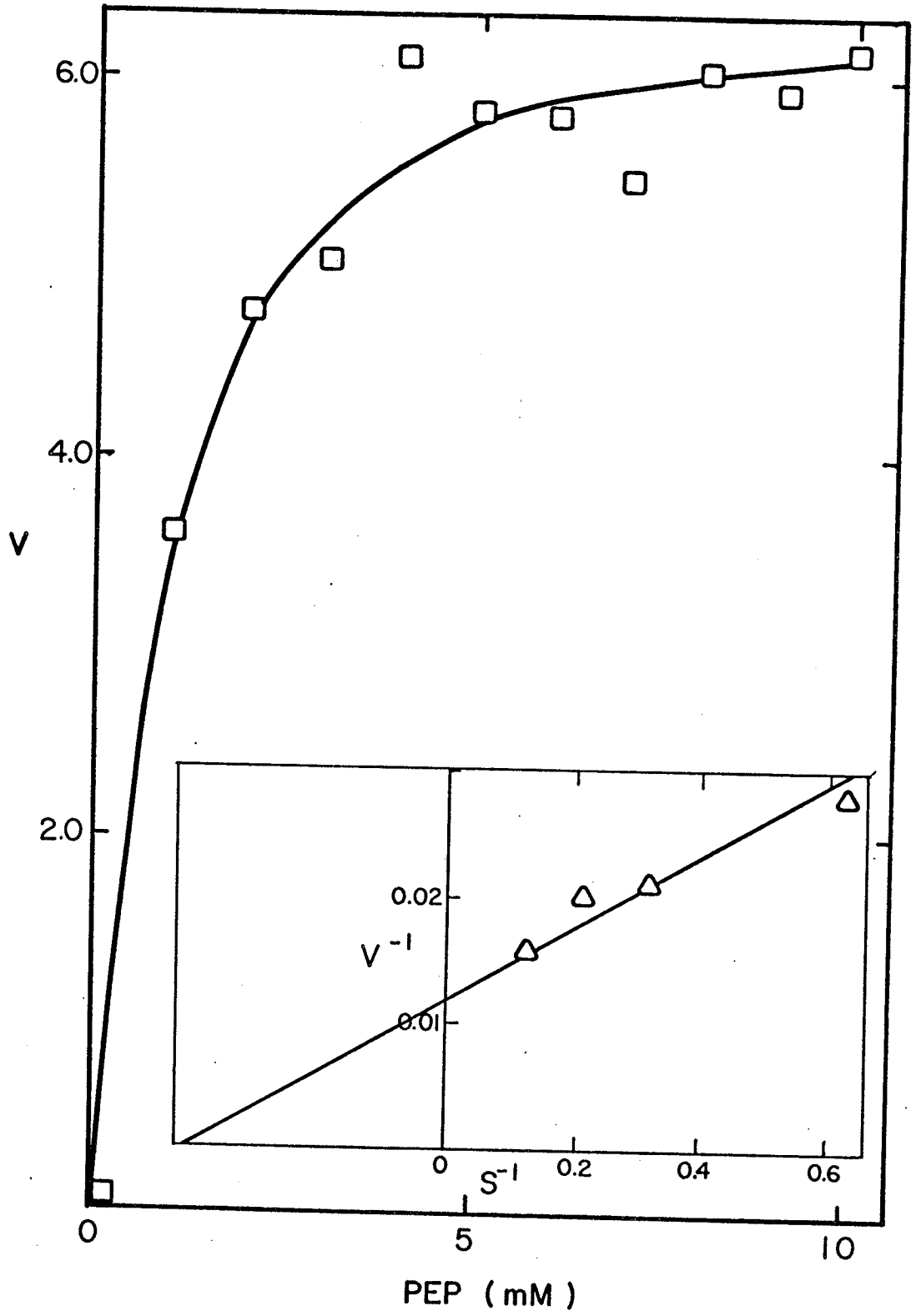

6.0
4.0
2.0
V
0
5
10
PEP (mM)
0.02
V⁻¹
0.01
0
S⁻¹
0.2
0.4
0.6

Figure 24. Effect of sodium bicarbonate concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize secondary leaves. Reaction systems (0.5 ml) contained in μmoles the following: PEP,8; $MgCl_2$, 5; Solution A, enough to make up 0.5 ml final volume; 0.4 mg crude supernatant protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S in in mM of $NaH^{14}CO_3$.

Km = 0.40 mM

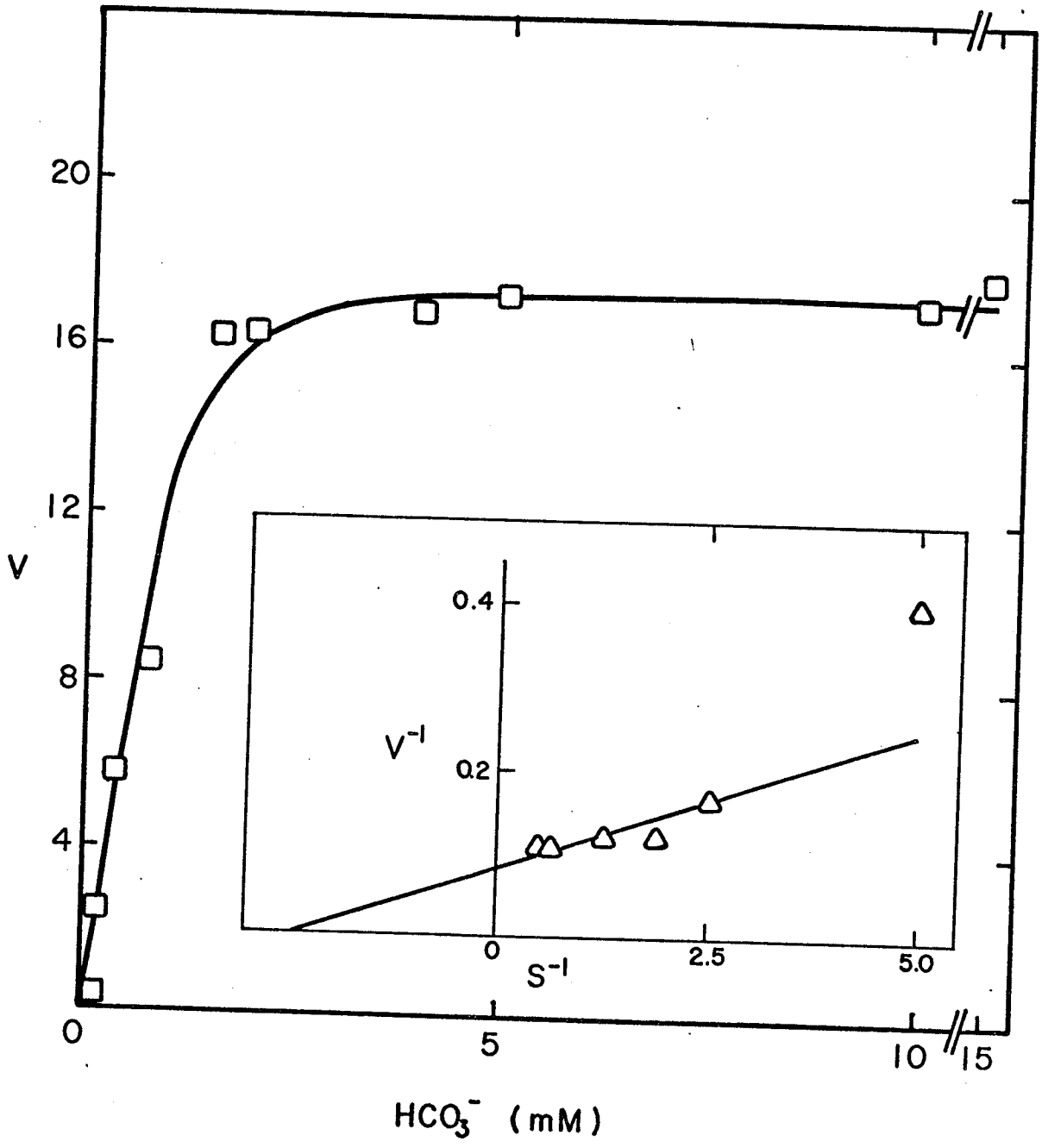

20
16
12
8
4
0
V
5
10
15
HCO_3^- (mM)
0.4
0.2
V^{-1}
0
2.5
5.0
S^{-1}

Figure 25. Effect of magnesium chloride concentration on the initial velocity of CO_2 fixation by the supernatant fraction of maize secondary leaves. Reaction systems (0.5 ml) contained in μmoles the following: PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); Solution A, enough to make up 0.5 ml final volume; 0.4 mg of crude supernatant protein and varying amounts of magnesium chloride as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $MgCl_2$.

Km = 0.35mM

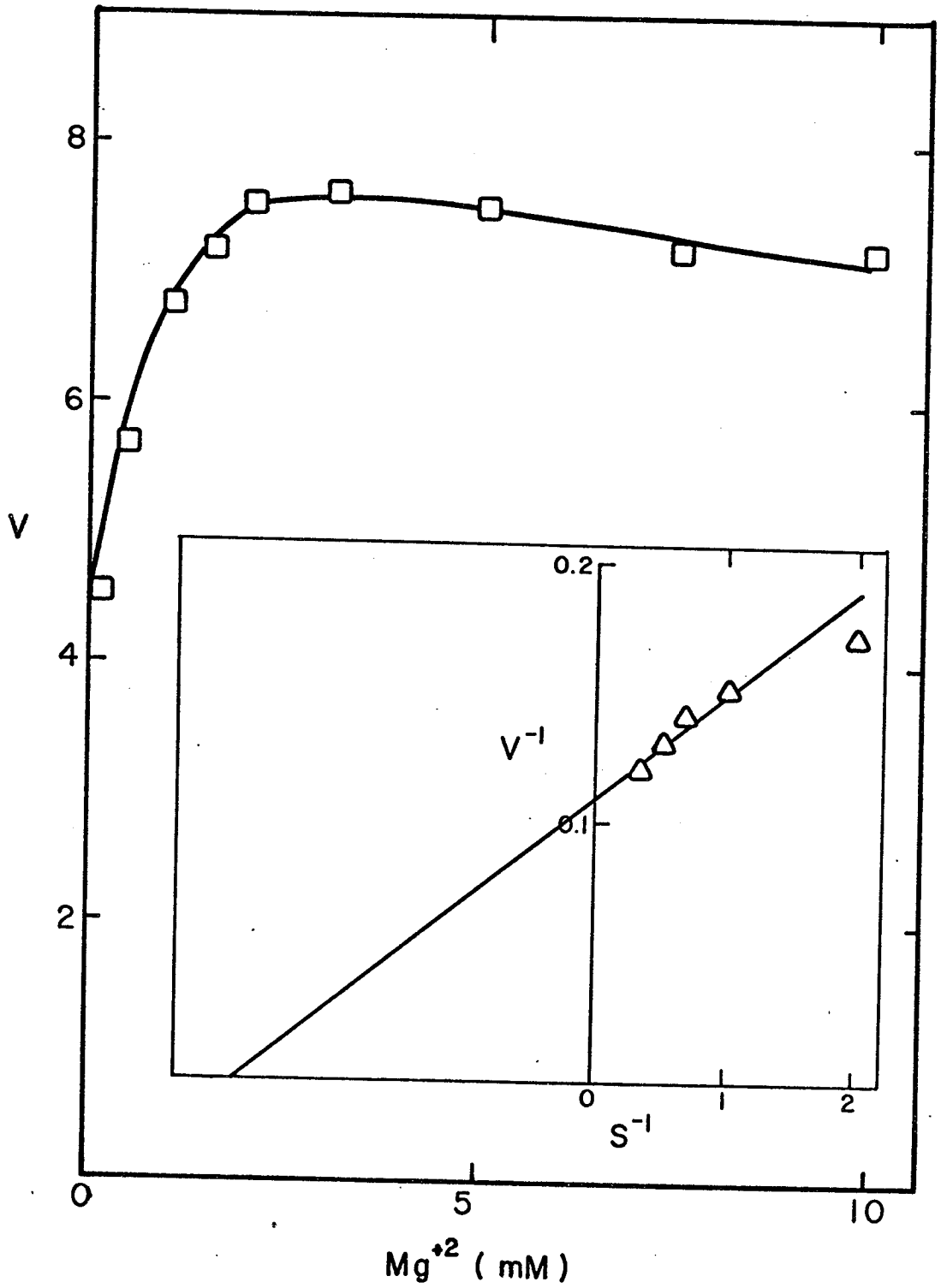
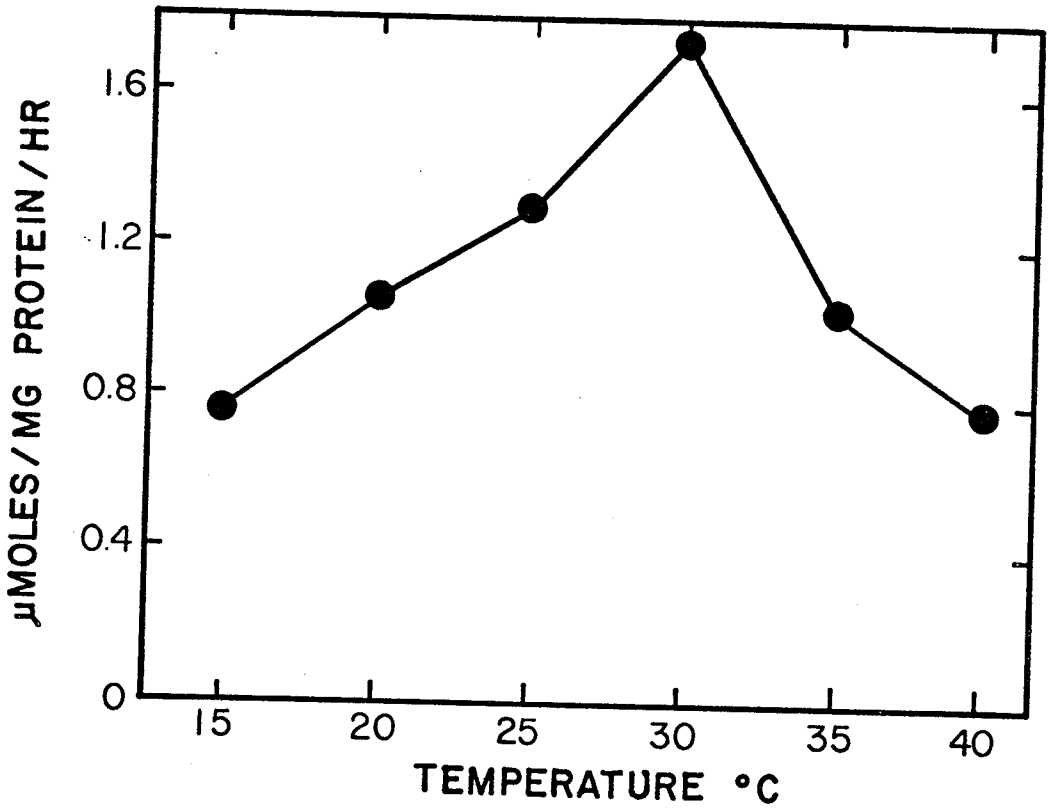

8
6
4
2
0
V
5
10
Mg+2 (mM)
0.2
0.1
V-1
0
1
2
S-1

Figure 26. Effect of temperature on CO_2 fixation capacity primary leaf cellular fractions of maize grown under 25°C day and 20° night temperatures.

a. Isolated chloroplasts

b. Supernatant


µMOLES / MG PROTEIN / HR
0
0.4
0.8
1.2
1.6
15
20
25
30
35
40
TEMPERATURE °C

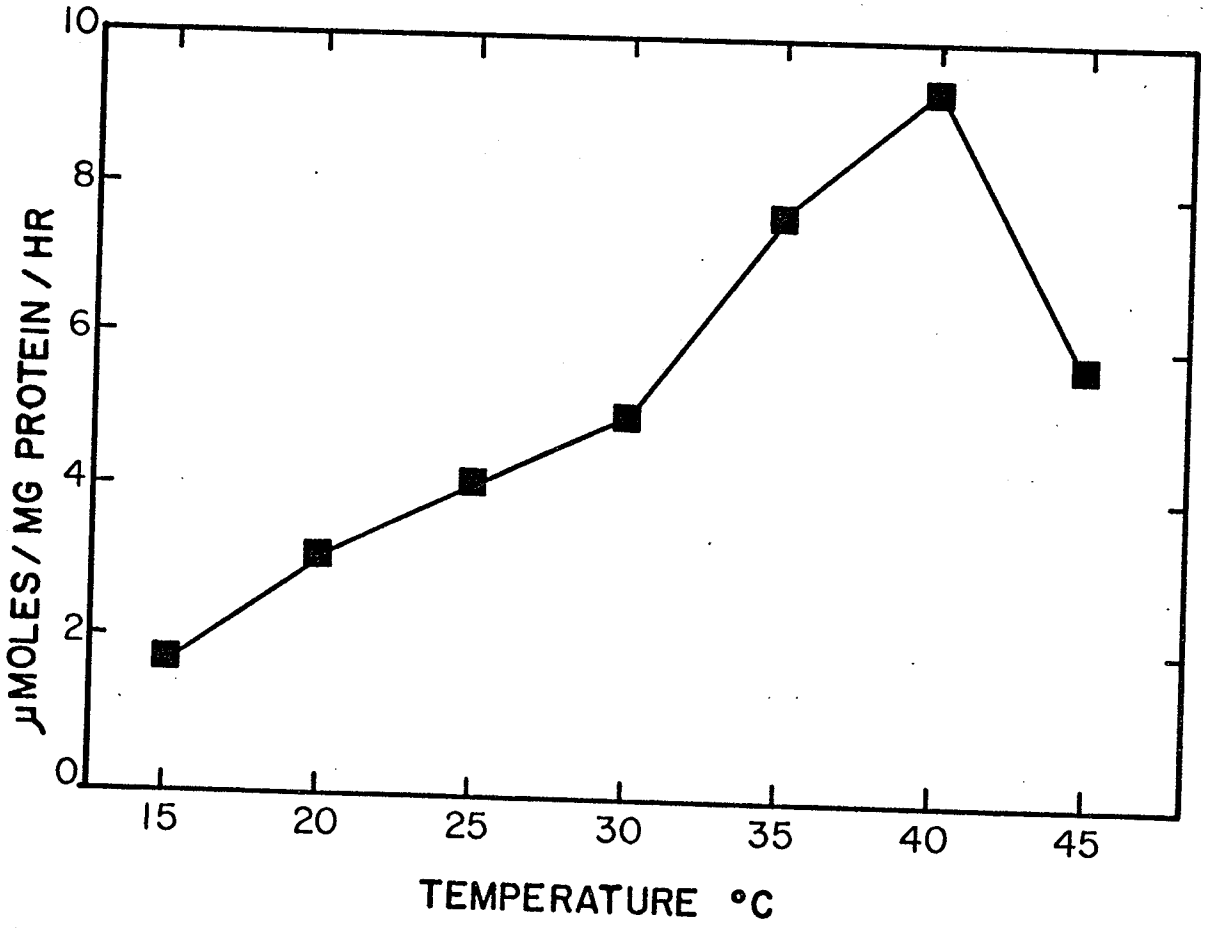
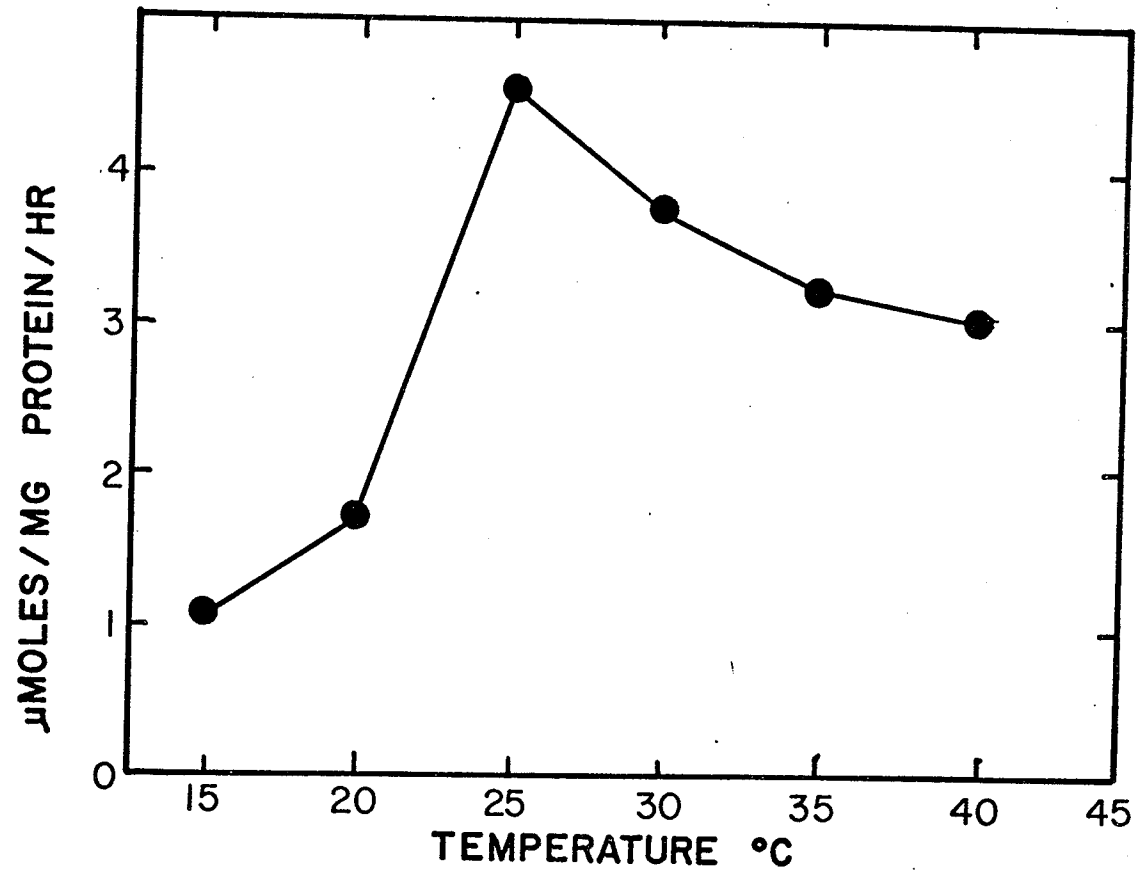

µMOLES / MG PROTEIN / HR
0
2
4
6
8
10
15
20
25
30
35
40
45
TEMPERATURE °C

Figure 27. Effect of temperature on CO_2 fixation capacity of primary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.

a. Isolated chloroplasts

b. Supernatant


µMOLES/MG PROTEIN/HR
0
1
2
3
4
15
20
25
30
35
40
45
TEMPERATURE °C

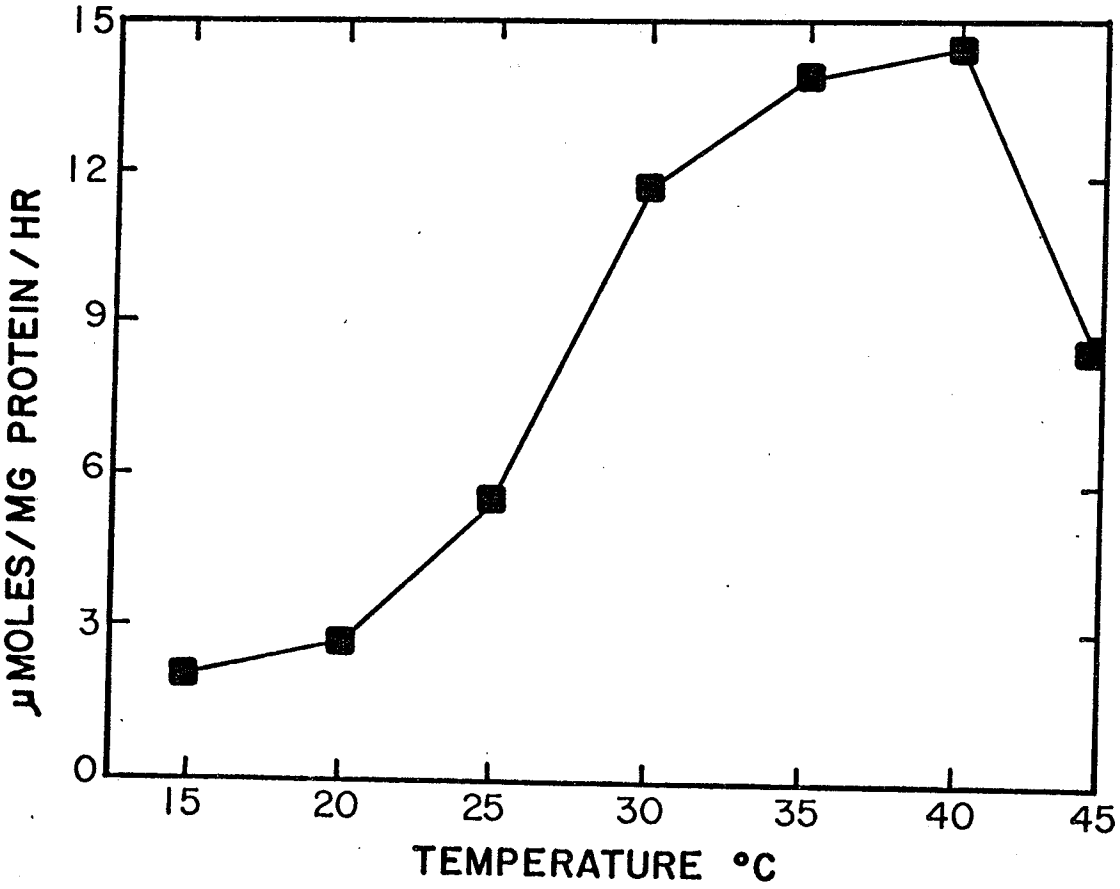
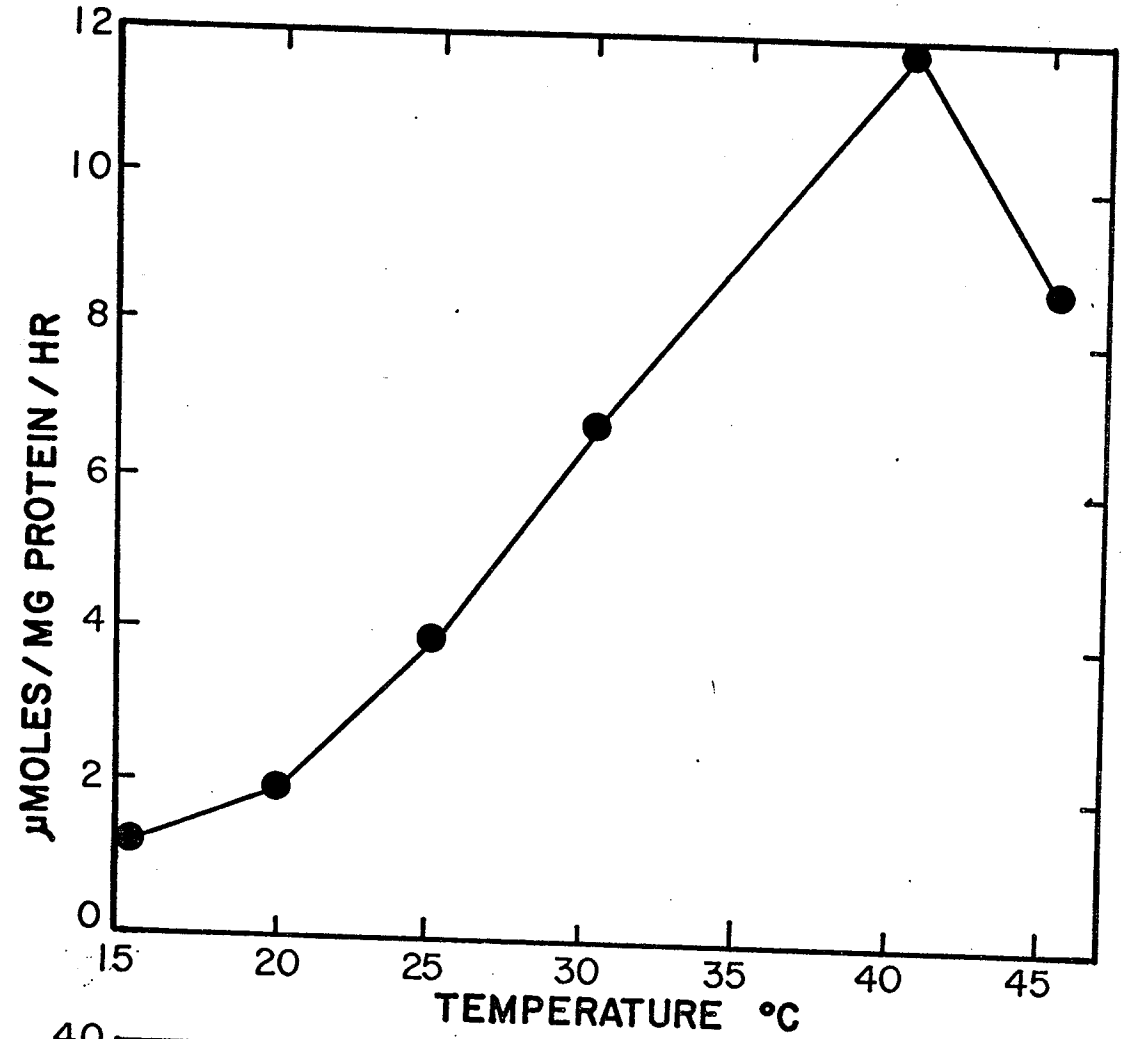

µMOLES/MG PROTEIN/HR
0
3
6
9
12
15
15
20
25
30
35
40
45
TEMPERATURE °C

Figure 28. Effect of temperature on CO_2 fixation capacity of secondary leaf cellular fractions of maize grown under 25°C day and 20°C night temperatures.

a. Isolated chloroplasts

b. Supernatant


12
10
8
6
4
2
0
µMOLES/MG PROTEIN/HR
15
20
25
30
35
40
45
TEMPERATURE °C

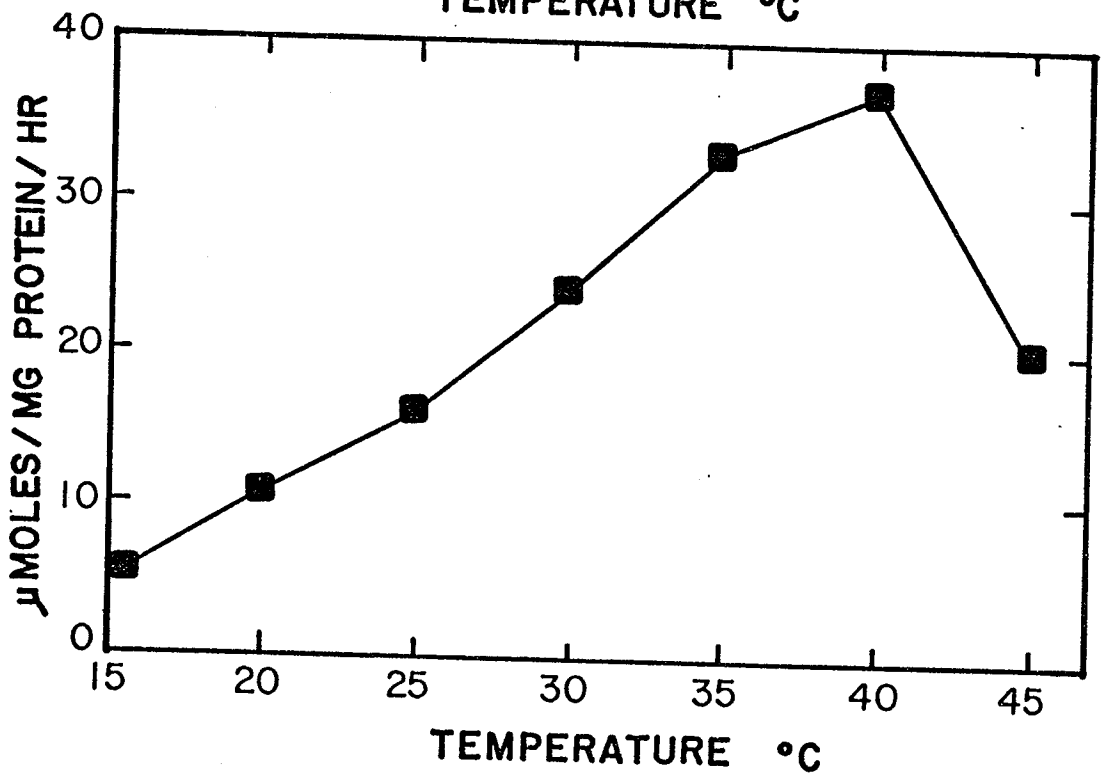
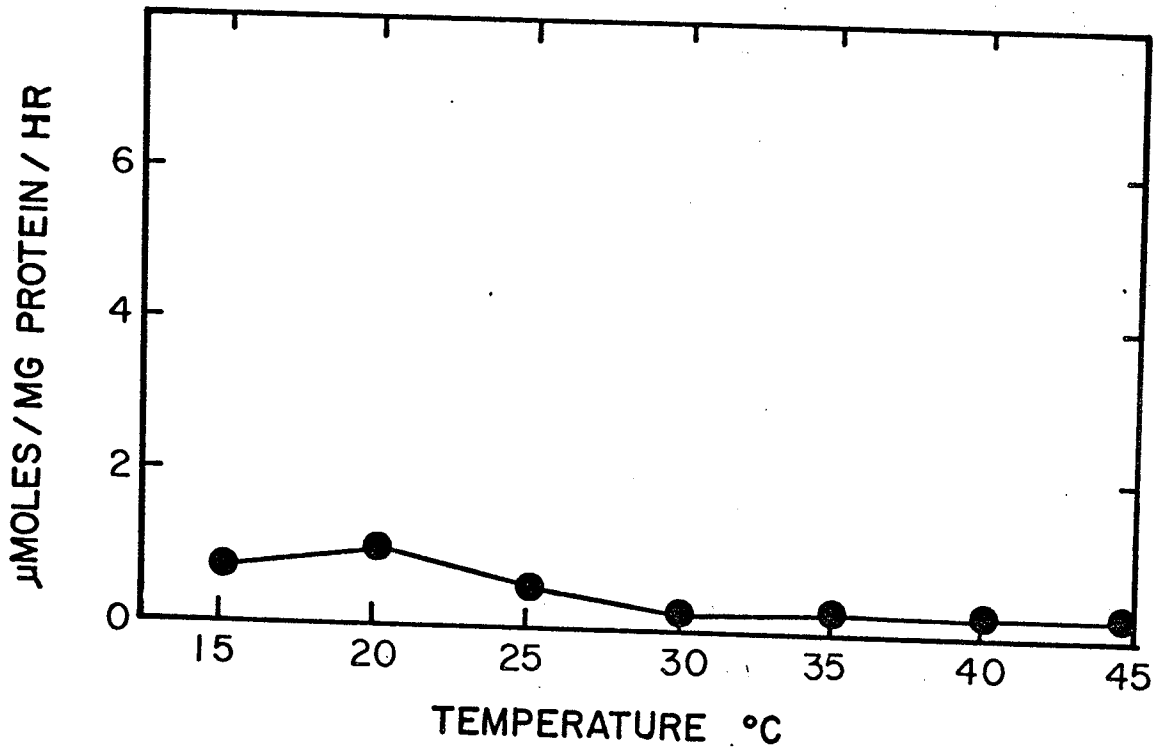

40
30
20
10
0
µMOLES/MG PROTEIN/HR
15
20
25
30
35
40
45
TEMPERATURE °C

Figure 29. Effect of temperature on CO_2 fixation capacity of secondary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.

a. Isolated chloroplasts

b. Supernatant


µMOLES / MG PROTEIN / HR
0
2
4
6
15
20
25
30
35
40
45
TEMPERATURE °C

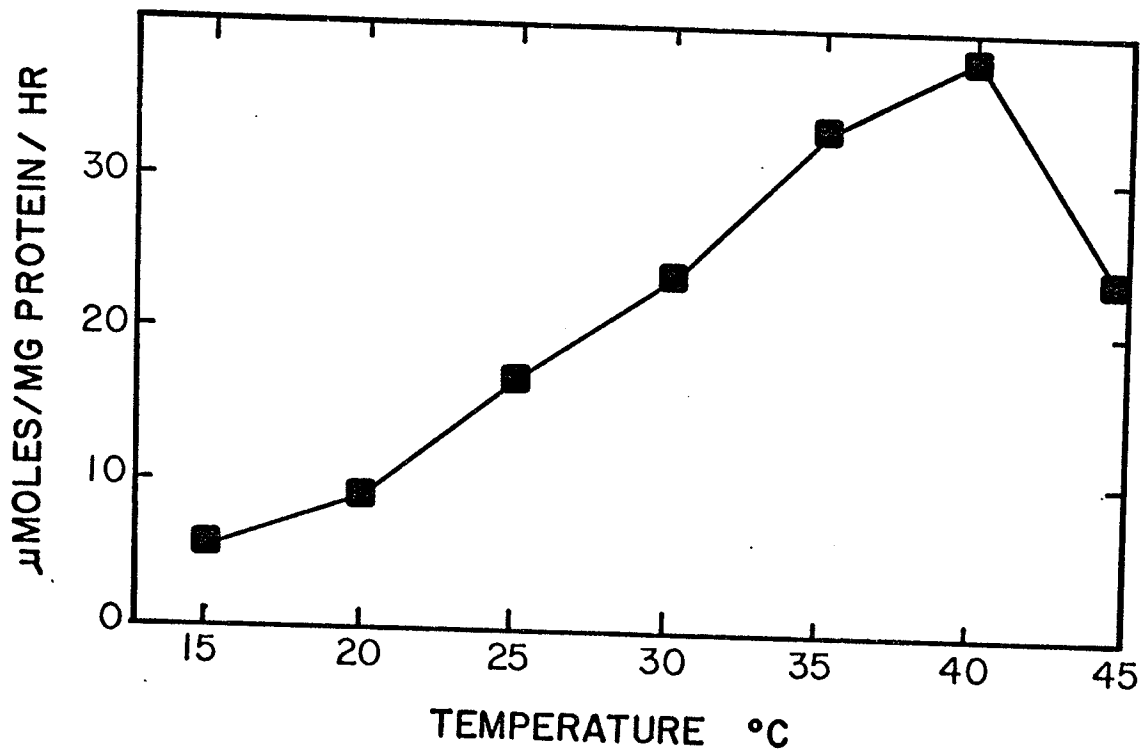

µMOLES/MG PROTEIN/ HR
0
10
20
30
15
20
25
30
35
40
45
TEMPERATURE °C

Figure 30. Effect of PEP concentration on the initial velocity of PEP carboxylase in isolated chloroplasts of maize primary leaves. Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); $MgCl_2$, 5; NADH,2; malate dehydrogenase, excess, 0.6 mg crude protein and varying amounts of PEP as indicated. v is calculated as μm CO_2/mg protein/hr, S is in mM of PEP.

Km = 10.00 mM

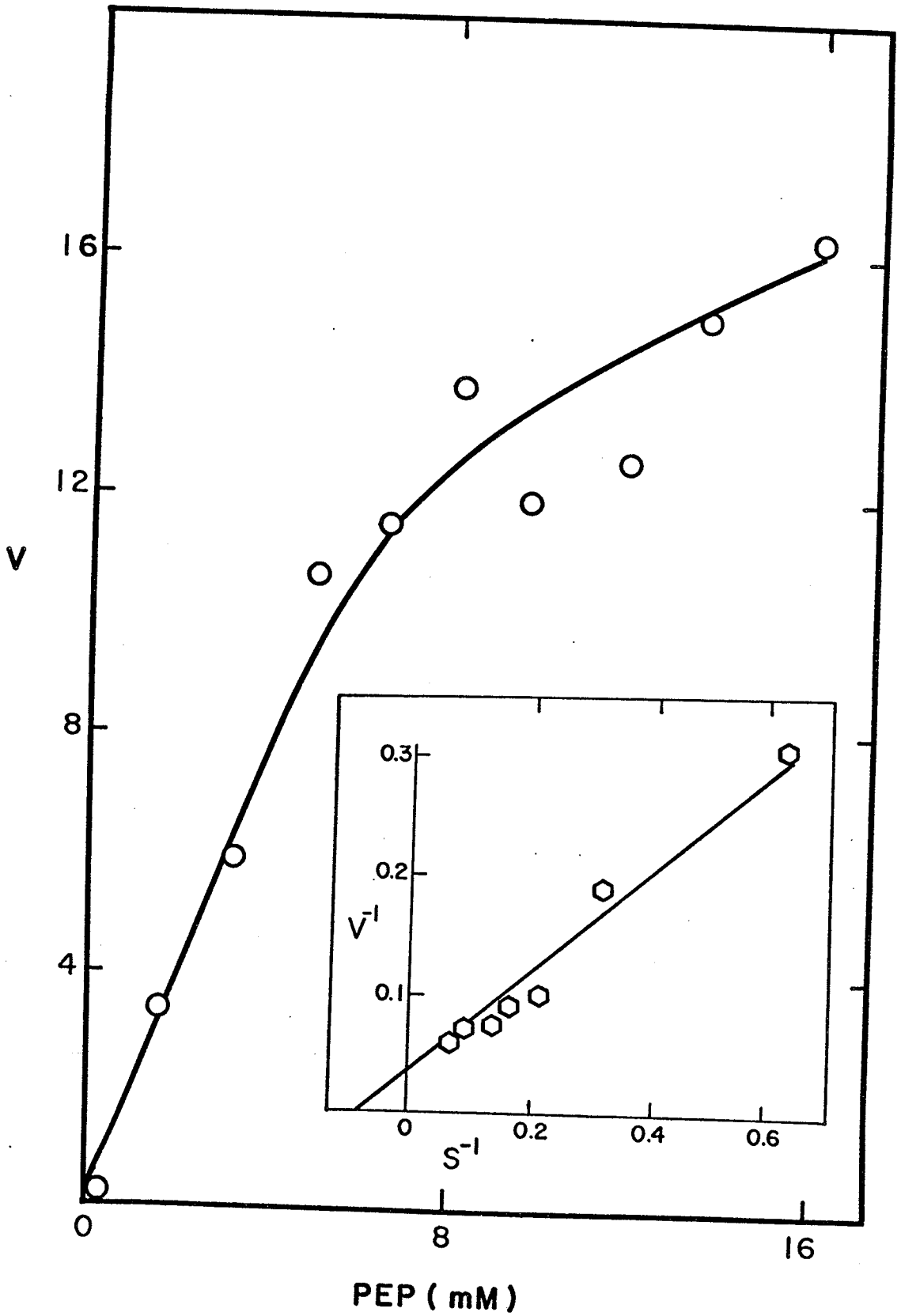

16
12
8
4
V
0
8
16
PEP (mM)
0.3
0.2
0.1
V⁻¹
0
0.2
0.4
0.6
S⁻¹

Figure 31. Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in isolated chloroplasts from maize primary leaves. Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; PEP, 8; $MgCl_2$, 5; NADH, 2; malate dehydrogenase, excess; 0.6 mg crude protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$.

Km = 0.43 mM

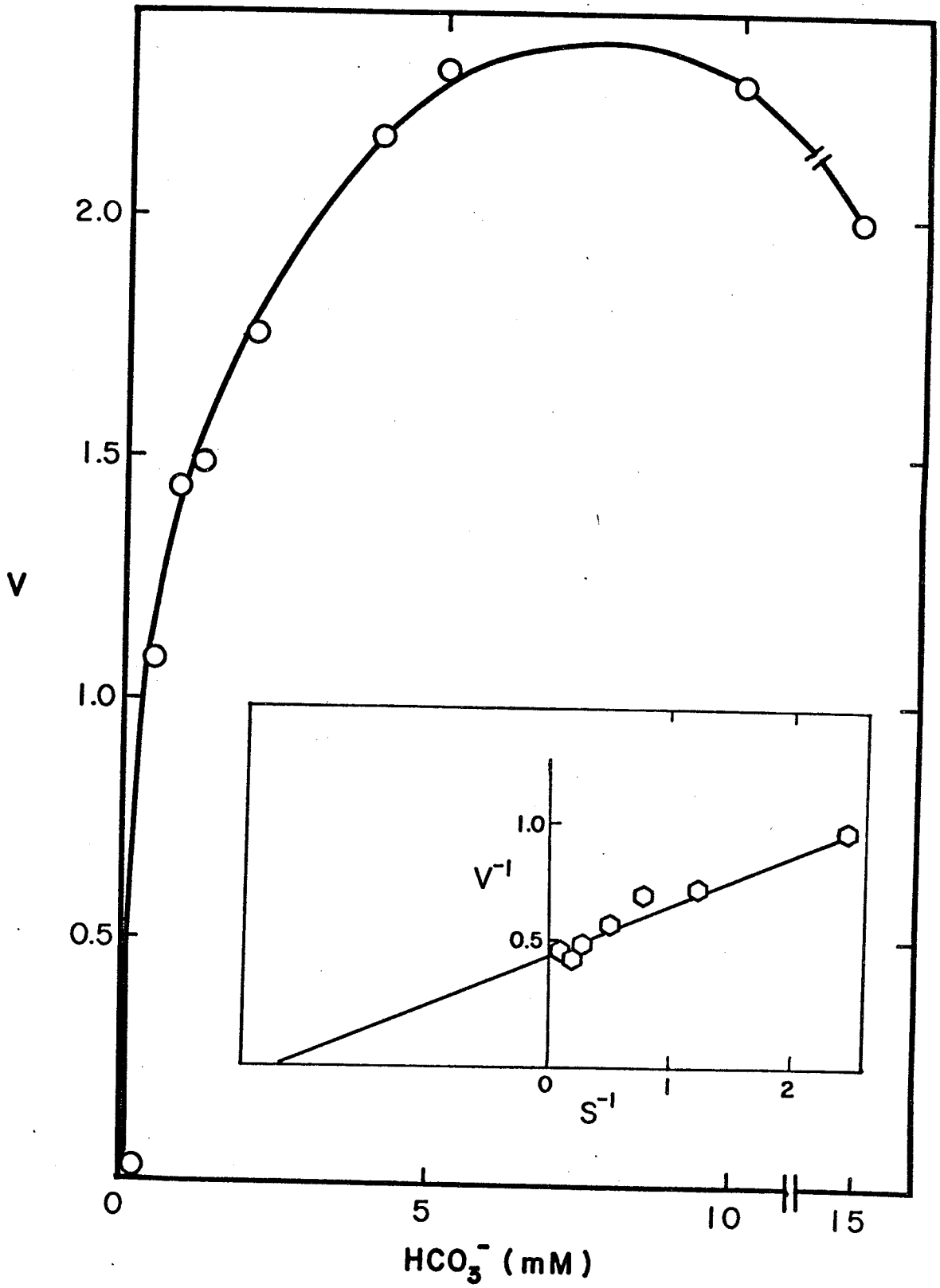

2.0
1.5
V
1.0
0.5
0
5
10
15
HCO3- (mM)
1.0
V-1
0.5
0
1
2
S-1

Figure 32. Effect of magnesium chlroide concentration on the initial velocity of PEP carboxylase in isolated chloroplasts from primary leaves of maize. Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm), NADH, 2; malate dehydrogenase, excess; 0.6 mg of crude protein and varying amounts of $MgCl_2$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $MgCl_2$.

Km = 4.00 mM

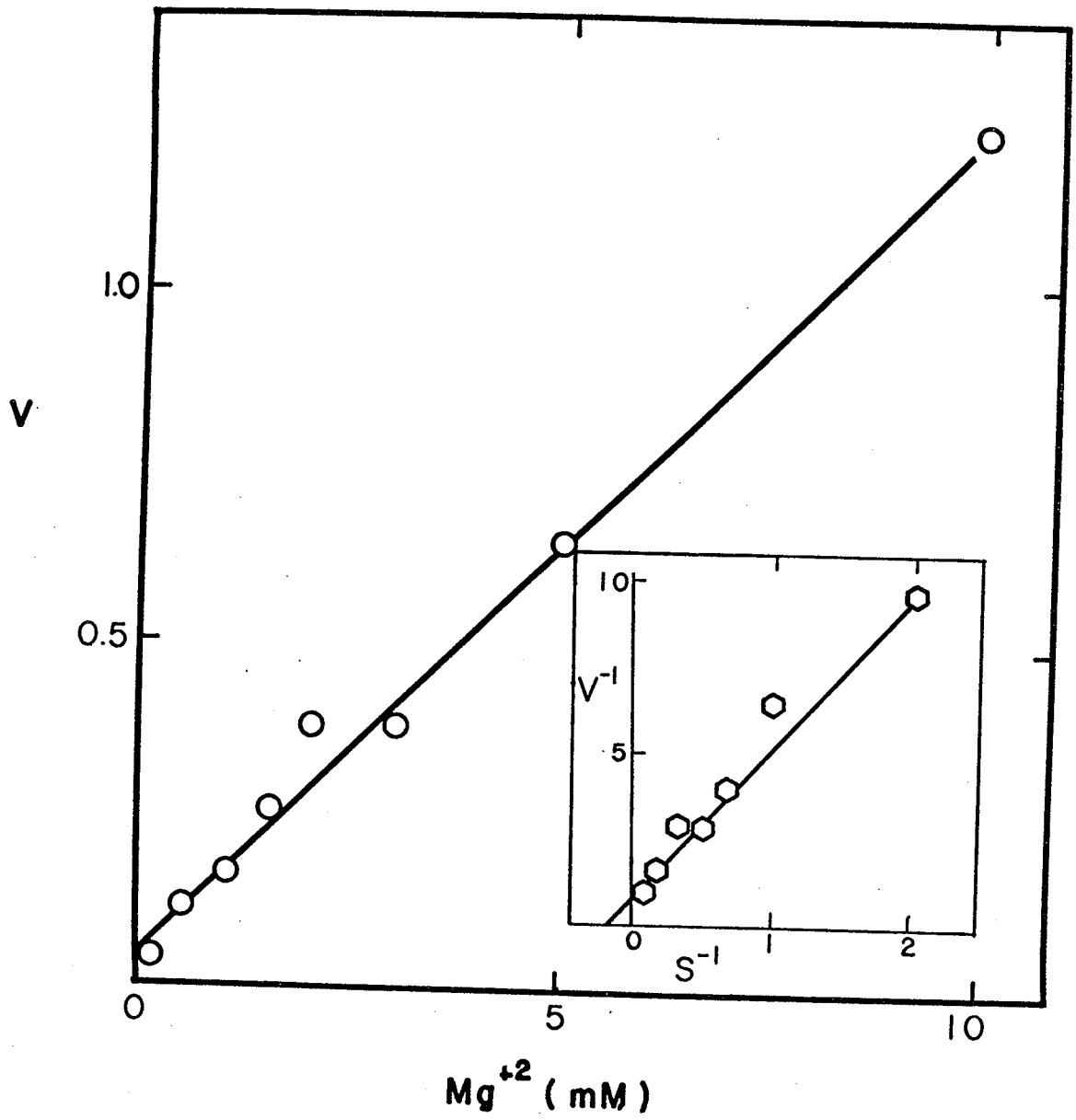

V
1.0
0.5
0
5
10
Mg+2 (mM)
V-1
10
5
0
1
2
S-1

Figure 33. Effect of PEP concentration on the initial velocity of PEP carboxylase in supernatant of maize primary leaves.
Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm), $MgCl_2$,5; NADH, 2; malate dehydrogenase, excess; 0.4 mg crude protein and varying amounts of PEP as indicated. v is in μm CO_2/mg protein/hr. S is in mM of PEP.

Km = 10.00 mM

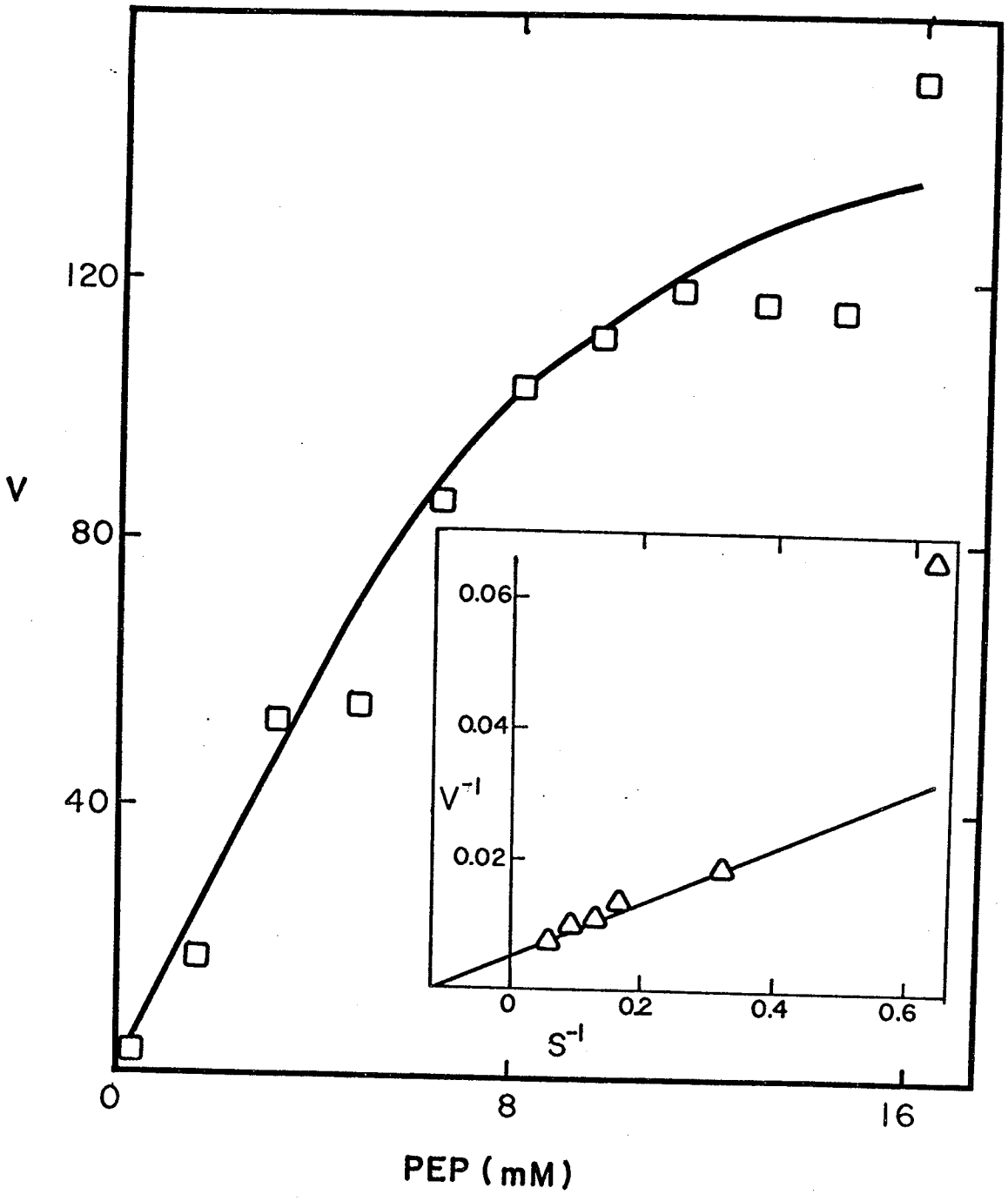

120
80
40
V
0
8
16
PEP (mM)
0.06
0.04
V^{-1}
0.02
0
0.2
0.4
0.6
S^{-1}

Figure 34. Effect of sodium bicarbonate concentration of the initial velocity of PEP carboxylase in supernatant of maize primary leaves. Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; PEP, 8; $MgCl_2$, 5; NADH, 2; malate dehydrogenase, excess; 0.4 mg of crude protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$.

Km = 0.44 mM

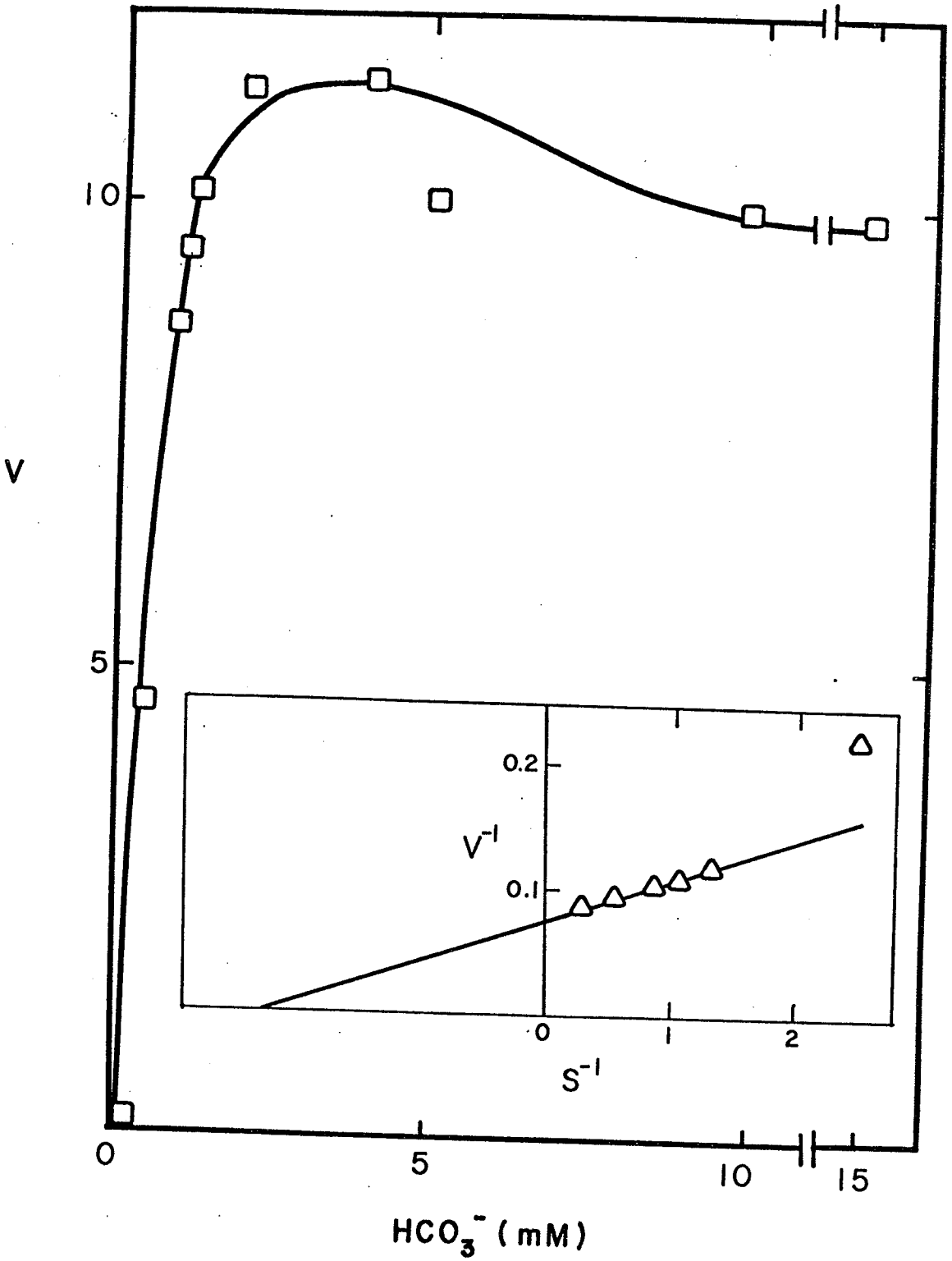

V
10
5
0
5
10
15
HCO_3^- (mM)
0.2
V^{-1}
0.1
0
1
2
S^{-1}

Figure 35. Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in supernatant of maize primary leaves.
Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; PEP, 8; NADH, 2; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); malate dehydrogenase, excess, 0.4 mg crude protein and varying amounts of $MgCl_2$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $MgCl_2$.

Km = 1.14 mM

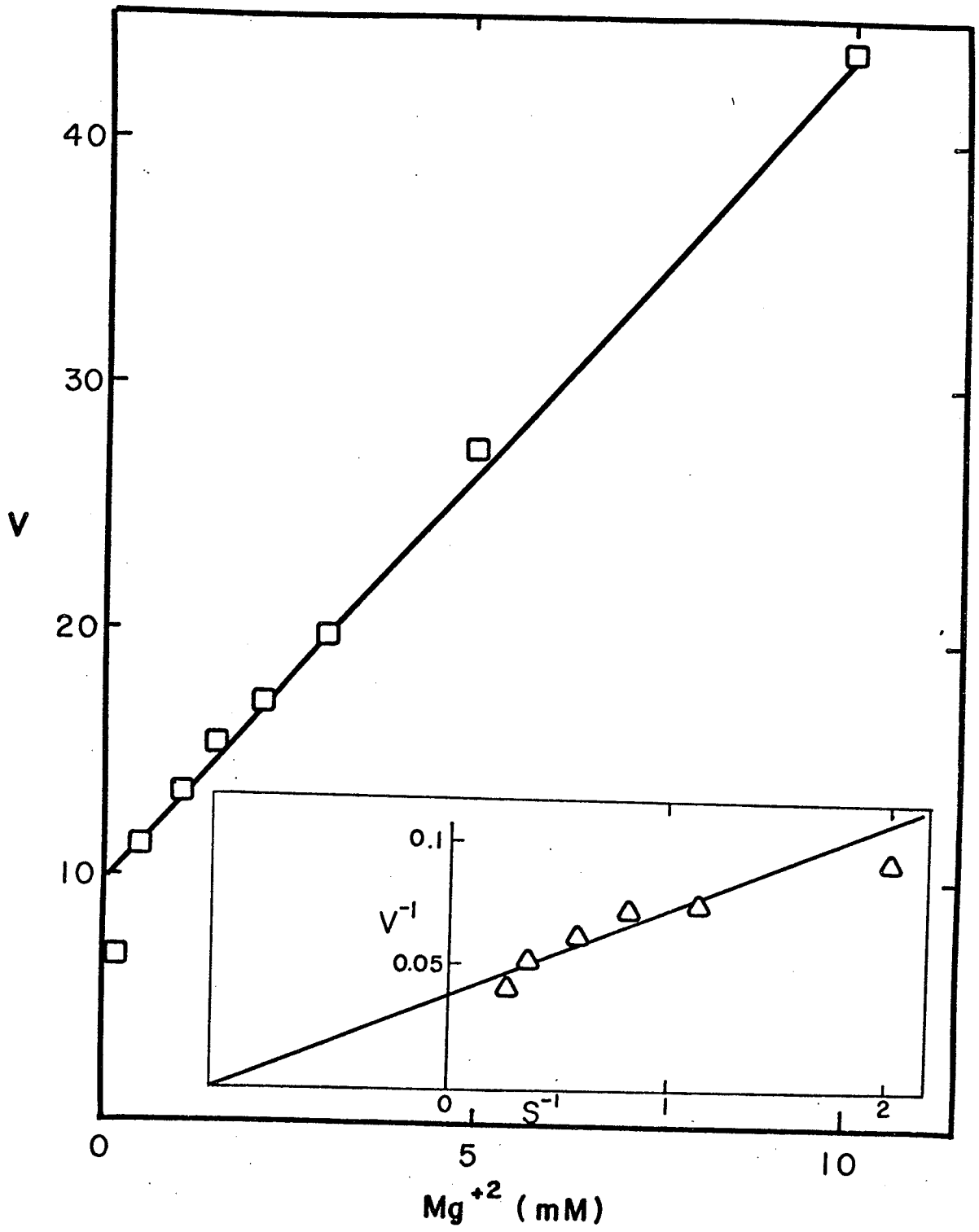

40
30
20
10
V
0
5
10
Mg+2 (mM)
0.1
V-1
0.05
0
S-1
1
2

Figure 36. Effect of PEP concentration on the initial velocity of PEP carboxylase in isolated chloroplasts from maize secondary leaves.
Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; $MgCl_2$,5; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); NADH, 2; malate dehydrogenase, excess, 0.6 mg crude protein and varying amounts of PEP as indicated. v is is μm CO_2/mg protein/hr. S is in mM of PEP.

Km = 1.40 mM

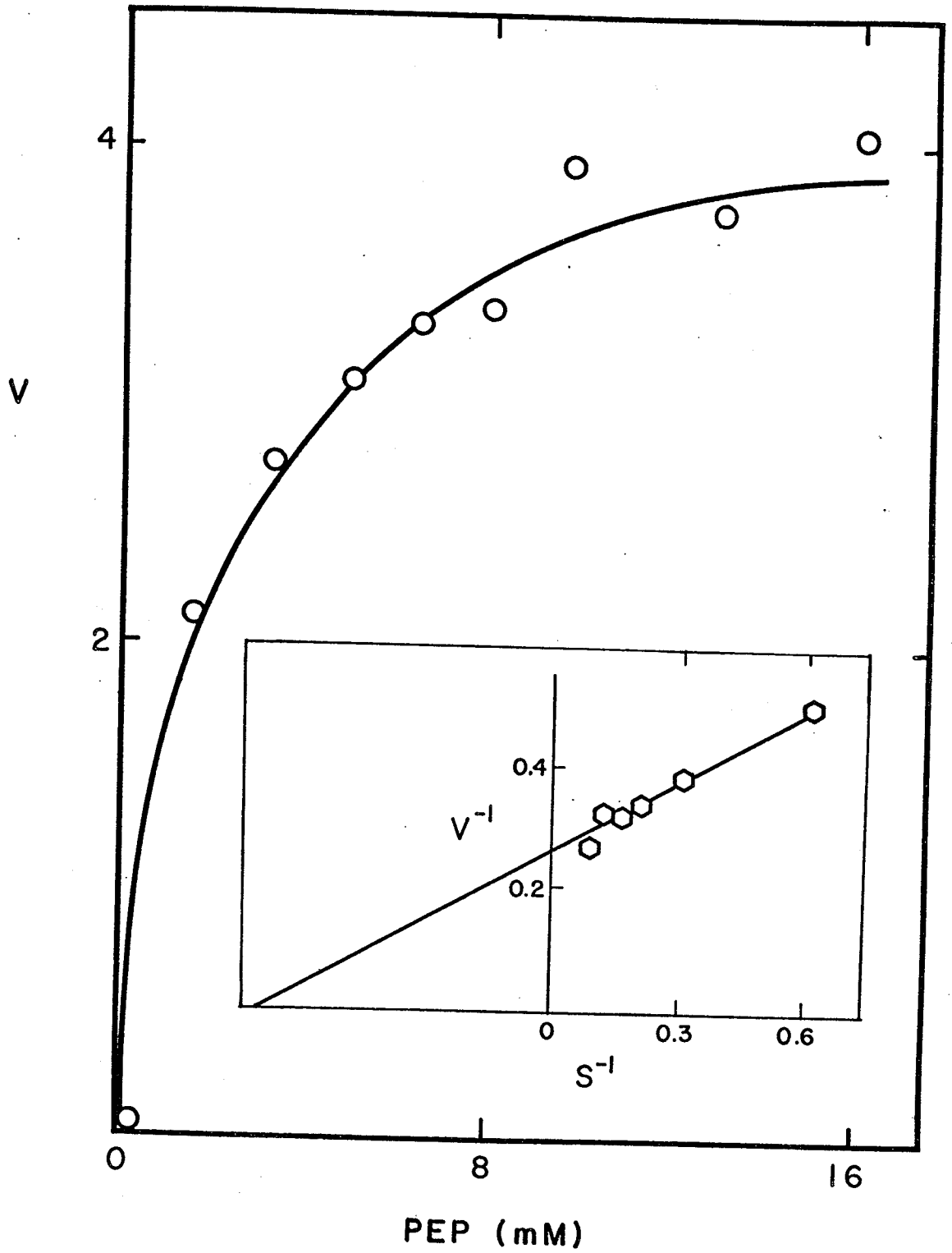

V
4
2
0
8
16
PEP (mM)
0.4
V^{-1}
0.2
0
0.3
0.6
S^{-1}

Figure 37. Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in isolated chloroplasts from maize secondary leaves. Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8, 100; PEP,8; $MgCl_2$, 5; NADH, 2; malate dehydrogenase, excess; 0.6 mg of crude protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$.

Km = 0.27 mM

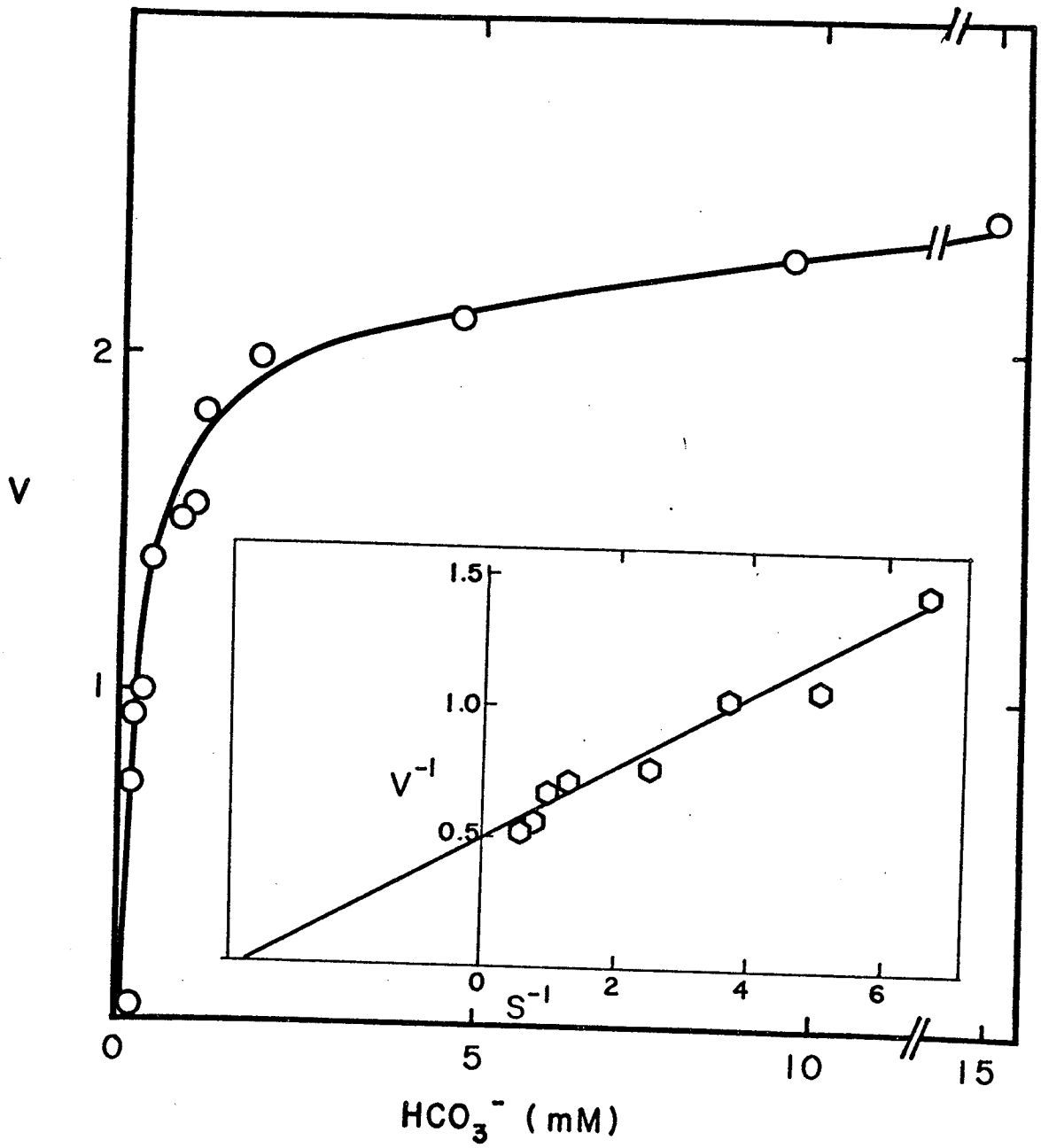

V
2
1
0
5
10
15
HCO_3^- (mM)
1.5
1.0
V^{-1}
0.5
0
S^{-1}
2
4
6

Figure 38. Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in isolated chloroplasts from maize secondary leaves. The reaction systems (0.5 ml) contained in μmoles the following: Tris-HCl pH 7.8, 100; PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); NADH, 2; malate dehydrogenase, excess; 0.6 mg crude protein and varying amounts of $MgCl_2$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $MgCl_2$.

Km = 5.71 mM

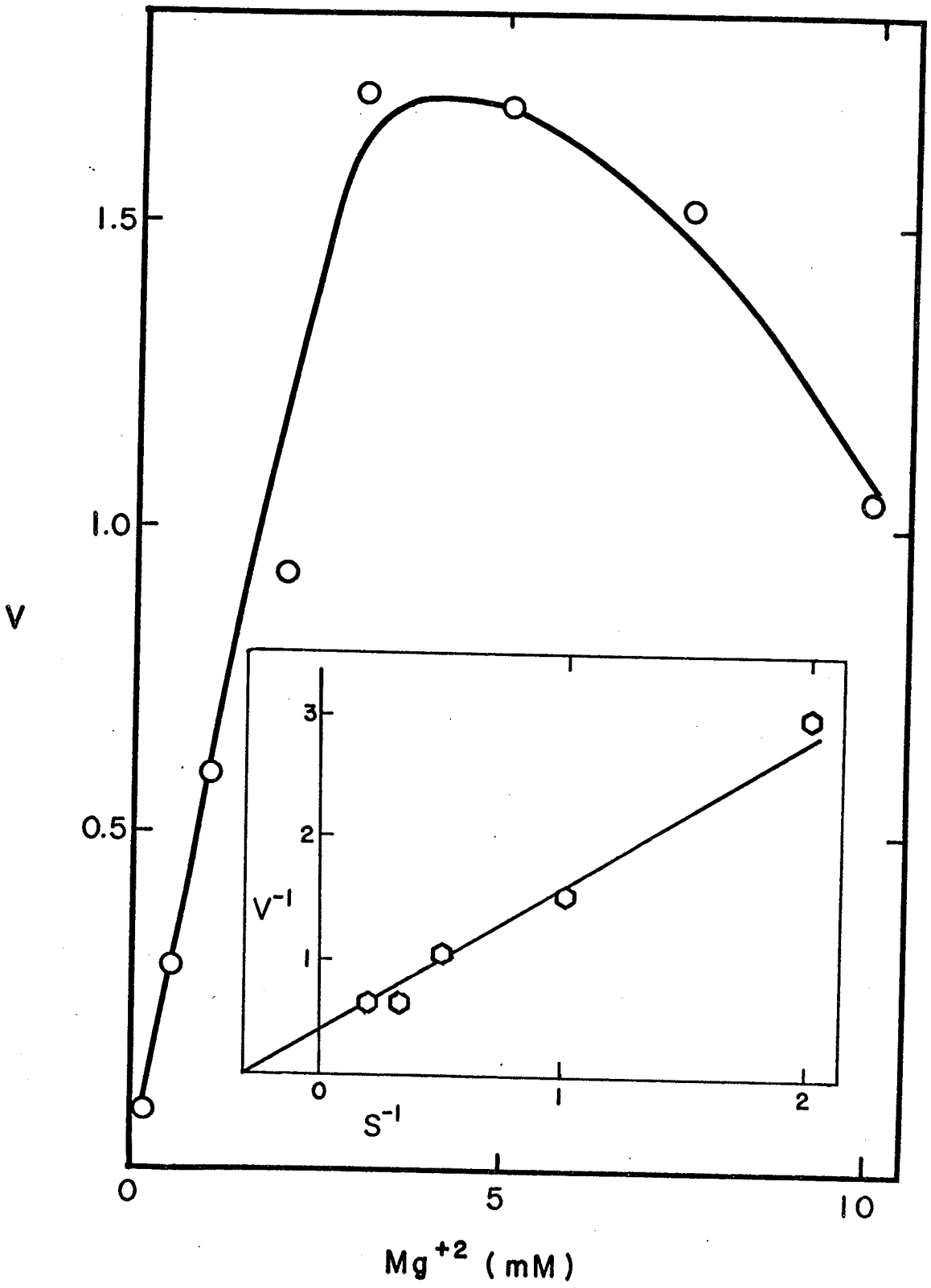

1.5
1.0
0.5
V
0
5
10
Mg+2 (mM)
3
2
V-1
1
0
1
2
S-1

Figure 39. Effect of PEP concentration on the initial velocity of PEP carboxylase in supernatant of maize secondary leaves.
Reaction systems (0.5 ml) contained in μmoles the following: Tris-HCl pH 7.8, 100; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); $MgCl_2$, 5; NADH, 2; malate dehydrogenase, excess; 0.4 mg crude protein and varying amounts of PEP as indicated. v is in μm CO_2/mg protein/hr. S is in mM of PEP.

Km = 3.85 mM

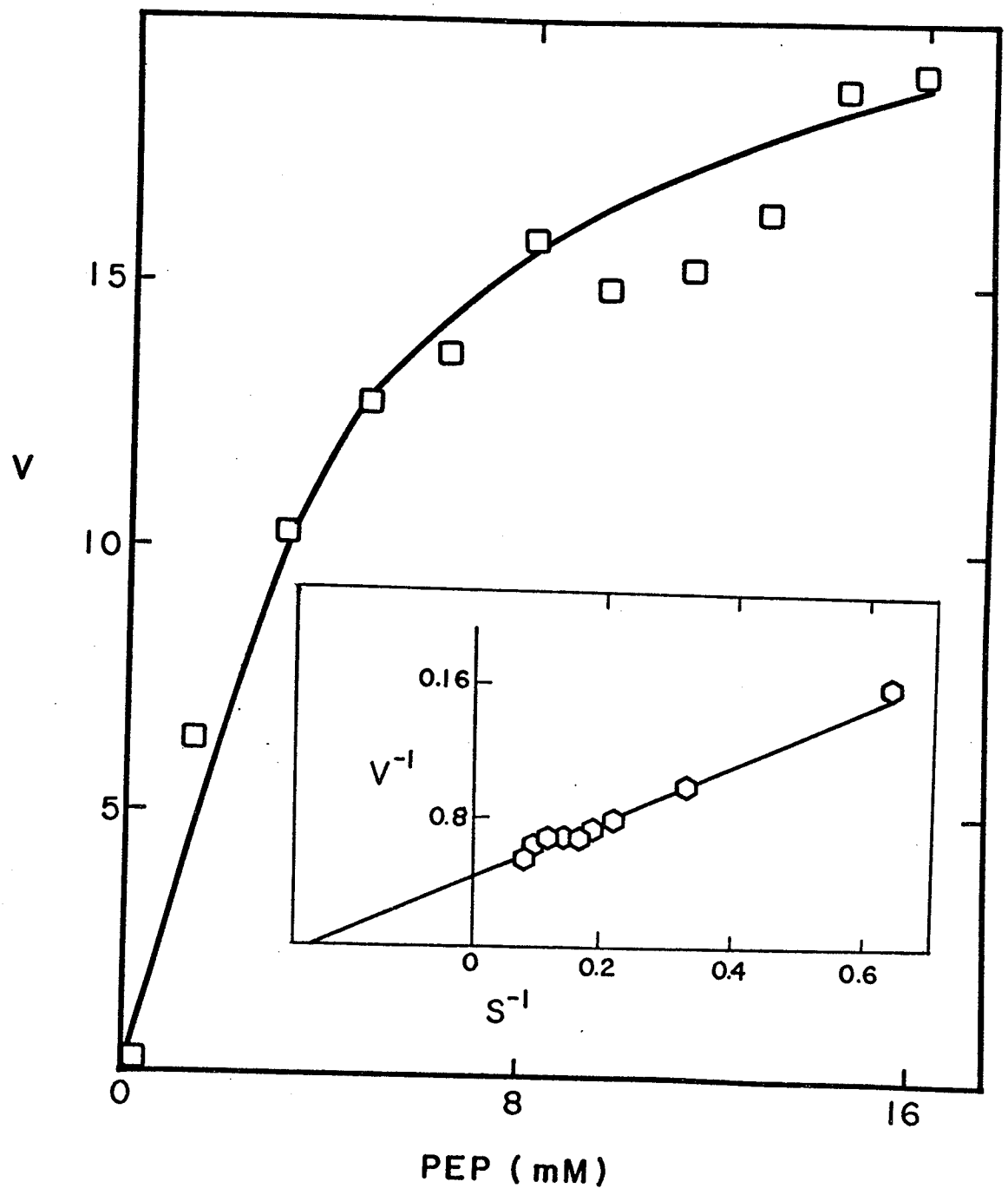

V
15
10
5
0
8
16
PEP (mM)
0.16
V^{-1}
0.8
0
0.2
0.4
0.6
S^{-1}

Figure 40. Effect of sodium bicarbonate concentration on the initial velocity of PEP carboxylase in supernatant of maize secondary leaves. Reaction systems (0.5 ml) contained in μmoles the folllowing: Tris-HCl pH 7.8, 100; PEP, 8; $MgCl_2$, 5; NADH, 2; malate dehydrogenase, excess; 0.4 mg of crude protein and varying amounts of $NaH^{14}CO_3$ as indicated. v is in μm CO_2/mg protein/hr. S is in mM of $NaH^{14}CO_3$.

Km = 1.33 mM

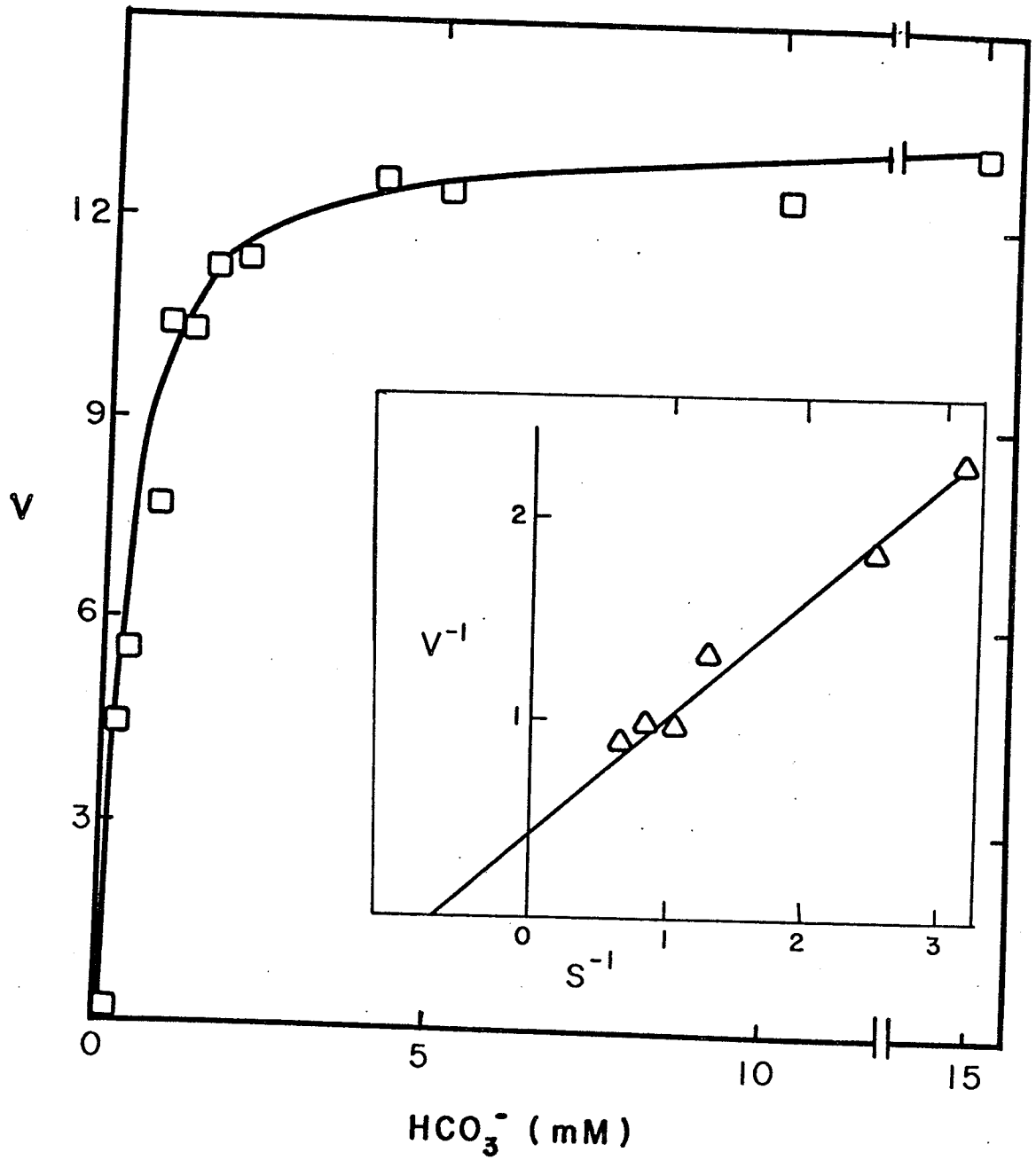

V
12
9
6
3
0
5
10
15
HCO_3^- (mM)
V^{-1}
2
1
0
1
2
3
S^{-1}

Figure 41. Effect of magnesium chloride concentration on the initial velocity of PEP carboxylase in supernatant of maize secondary leaves.
Reaction systems (0.5 ml) contained in μmoles of the following: Tris-HCl pH 7.8; PEP, 8; $NaH^{14}CO_3$, 25 (>6 x 10^6 dpm); NADH, 2; malate dehydrogenase, excess; 0.4 mg of crude protein and varying amounts of $MgCl_2$ as indicated. v is in μm CO_2/ mg protein/hr. S is in mM of $MgCl_2$.

Km = 0.21 mM

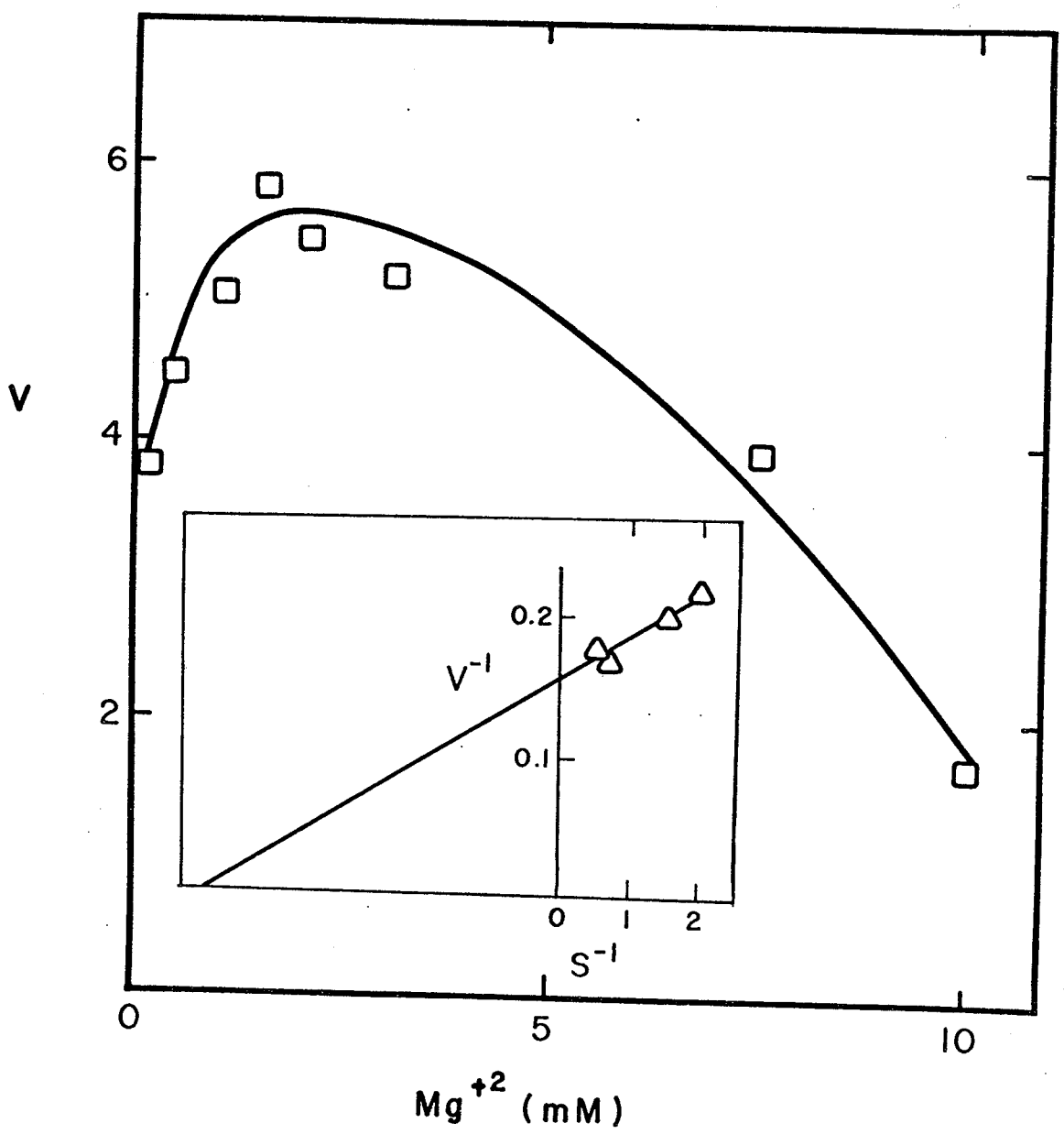

V
6
4
2
0
5
10
Mg^{+2} (mM)
0.2
0.1
V^{-1}
0
1
2
S^{-1}

Figure 42. Effect of incubation temperature on PEP carboxylase and RuDP carboxylase activities in primary leaf cellular fractions of maize grown under 25°C day and 20°C night temperatures.

a. Isolated Chloroplasts

○ = PEP carboxylase activity

⬡ = RuDP carboxylase activity

b. Supernatant

□ = PEP carboxylase activity

△ = RuDP carboxylase activity

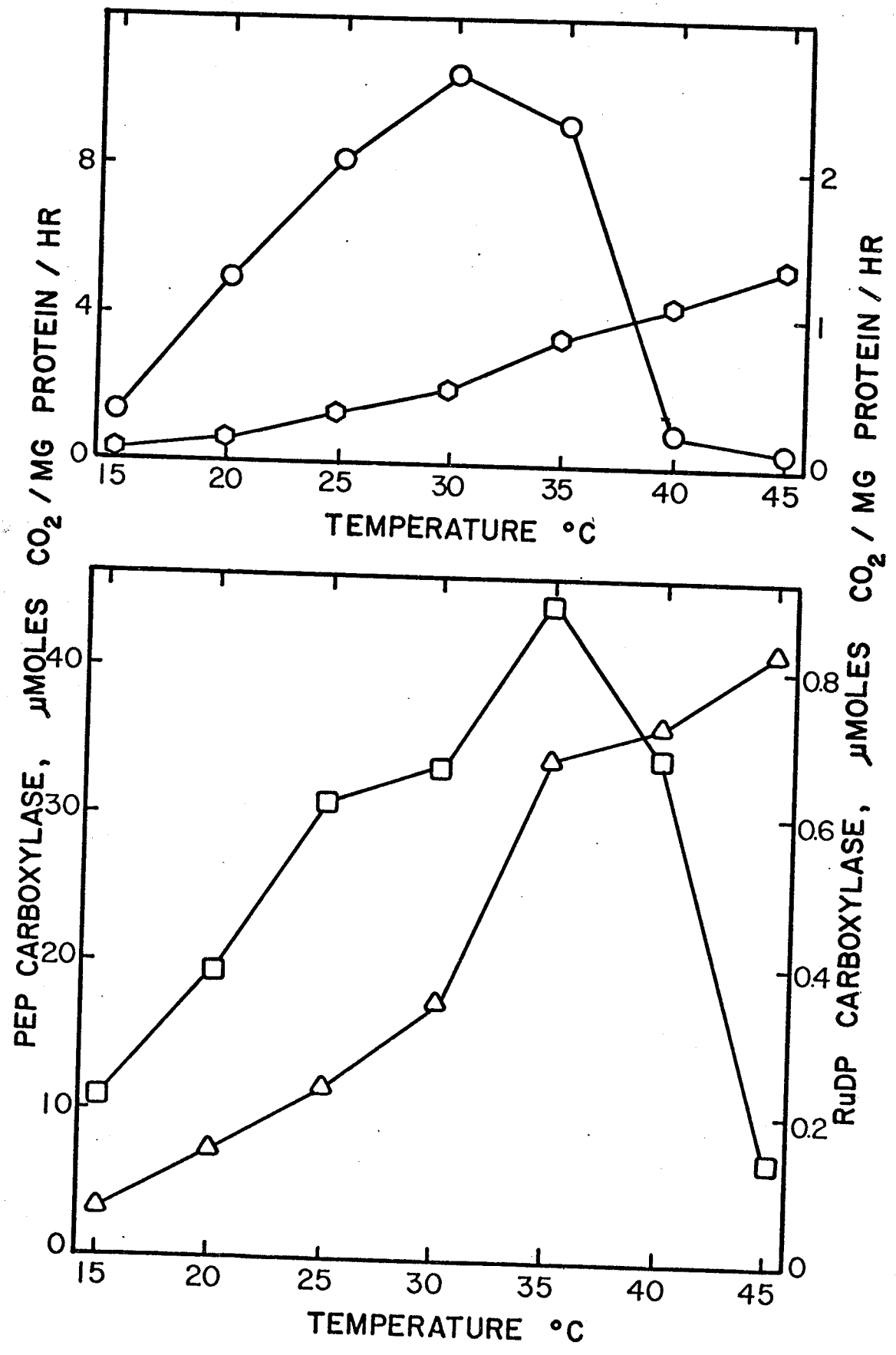

8
4
0
2
1
0
15
20
25
30
35
40
45
TEMPERATURE °C
PEP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
RuDP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
40
30
20
10
0
0.8
0.6
0.4
0.2
0
15
20
25
30
35
40
45
TEMPERATURE °C

Figure 43. Effect of incubation temperature on PEP carboxylase and RuDP carboxylase activities in primary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.

a. Isolated chloroplasts

○ = PEP carboxylase activity

⬡ = RuDP carboxylase activity

b. Supernatant

□ = PEP carboxylase activity

△ = RuDP carboxylase activity

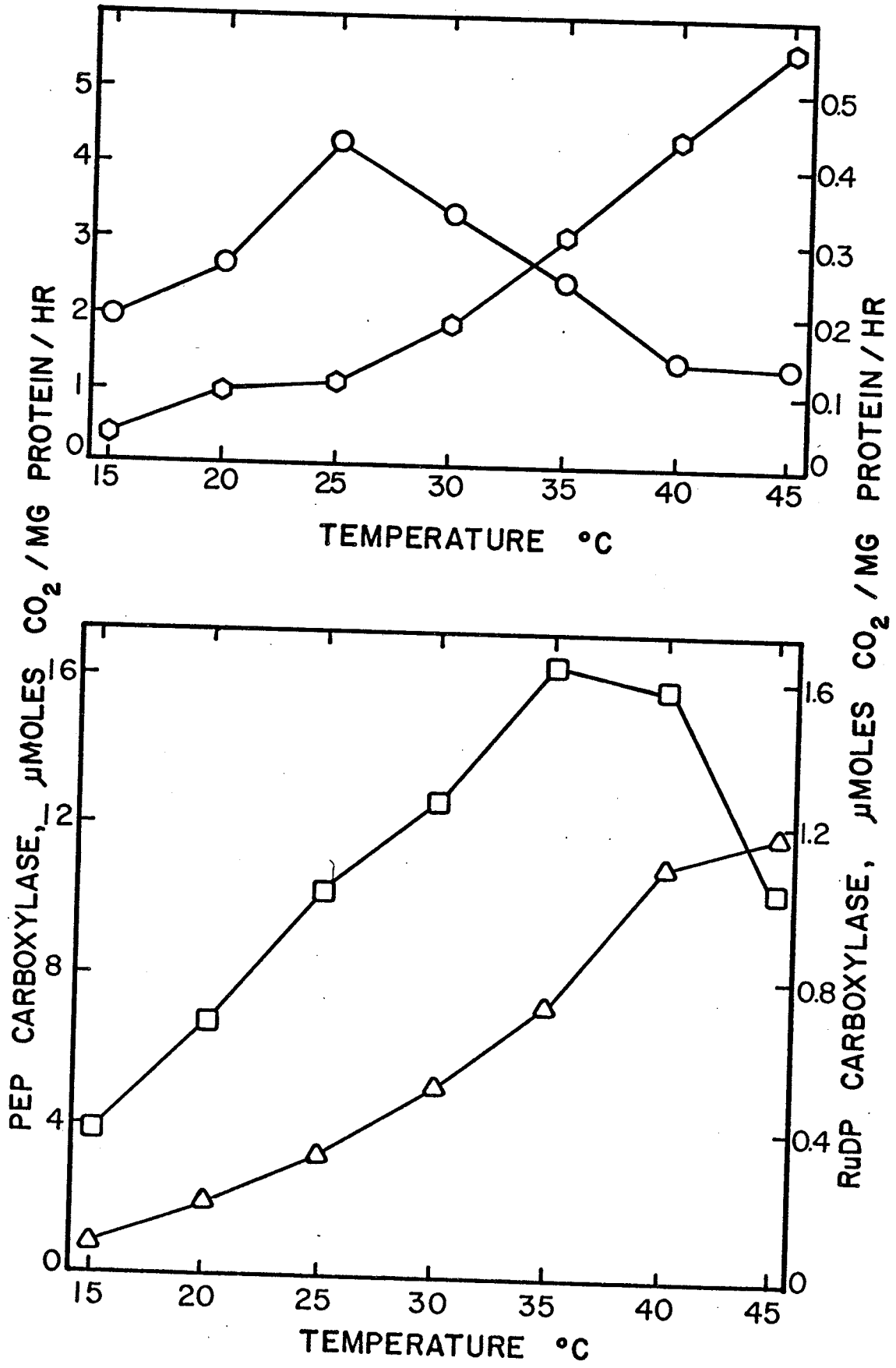

PEP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
RuDP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
TEMPERATURE °C
TEMPERATURE °C

Figure 44. Effect of incubation temperature on PEP carboxylase and RuDP carboxylase activities of secondary leaf cellular fractions of maize grown under 25°C day and 20°C night temperatures.

a. Isolated Chloroplasts

○ = PEP carboxylase activity

⬡ = RuDP carboxylase activity

b. Supernatant

□ = PEP carboxylase activity

△ = RuDP carboxylase activity

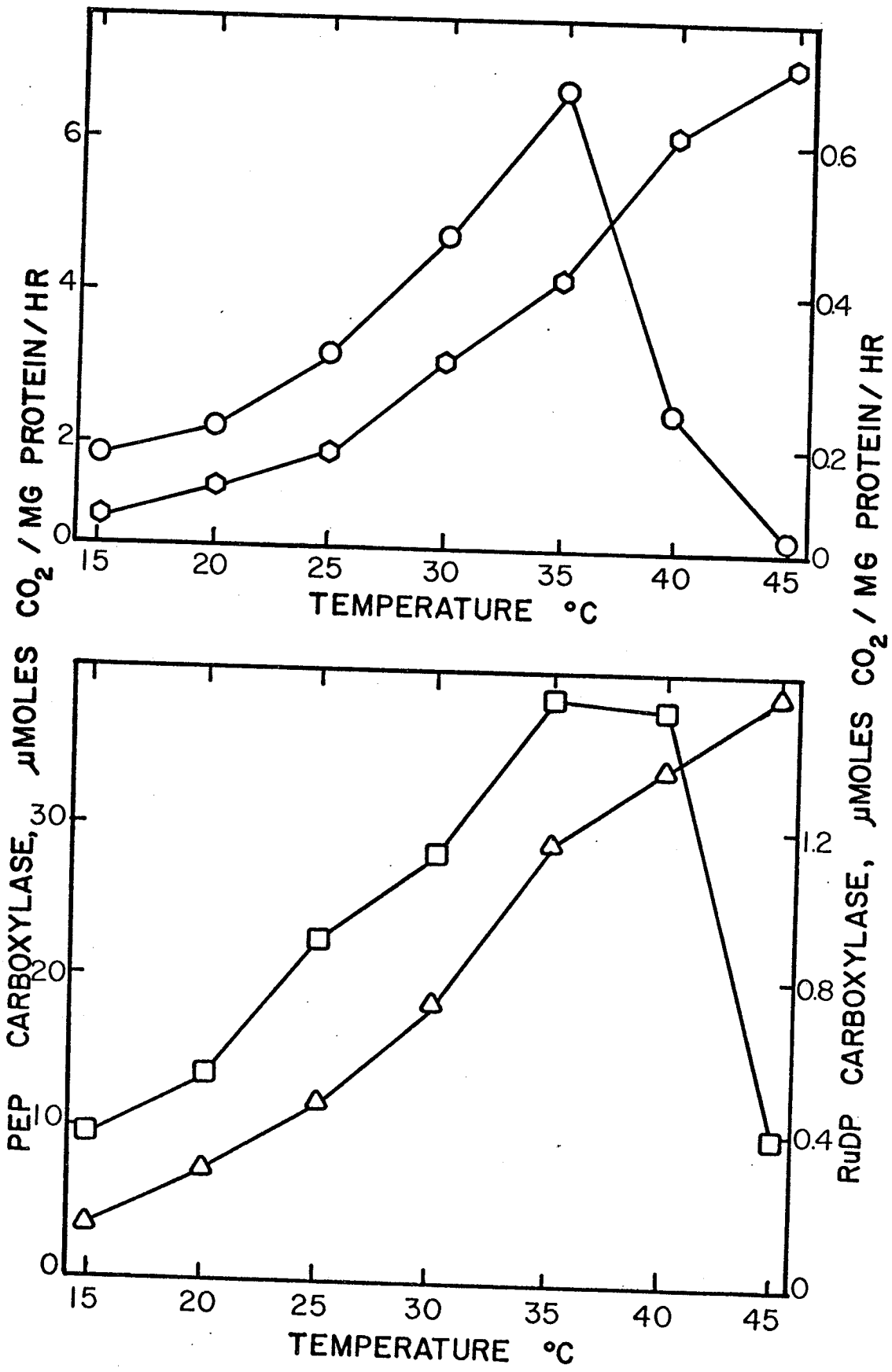

PEP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
RuDP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
TEMPERATURE °C
TEMPERATURE °C
0
2
4
6
0.2
0.4
0.6
10
20
30
0.4
0.8
1.2
15
20
25
30
35
40
45

Figure 45. Effect of incubation temperature on PEP carboxylase and RuDP carboxylase activities of secondary leaf cellular fractions of maize grown under 15°C day and 10°C night temperatures.

a. Isolated Chloroplasts

○ = PEP carboxylase activity

⬡ = RuDP carboxylase activity

b. Supernatant

□ = PEP carboxylase activity

△ = RuDP carboxylase activity

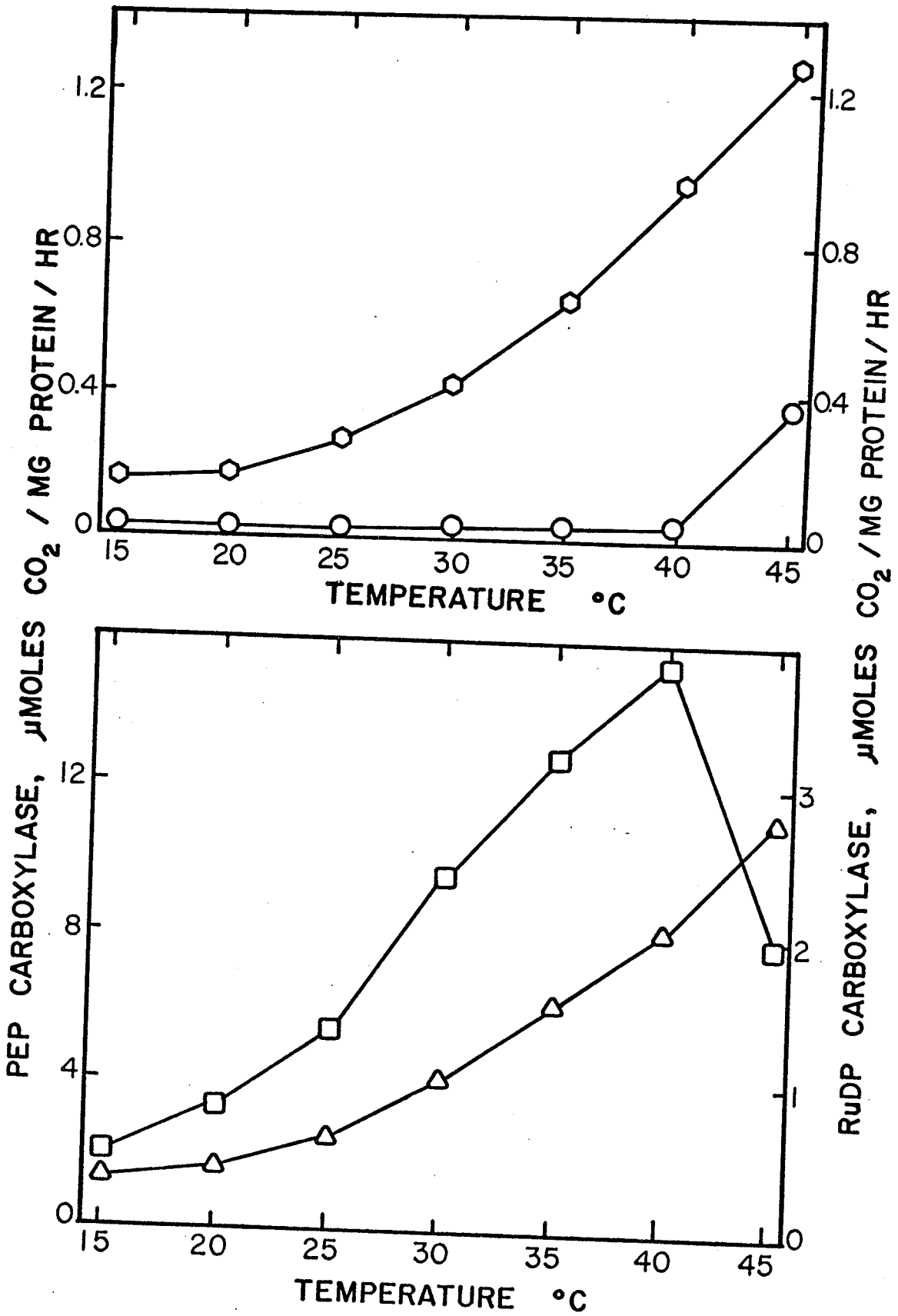

1.2
0.8
0.4
0
15
20
25
30
35
40
45
TEMPERATURE °C
PEP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
RuDP CARBOXYLASE, µMOLES CO2 / MG PROTEIN / HR
12
8
4
0
3
2
1

Plate 1. Sample radioautogram of the products in the organic acid fraction of leaf disc extracts.

ISL = Isocitrate lactone
MAL = Malate
PGA = 3-phosphoglyceric acid
SMP = Sugar monophosphates
SDP = Sugar diphosphates

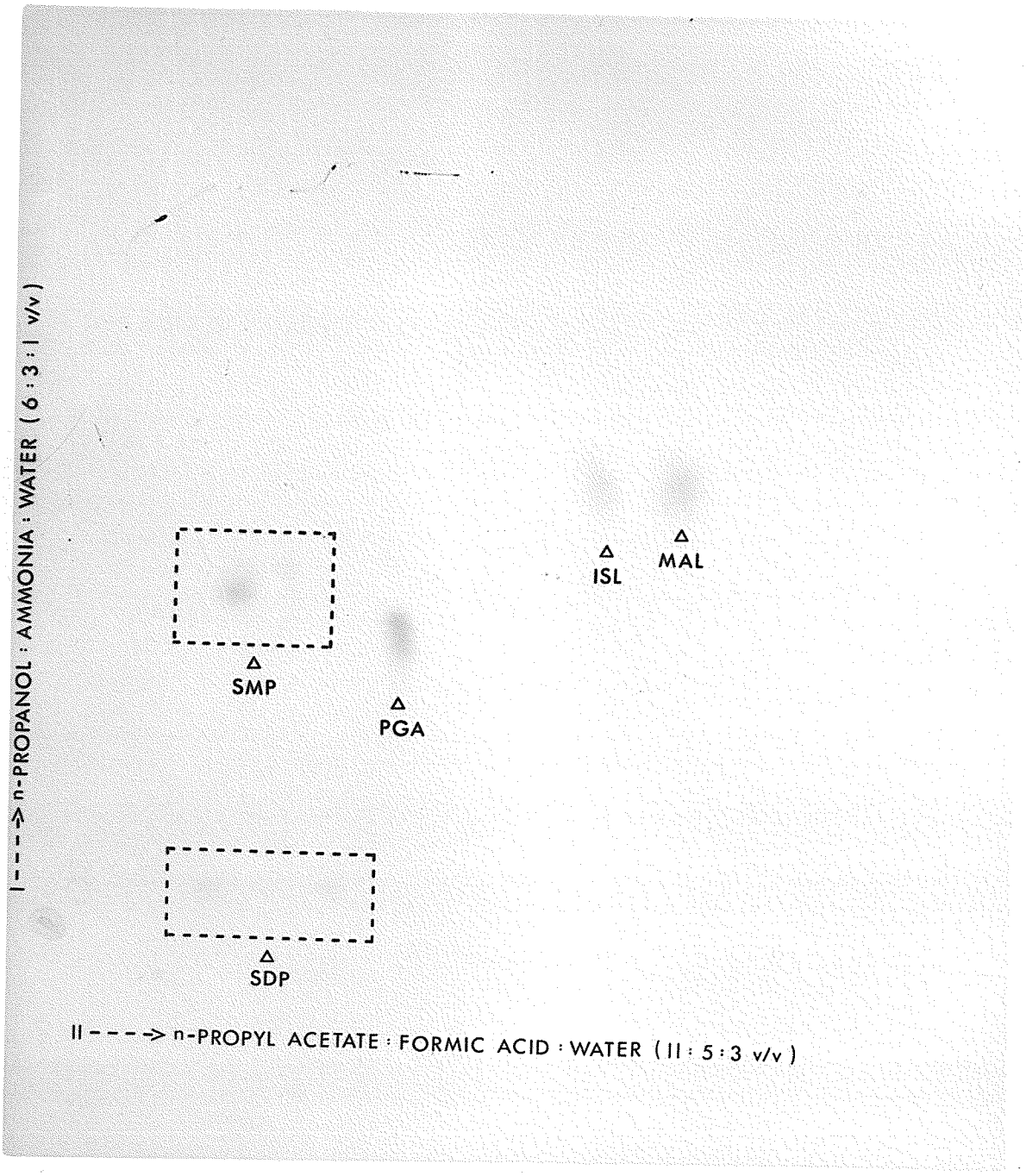

I - - - -> n-PROPANOL : AMMONIA : WATER (6 : 3 : 1 v/v)
ISL
MAL
SMP
PGA
SDP
II - - - -> n-PROPYL ACETATE : FORMIC ACID : WATER (11 : 5 : 3 v/v)

Plate 2. Sample radioautogram of the products in the amino acid fraction of leaf disc extracts.

ALA = Alanine
ASP = Aspartate
GLU = Glutamate
GLY = Glycine
SER = Serine
UN = Unidentified

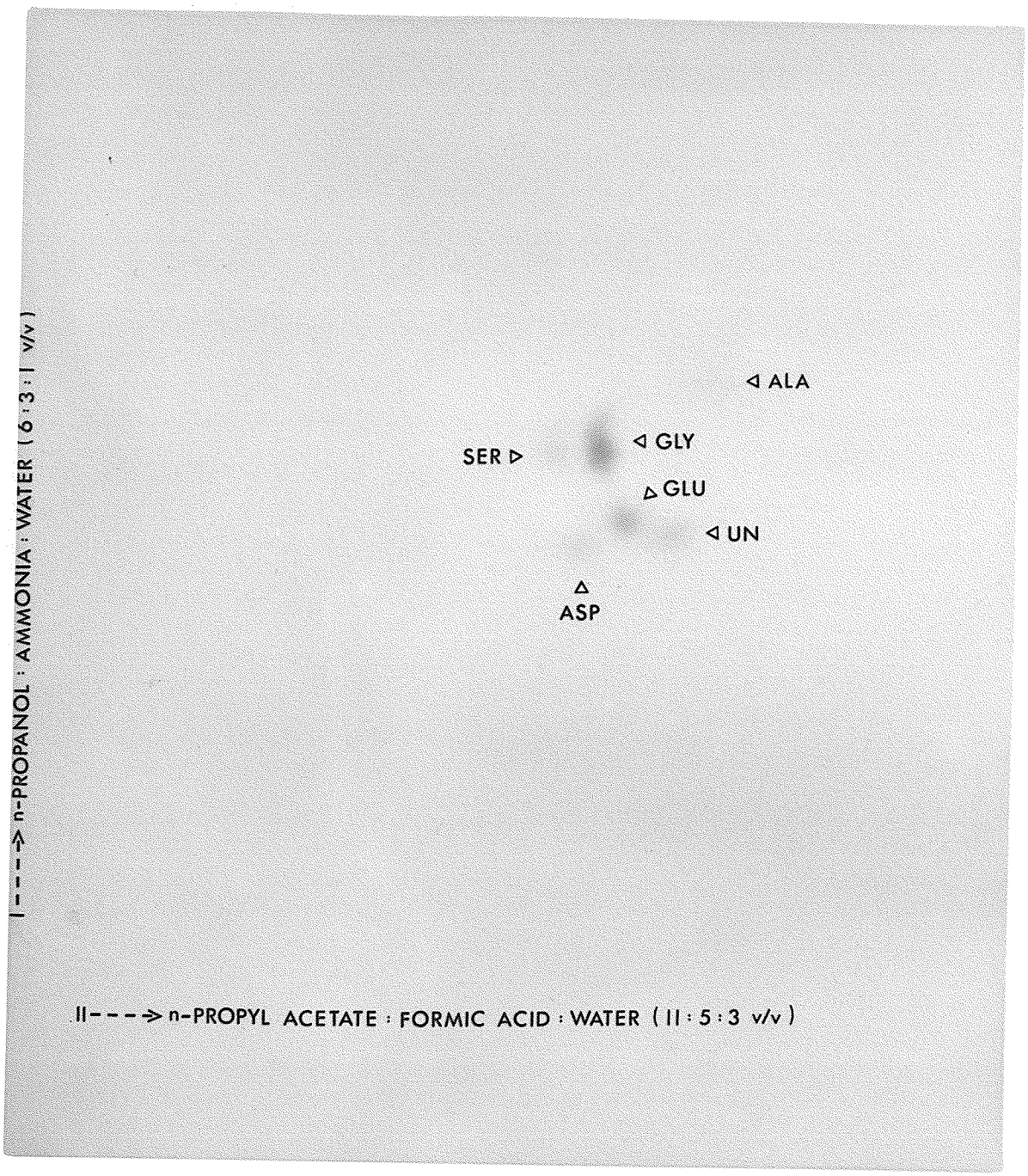

I - - - -> n-PROPANOL : AMMONIA : WATER (6 : 3 : 1 v/v)
II - - - -> n-PROPYL ACETATE : FORMIC ACID : WATER (11 : 5 : 3 v/v)
ALA
GLY
SER
GLU
UN
ASP

Plate 3. Sample radioautogram of the products in the sugar fraction of leaf disc extracts.

GLU = Glucose
FRU = Fructose
SUC = Sucrose

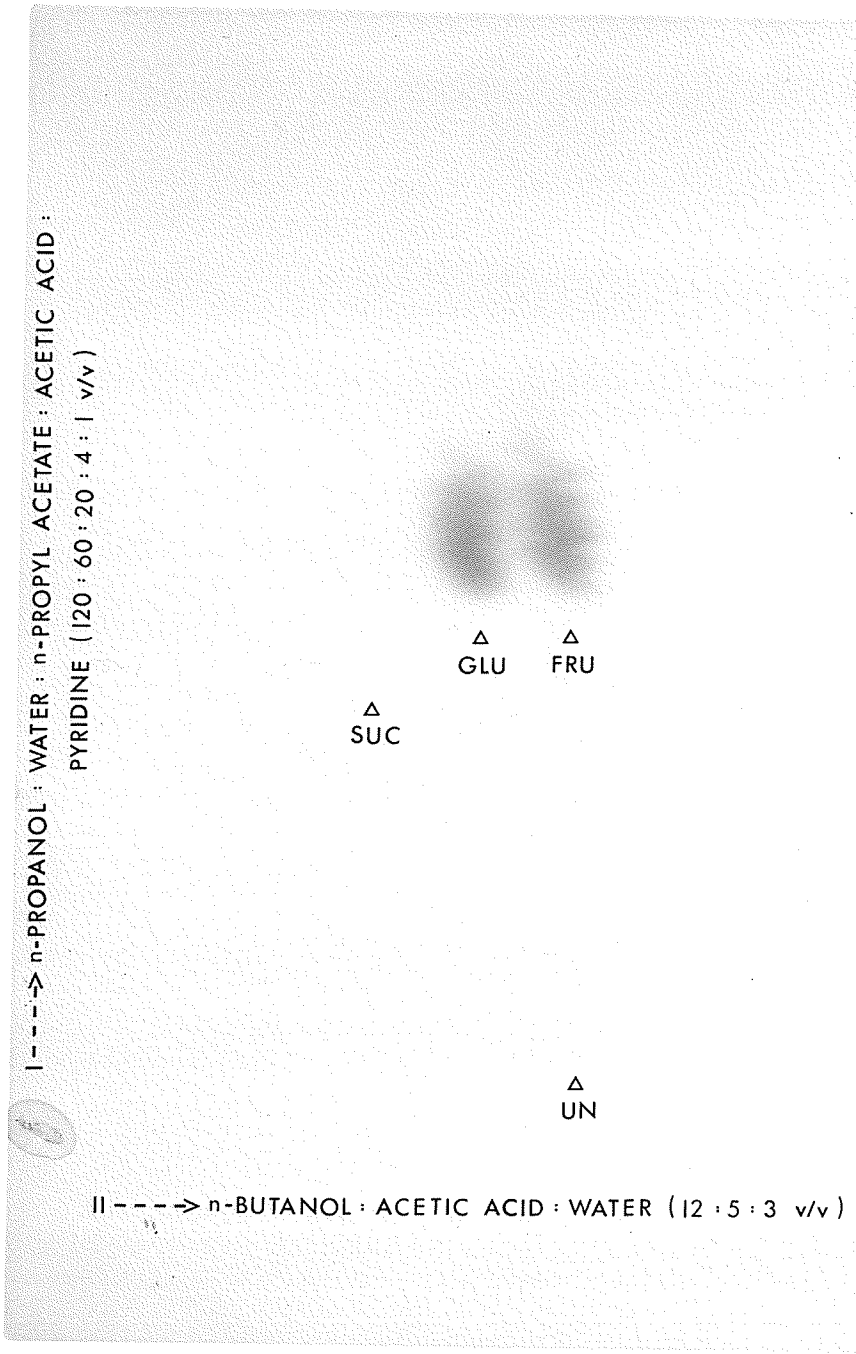

I - - - -> n-PROPANOL : WATER : n-PROPYL ACETATE : ACETIC ACID :
PYRIDINE (120 : 60 : 20 : 4 : 1 v/v)
GLU
FRU
SUC
UN
II - - - -> n-BUTANOL : ACETIC ACID : WATER (12 : 5 : 3 v/v)

Plate 4. Sample radioautogram of the products in the organic acid fraction of chloroplast extracts.

GLYCE = Glycerate
GLYCO = Glycolate
SMP = Sugar monophosphates

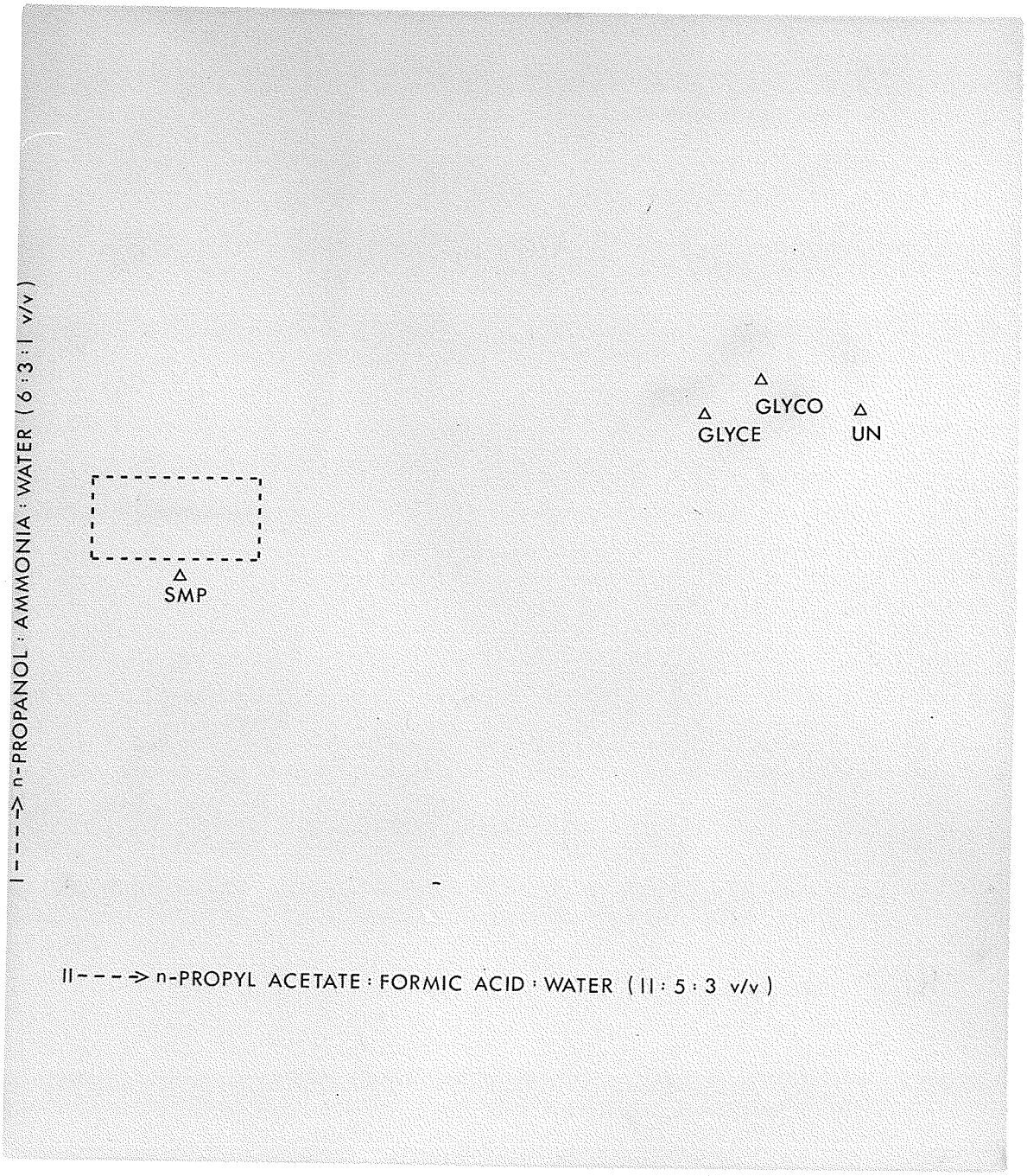

I - - - -> n-PROPANOL : AMMONIA : WATER (6 : 3 : 1 v/v)
II - - - -> n-PROPYL ACETATE : FORMIC ACID : WATER (11 : 5 : 3 v/v)
SMP
GLYCO
GLYCE
UN

Plate 5. Sample radioautogram of the products in the amino acid fraction of chloroplast extracts.

ASP = Aspartate

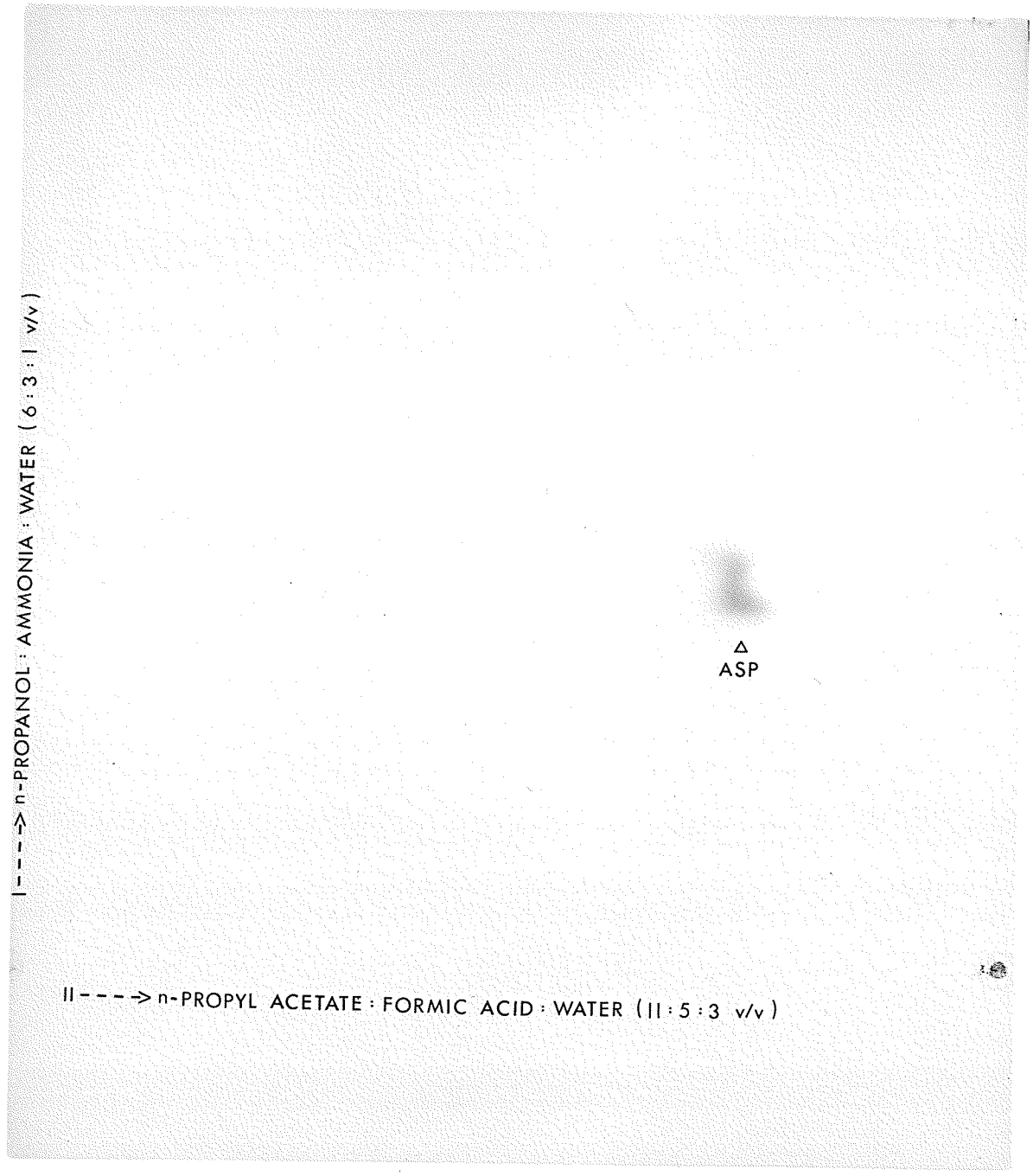
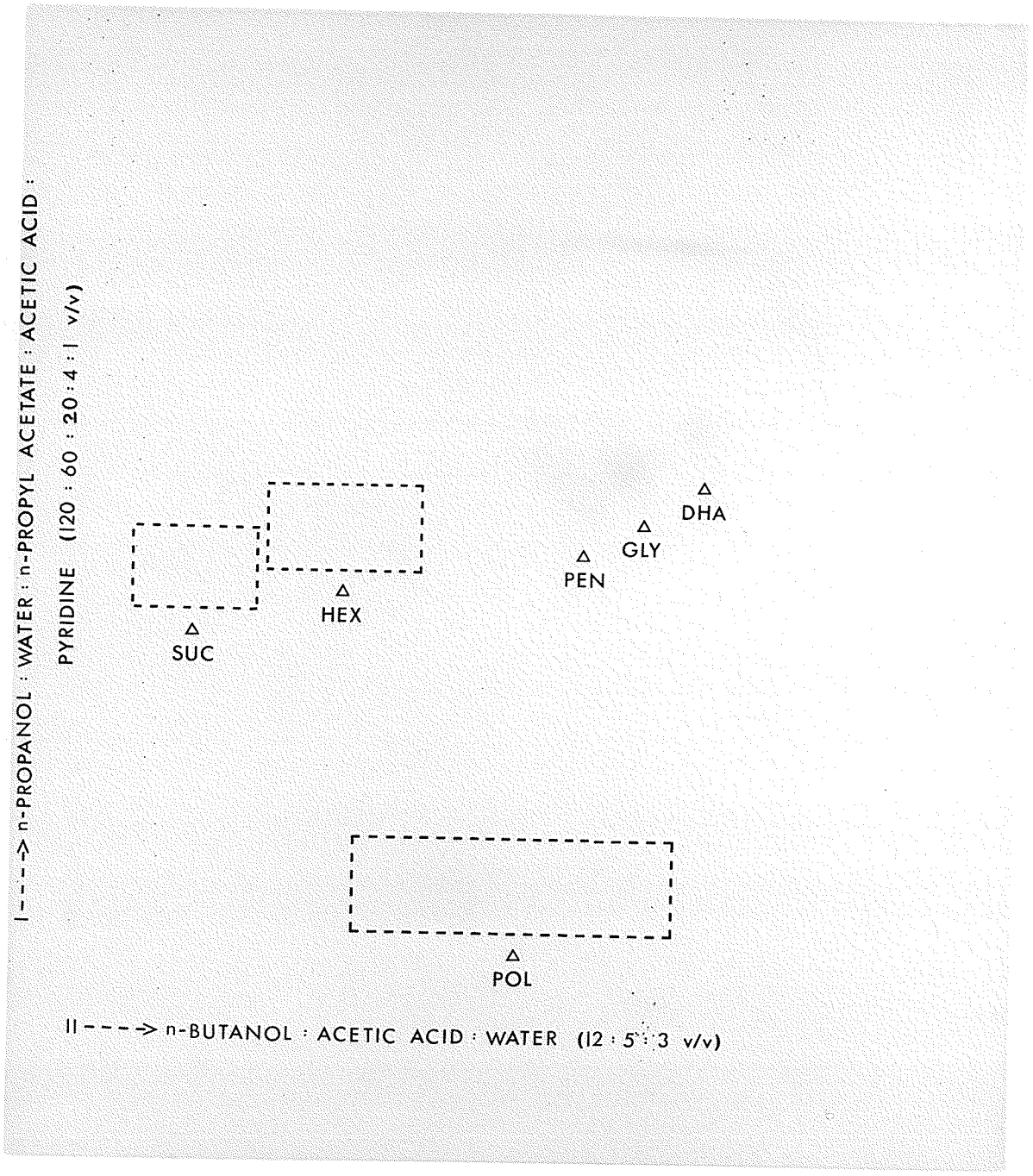

I - - - -> n-PROPANOL : AMMONIA : WATER (6 : 3 : 1 v/v)
II - - - -> n-PROPYL ACETATE : FORMIC ACID : WATER (11 : 5 : 3 v/v)
△
ASP

Plate 6. Sample radioautogram of the products in the sugar fraction of chloroplast extracts.

DHA = Dihydroxyacetone
GLY = Glyceraldehyde
PEN = Pentose
HEX = Hexoses
SUC = Sucrose
POL = Polysaccharide


I ----> n-PROPANOL : WATER : n-PROPYL ACETATE : ACETIC ACID :
PYRIDINE (120 : 60 : 20 : 4 : 1 v/v)
SUC
HEX
PEN
GLY
DHA
POL
II ----> n-BUTANOL : ACETIC ACID : WATER (12 : 5 : 3 v/v)