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The metabolism of [illegible] and bicarbonate in isolated bundle sheath cells of Zea mays L. and Sorghum bicolor L.

University — Université

University of Alberta

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

M.Sc. ([illegible] Botany)

Year this degree conferred — Année d'obtention de ce grade

Name of Supervisor — Nom du directeur de thèse

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NL-91 (4/77)

THE UNIVERSITY OF ALBERTA

The metabolism of glycollate and bicarbonate in isolated bundle sheath cells of *Zea mays* L. and *Sorghum bicolor* L.

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

PLANT BIOCHEMISTRY

Department of Botany

EDMONTON, ALBERTA

Fall 1983

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR Rickey Andrew Scott

TITLE OF THESIS The metabolism of glycollate and bicarbonate in isolated bundle sheath cells of *Zea mays* L. and *Sorghum bicolor* L.

DEGREE FOR WHICH THESIS WAS PRESENTED MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED Fall 1983

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The metabolism of glycollate and bicarbonate in isolated bundle sheath cells of *Zea mays* L. and *Sorghum bicolor* L. submitted by Rickey Andrew Scott in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in PLANT BIOCHEMISTRY.

Edwin A. Cossins

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David W. Ow

Date Sept 14 1983

Abstract

Metabolic studies were undertaken to examine the glycollate pathway in C_4 plants. *Zea mays* L. and *Sorghum bicolor* L. were grown at 25 °C under a 12 hour day of 200 uE m^{-2} s^{-1} in a controlled environment chamber. Excised leaves of corn, pretreated for 4.5 hours with 20mM aminoacetonitrile (AAN), an inhibitor of glycine decarboxylase and serine hydroxymethyl transferase, were incubated for 2 min in air containing $^{14}CO_2$. Ion exchange, thin layer, and high performance liquid chromatography were used to identify the labelled products. The labelling pattern suggested that a glycollate pathway operates in corn. The incorporation by inhibitor-treated leaves was only 60% of control tissues. The percentage of total label incorporated into glycine and glycollate increased from 4.8% to 10% and 0.8% to 6.4% respectively. Serine labelling decreased from 4% to 0.8%. Bundle sheath cells of both corn (midrib removed) and sorghum (intact) leaves were isolated using a combined cellulase-grinding method (Chapman et al., 1980). Cell suspensions, pretreated for 15 minutes in 20mM AAN, were supplied 0.11 mM $NaH^{14}CO_3$ and 10mM malate for 10 minutes in the light. The inhibitor reduced incorporation of bicarbonate in corn by 8% and in sorghum by 64%. In inhibitor-treated cells, the amount of glycine-^{14}C was double that of the control, but only a minimal decrease in serine labelling occurred. Glycine labelling was also substantially increased in sorghum bundle sheath cells but serine-^{14}C was not detected in either the control or AAN-treated cells.

Glycollate-2-^{14}C was also supplied to illuminated bundle sheath cells. A 15 minute pretreatment with 20mM AAN reduced the amount of glycollate metabolized. The major products of glycollate metabolism in both species were glyoxylate, glycine and formate. No ^{14}C was detected in serine or in intermediates of the glycerate pathway. AAN treatment unexpectedly reduced the amount of label incorporated into glycine, both in absolute amount and as a percentage of total glycollate metabolized. It is suggested that the inhibitor reduced the availability of amino groups needed for glycine synthesis. Glycollate-2-^{14}C supplied to bundle sheath cells with added serine (1mM) resulted in an increase in labelled glycine. No serine labelling was detected in sorghum with added serine but a small amount was detected in corn (0.5% of the total metabolized). The production of formate-^{14}C from glycollate-2-^{14}C and the absence of serine-^{14}C indicated that C-2 of glycollate was not flowing through a glycollate-glycerate pathway in these isolated bundle sheath cells.

Formate production increased in AAN-treated cells of sorghum. This implied that a direct decarboxylation of glyoxylate was occurring. In contrast, formate production was decreased in AAN-treated corn bundle sheath cells. This result suggested that some formate arose via glycine decarboxylation. Methylene tetrahydrofolic acid is suggested as a possible precursor of formate in this system. The absence of serine labelling from glycollate-2-^{14}C in corn bundle sheath cells may be due to rapid removal of methylene tetrahydrofolic acid for formate production.

The results of the present study and published data are discussed in relation to a glycollate pathway in C$_4$ species.

Acknowledgements

I am indebted to Dr. E.A. Cossins for allowing me the opportunity to work in his lab and for his guidance and help in the preparation of this thesis. I would also like to thank Mr. Ben Blawacky for the amino acid analyses he performed and Mrs. Wilda Yuill and Miss Helena Imeson for their help in typing the manuscript. A warm appreciation is extended to those in the lab and in the department with whom I had many enjoyable and varied discussions during the course of my studies.

This work was supported by funds awarded to Dr. E.A. Cossins by the Natural Sciences and Engineering Research Council of Canada.

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AAN	aminoacetonitrile
dpm	disintegrations per minute
HBA	hydroxy-2,3-butynoic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HPLC	high performance liquid chromatography
INH	isonicotinylhydrazide
a-HPMS	*a*-hydroxy-2-pyridinemethane sulfonate
MSO	methionine sulfoximine
MHB	methyl-2-hydroxy-3-butynoate
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
PEP	phospoenolpyruvate
3-PGA	3-phosphoglycerate
RuBP	ribulose-1,5-bisphosphate
THFA	tetrahydrofolic acid
TLC	thin layer chromatography
Tricine	N-tris[hydroxymethyl]methyl glycine
Tris	Tris (hydroxymethyl) aminomethane

I. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39) is the enzyme found in all photosynthetic plants responsible for fixing CO_2 before it can be reduced to carbohydrate in the Calvin cycle, also known as the photosynthetic carbon reduction cycle (Jenson and Bahr, 1977). In addition to the carboxylation reaction, this dually active enzyme catalyzes an oxygenation reaction. One of the products of the oxygenation reaction is the toxic compound phosphoglycollate. Plants contain a specific phosphatase (phoshoglycollate phosphatase, E.C. 3.1.3.18), the action of which results in glycollate production (Halliwell, 1978). This glycollate is then metabolized via the glycollate pathway. It is the competition by O_2 with CO_2 that is thought to be the major source of glycollate in plants (Lorimer, 1981). In C_4 plants, it has been suggested that a CO_2 concentrating mechanism reduces the oxygenation reaction and thus also reduces the formation of glycollate (Hatch and Osmond, 1976). However, the enzymes associated with the glycollate pathway are found in this group of plants (Halliwell, 1978). It is the intention of this thesis to examine the potential role of the glycollate pathway in C_4 plants by reviewing the literature on this subject and by presenting new experimental results that pertain to this question.

The C_3 and C_4 Classification of Plants

The two categories of photosynthetic plants known as C_3 and C_4 are distinguished by the nature of their primary carboxylation products. The first major product of C_3 metabolism is the 3-carbon compound, 3-phosphoglyceric acid. As illustrated in fig. 1, this compound arises when one molecule of ribulose-1,5-bisphosphate (RuBP) and one of CO_2 combine to form two molecules of 3-phosphoglyceric acid. C_4 plants also use RuBP carboxylase/oxygenase in assimilating CO_2, but have a preliminary carboxylation reaction resulting in a 4-carbon acid. The enzyme responsible for this reaction is phosphoenolpyruvate carboxylase (E.C. 4.1.1.31). Carbon dioxide and phosphoenolpyruvate (PEP) combine to form oxaloacetic acid. This 4-carbon acid is converted to either malate or aspartate depending on the C_4 species, before it is decarboxylated to release the CO_2 that is utilized by RuBP carboxylase. An anatomical manifestation, termed Kranz anatomy, facilitates this complex flow of carbon (see fig. 2).

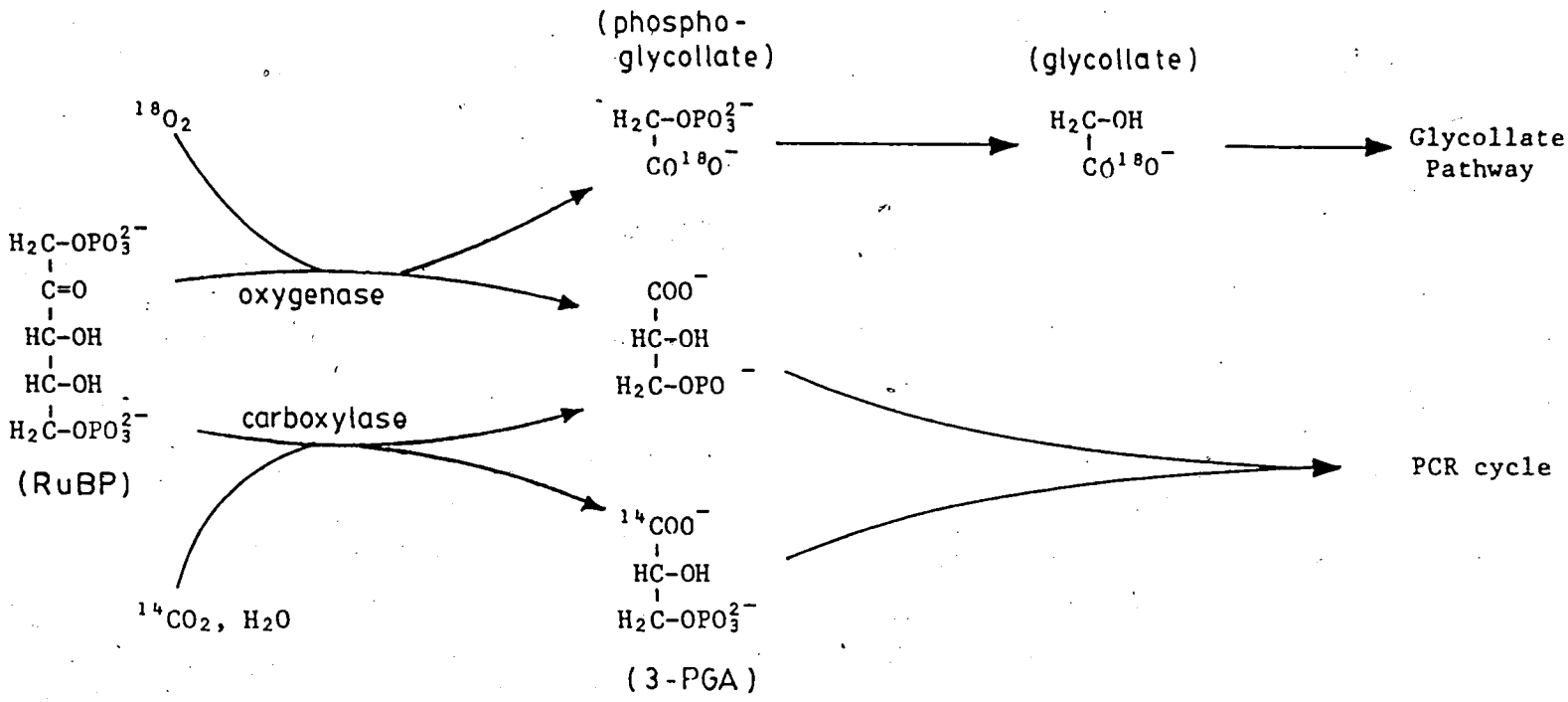


Fig. 1 The dual action of RuBP carboxylase/oxygenase.
(Adapted from Jensen and Bahr (1977))

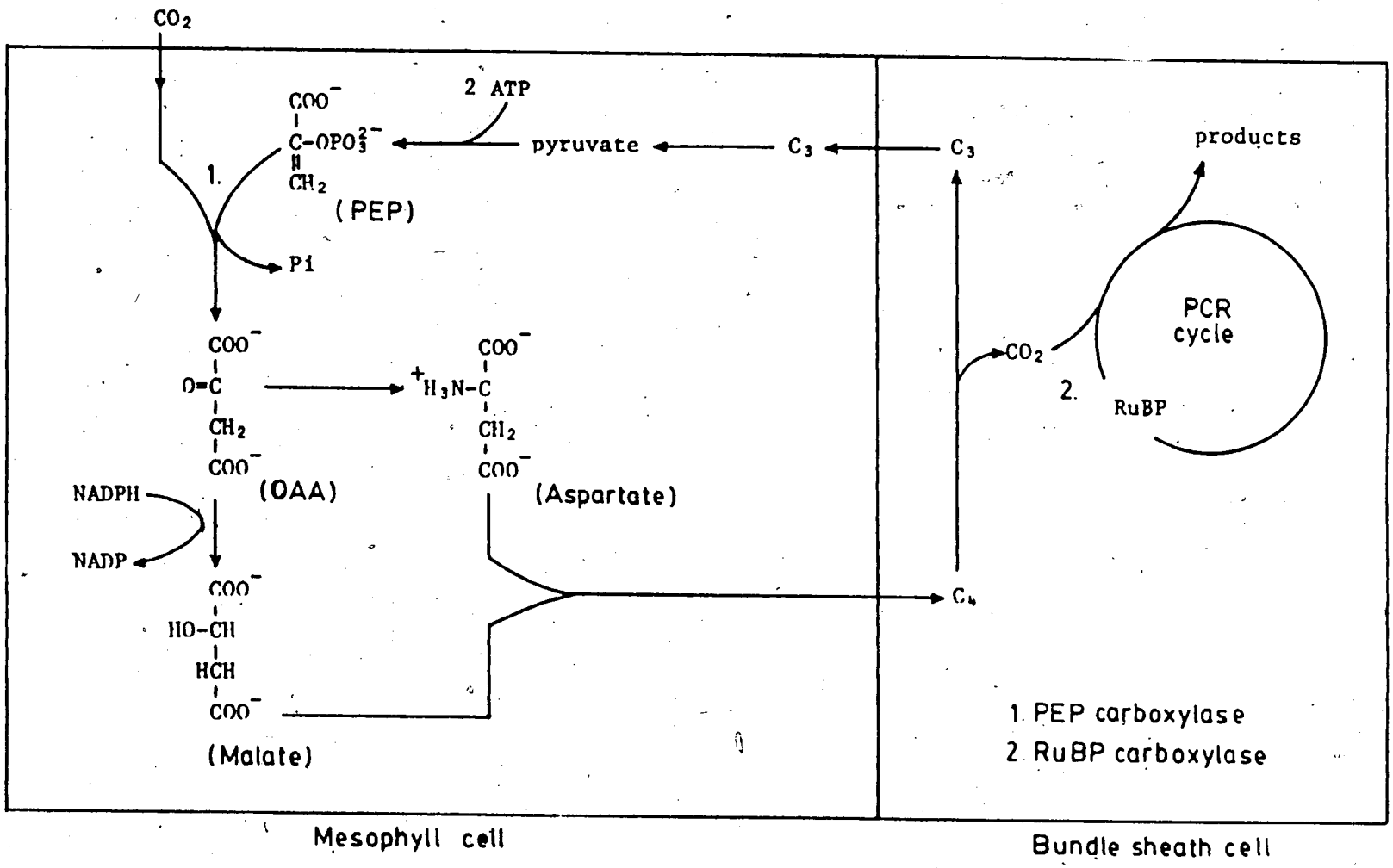


Fig 2 Illustrating the compartmentation of the two carboxylation enzymes and the cyclic nature of the C_4 pathway. (Adapted from Edwards and Huber 1981)

It is clear that there are two types of photosynthetic cells in C_4 plants. The bundle sheath cells form a circle or wreath around the vascular strands. In addition, the mesophyll cells encircle the bundle sheath cells and have conspicuous intercellular air spaces. In C_4 plants, mesophyll cells contain PEP carboxylase whereas the bundle sheath cells contain RuBP carboxylase (Edwards and Huber, 1981). Carbon dioxide, fixed by PEP carboxylase in the mesophyll cells, is transported as malate or aspartate into the bundle sheath cells where decarboxylation occurs releasing the CO_2 that is refixed by RuBP carboxylase. The photosynthetic carbon reduction cycle which operates in all photosynthetic cells of C_3 plants, only operates in the bundle sheath cells of C_4 plants. Detailed reviews of this subject have appeared (Hatch and Osmond, 1976; Hatch, 1976 and Edwards and Huber, 1981).

Another characteristic of C_4 plants which distinguishes them from C_3 plants can be found in their reduced $^{13}CO_2$ discrimination. This discrimination is due to the difference in carboxylating enzymes in the two groups of plants. The $^{13}C/^{12}C$ ratio of a sample is compared to a standard and this ratio is given the symbol delta. The delta ^{13}C value for atmospheric CO_2 is −0.67‰. C_3 plants discriminate against $^{13}CO_2$ and their average delta value is −2.78 ± 0.28‰ (Troughton, 1979). C_4 plants are less discriminating and their value is −1.36 ± 0.16‰.

Photorespiration and the Glycollate Pathway

In addition to the differences between C_3 and C_4 plants already described, there is another major physiological difference. Unlike C_3 plants, C_4 plants do not exhibit the external manifestations of photorespiration. Photorespiration is externally expressed by plants which have a high CO_2 compensation point (Γ) (the level of CO_2 remaining constant under closed system illumination) and which exhibit a post-illumination CO_2 burst (Canvin, 1979). C_4 plants have a zero or very low Γ while C_3 plants have a CO_2 compensation point ranging from 40 μl CO_2 l^{-1} or higher (Canvin, 1979). C_4 plants do not exhibit the CO_2 post-illumination burst seen in C_3 plants.

Photorespiration is an indirect result of the oxygenase activity of RuBP carboxylase/oxygenase (fig. 1). Phosphoglycollate, arising from this reaction, is inhibitory to the triosephosphate isomerase (E.C.5.3.1.1) of the photosynthetic carbon reduction

cycle and thus must be metabolized (Halliwell, 1978). In this regard, a specific phosphatase, acting on phosphoglycollate, gives rise to glycollate (Randal et al., 1971). Glycollate is metabolized via the glycollate pathway, or as termed by Lorimer (1981), the C_2-photorespiratory oxidation cycle (PCO cycle).

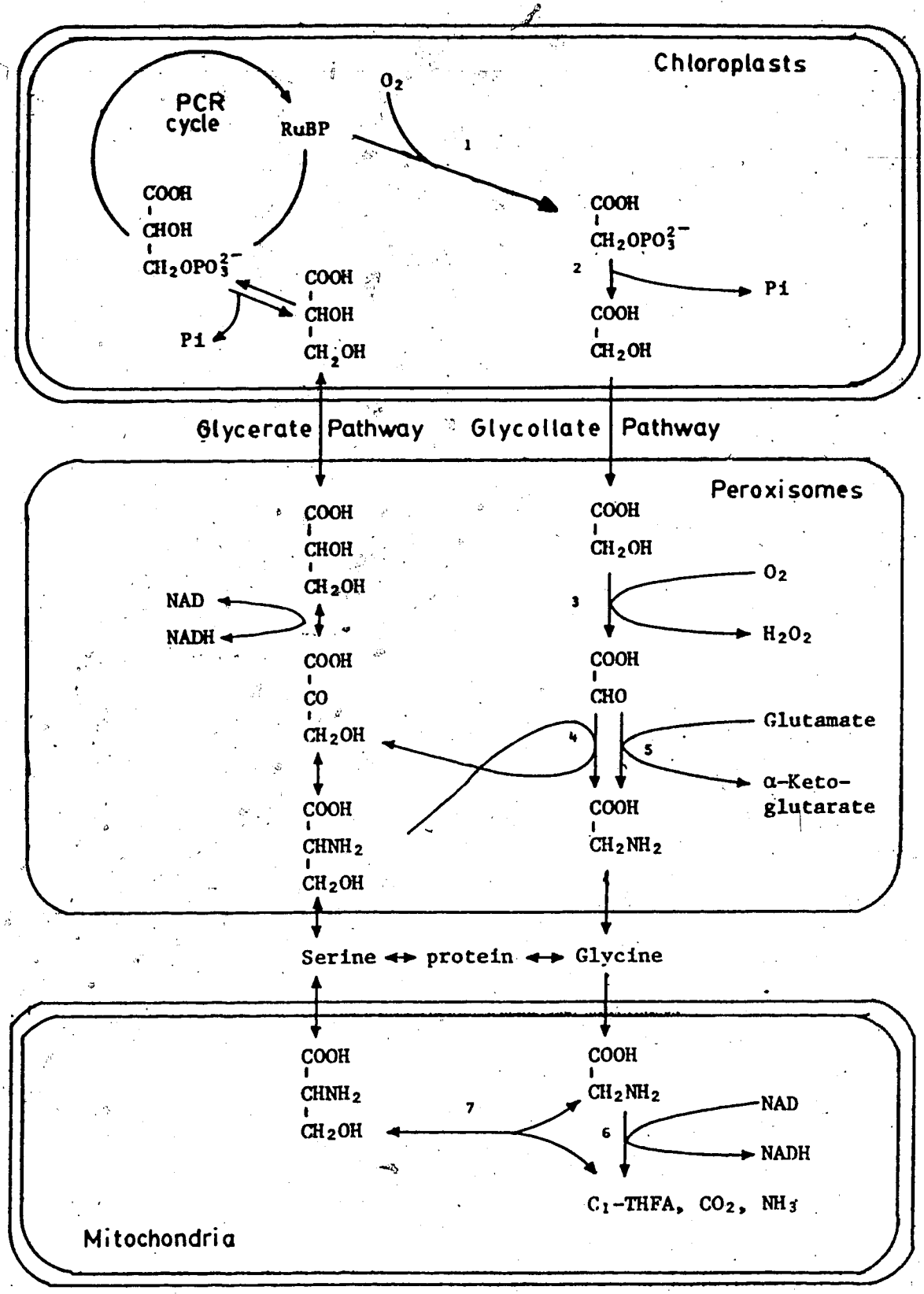
The glycollate pathway utilizes oxygen and produces CO_2 in the light and it is these properties which give the process of photorespiration its name (Tolbert, 1981). As illustrated in Fig. 3, the first step in glycollate metabolism occurs in the peroxisome and is catalyzed by glycollate oxidase (E.C. 1.1.3.1). This results in the production of glyoxylate and H_2O_2. Glyoxylate is readily converted to glycine by action of glycine aminotransferase (E.C. 2.6.1.4). The glycine is then transported to the mitochondria where glycine decarboxylase (E.C. 2.1.2.10) catalyzes its breakdown to CO_2, NH_3 and methylene tetrahydrofolic acid (Keys, 1980). This reaction has been demonstrated to be the principle source of CO_2 in photorespiration (Tolbert and Ryan, 1976; Oliver, 1981). Methylene THFA and another glycine give rise to serine catalyzed by serine hydroxymethyl transferase (E.C. 2.1.2.1). In C_3 plants, it has been demonstrated that such serine can give rise to 3-phosphoglycerate and contribute to the regeneration of RuBP. In this case, the necessary reactions also occur within the peroxisome and are collectively known as the glycerate pathway (Tolbert, 1981).

In early literature, the origins of glycollate were debated (Lorimer and Andrews, 1981; Beck, 1979; Tolbert and Ryan, 1976). Work by Bowes et al., (1971) using isolated RuBP carboxylase established the bifunctional nature of this enzyme. Experiments with $^{18}O_2$ demonstrated that the carboxyl group of glycollate and glycine became labelled (Andrews et al., 1971). More recently Somerville and Ogren (1979) demonstrated that phosphoglycollate accumulated and glycine and serine decreased in a mutant of *Arabidopsis thaliana* that lacked phosphoglycollate phosphatase. Hydroxybutanoic acid, an inhibitor of glycollate oxidase, induced the accumulation of glycollate in the wild type but not in the mutant, suggesting that glycollate normally arises from phosphoglycollate. It is notable that the mutant had no post-illumination CO_2 burst.

In C_3 plants 25 to 50% of recently fixed CO_2 may pass through the glycollate pathway and be lost as photorespiratory CO_2 (Chollet, 1977). Thus there is a significant proportion of energy being utilized by the C_3 plant in generating the glycollate which

Figure 3. The compartmentation of the glycollate and glycerate pathways. (Adapted from Tolbert, 1981)

E.C. No.	Reaction	Trivial Name	Systematic Name
4.1.1.39	1	RuBP oxygenase	3-phospho-D-glycerate carboxy-lyase
3.1.3.18	2	Phosphoglycollate phoshatase	2-phosphoglycollate phosphohydrolase
1.1.3.1	3	Glycollate oxidase	Glycollate:oxygen oxido-reductase
2.6.1.45	4	Serine-glyoxylate aminotransferase	L-serine:glyoxylate aminotransferase
2.6.1.4	5	Glutamate-glyoxylate amino-transferase	Glycine:2-oxoglutarate aminotransferase
2.1.2.10	6	Glycine decarboxylase	5-10-methylenetetrahydrofolate:glycine hydroxymethyltransfease
2.1.2.1	7	Serine hydroxymethyl transferase	5-10-methylenetetrahydrofolate:ammonia hydroxymethyltransferase


Chloroplasts
PCR cycle
RuBP
O_2
1
COOH
CHOH
$CH_2OPO_3^{2-}$
COOH
CHOH
CH_2OH
Pi
COOH
$CH_2OPO_3^{2-}$
2
Pi
COOH
CH_2OH
Glycerate Pathway
Glycollate Pathway
Peroxisomes
COOH
CHOH
CH_2OH
NAD
NADH
COOH
CO
CH_2OH
COOH
$CHNH_2$
CH_2OH
COOH
CH_2OH
3
O_2
H_2O_2
COOH
CHO
4
5
Glutamate
α-Keto-glutarate
COOH
CH_2NH_2
Serine ↔ protein ↔ Glycine
COOH
$CHNH_2$
CH_2OH
7
COOH
CH_2NH_2
6
NAD
NADH
C_1-THFA, CO_2, NH_3
Mitochondria

passes through the glycollate pathway. This is viewed by some authors as unproductive and energetically wasteful (Andrews and Lorimer, 1978). C_3 plants grown in 2% O_2 and normal CO_2 levels show increased photosynthetic capacity presumably because there is reduced O_2 competition with CO_2 for RuBP carboxylase/oxygenase (Halliwell, 1978; Lorimer and Andrews, 1981).

There is to date no firm evidence in the literature which supports a necessary function for photorespiration other than metabolizing the excess glycollate. In this sense, it can be calculated that the pathway can salvage 50% of the carbon from glycollate and generate an NADH in cycling the carbon back to 3-PGA (Chollet, 1977).

One theory for the presence of photorespiration is that it is unavoidable because of the dual nature of RuBP carboxylase/oxygenase (Andrews and Lorimer, 1978). It has been hypothesized that the enzyme evolved at a time when atmospheric CO_2 levels were higher but O_2 levels were lower thus minimizing O_2 competition. Another theory (Osmond and Bjorkman, 1972) for the operation of the glycollate pathway is that it provides a constant source of CO_2 even during periods of environmental stress when the stomates are closed. Without a CO_2 source the energy from NADPH and ATP produced during photosynthesis may cause photoinhibition of the cholorophyll apparatus. The review by Lorimer and Andrews (1981) gives an evaluation of these theories.

With the discovery that certain plants do not photorespire it was hoped that the productivity of crop plants could be improved. The non-photorespiratory plants are the C_4 plants and it has come to be generally accepted that it is the unique anatomy and the complementary carboxylating systems which prevent any observed photorespiration in this group of plants. Attempts to find plants with reduced oxygenase activity of RuBP carboxylase/oxygenase have been unsuccessful (Anderson et al, 1978) Even the enzyme isolated from a C_4 plant had an oxygenase activity (Bowes et al., 1971). Somerville and Ogren (1980) have postulated that it may be possible to genetically improve the carboxylation reaction. They have recently isolated photorespiratory mutants which may enable screening for secondary mutants with reduced RuBP oxygenase activity. In previous attempts by Andersen et al. (1978) with five mutant strains of a photosynthetic bacteria with a RuBP carboxylase/oxygenase similar to higher plants there was a parallel change in oxygenase activity and carboxylase activity. The activity of RuBP carboxylase was not

increased and where it was decreased there was a corresponding decrease in oxygenase activity.

Several excellent review articles on photorespiration have appeared, most notably those of Lorimer and Andrews (1981), Tolbert (1981), Zelitch (1980), Andrews and Lorimer (1978), Chollet (1977), Schnarrenberger and Fock (1976), and Canvin et. al. (1976). The review by Somerville and Ogren (1982) considers genetic attempts to modify photorespiration.

Alternate Sources of Photorespiratory Carbon Dioxide

Although the majority of evidence in the literature suggests that the CO_2 arising during photorespiration comes from the decarboxylation of glycine there are reports of other mechanisms. The possibility exists that glyoxylate may be non-enzymatically decarboxylated by the H_2O_2 produced during the glycollate oxidase reaction (Zelitch, 1972b; Halliwell and Butt, 1974). This would result in the production of CO_2 and formate. Since this would be occuring in the peroxisome where there is a large amount of catalase (E.C. 1.11.1.16) it is generally assumed that the H_2O_2 would be broken down and thus the amount of CO_2 arising non-enzymatically would be minimal (Chollet, 1977; Oliver, 1981). However early work by Zelitch (1972 a) indicated that glyoxylate decarboxylation may be a source of photorespiratory CO_2. Tobacco leaf disks floated in solutions containing glycollate-1-^{14}C or glycine-1-^{14}C readily produced $^{14}CO_2$. Carrier glycine added to the glycollate-1-^{14}C did not reduce the amount of $^{14}CO_2$ released nor did INH, an inhibitor of serine hydroxymethyl transferase and glycine decarboxylase. Zelitch suggested that these results indicated that the conversion of glycine to serine was not essential to photorespiratory CO_2 release. In another paper, Zelitch (1972 b) demonstrated that envelope-free chloroplasts of spinach were capable of decarboxylating glyoxylate to CO_2 and formate in the presence of O_2, Mn^{2+}, and light. A Mehler type reaction may provide the necessary H_2O_2. Mesophyll cells were isolated from soybean and incubated with inhibitors of the glycollate pathway in the presence of glycollate-1-^{14}C or glycine-1-^{14}C (Oliver, 1979). The experiments were carried out in the dark to prevent refixation of CO_2. INH inhibited glycine decarboxylation over 90% and KCN inhibited the same reaction by 80%. These same inhibitors only decreased the $^{14}CO_2$ release from glycollate-1-^{14}C by 50%.

Methyl-2-hydroxy-3-butynoate (MHB), an inhibitor of glycollate oxidase, prevented CO_2 release from glycollate while 2,3-epoxypropionate (glycidate), an inhibitor of glutamate:glyoxylate aminotransferase (E.C. 2.6.1.4), did not. These data suggest that some of the $^{14}CO_2$ derived from glycollate-1-^{14}C must be arising directly from glyoxylate. Added catalase also inhibited CO_2 release suggesting that the alternate decarboxylation is non-enzymatic. Oliver suggested that glycine decarboxylation could be regulated by the availability of NAD in the mitochondria.

Halliwell and Butt (1974) using a catalase inhibitor on peroxisomes isolated from spinach-beet showed an increase in $^{14}CO_2$ release from glycollate-1-^{14}C when compared to non-inhibited peroxisomes.

Photorespiratory mutants of *Arabidopsis thaliana* isolated by Somerville and Ogren (1981) were found lacking in serine hydroxymethyl transferase and glycine decarboxylase. These mutants evolved CO_2 slowly under conditions that were conducive to photorespiration. With added NH_3, CO_2 evolution was stopped and glycine continued to accumulate. They suggested that CO_2 could arise from glyoxylate. However, since NH_3 prevented this by providing sufficient amino groups for glycine synthesis glyoxylate decarboxylation may not be a normal source of photorespiratory CO_2.

A more recent paper by Oliver (1981) indicated that adding the amino donors glutamate or serine in the presence of glycollate decreased the amount of glyoxylate available for non-enzymatic decarboxylation since this compound would be aminated to form glycine. From his data, Oliver calculated that only 2.5% of the CO_2 would arise non-enzymatically *in vivo*. The use of varied amounts of exogenously added substrate could influence the extent of direct glyoxylate decarboxylation by H_2O_2 (Walton, 1982). High concentrations of glyoxylate resulted in direct decarboxylation. Low concentrations favored CO_2 production through glycine decarboxylation. Walton concluded that only under extreme conditions would the amount of direct glyoxylate decarboxylation approach that reported for photorespiratory CO_2 evolution. However, the possibility that CO_2 and formate arise non-enzymatically is not entirely discounted.

The Glycollate Pathway in C_4 Plants

The literature contains several reports that C_4 plants possess the enzymes associated with the glycollate pathway and that these enzymes are localized in the bundle sheath cells (Rehfeld et al., 1970; Osmond and Harris, 1971; Liu and Black, 1972; Chen et al., 1974; and Frederick et al., 1975). This is also where RuBP carboxylase/oxygenase is located. Studies on peroxisome distribution have shown that there are greater numbers of these organelles in the bundle sheath cells than in the mesophyll cells (Liu and Black, 1972; Frederick et al., 1975). The peroxisomes contain glycollate oxidase and other enzymes associated with the glycollate pathway (Tolbert, 1981). From these observations it would appear that the potential for an operational glycollate pathway exists in C_4 plants and it may be associated with the oxygenase activity of RuBP carboxylase/oxygenase.

There are currently two theories attempting to explain the apparent lack of photorespiration in C_4 plants. The first invokes a CO_2 concentrating mechanism within the bundle sheath cells. It is suggested that the CO_2/O_2 ratio in the bundle sheath cell favors the carboxylation of RuBP and hence carbon flow through glyoxylate and glycine will be severely reduced or eliminated (Hatch and Osmond, 1976). Evidence supporting this view is derived from experiments in which lowering the O_2 concentration for photosynthesizing C_4 plants did not increase the net photosynthesis as is the case for C_3 species (Hofstra and Hesketh, 1969). Hesketh (1967) found no enhancement of CO_2 assimilation in corn, a C_4 plant, in the absence of atmospheric O_2. This would suggest that at normal O_2 concentrations the RuBP oxygenase reaction does not decrease net photosynthesis in C_4 plants. In early experiments using elevated O_2 levels with corn there was no photorespiratory CO_2 release but there was a decrease in net photosynthesis (Forrester et al., 1966). More recent work using corn and a long equilibration time demonstrated that net photosynthesis becomes reduced at elevated O_2 levels and this reduction is not entirely due to changes in stomatal resistance but to a direct inhibition of CO_2 fixation (Gale and Tako, 1976). Ku and Edwards (1980), using the C_4 species *Amaranthus graecizans*, revealed that there was an increase in the CO_2 compensation point even with O_2 concentrations between 2 and 21%. This increase was no longer linear after a period in high O_2 concentration. The authors suggested that this characteristic is due to the diffusive resistance of gases and that *A. graecizans* may have a lower diffusive resistance than

other C_4 plants. The O_2 effect they observed took one hour to reach equilibrium unlike C_3 plants where such a response takes only minutes. The explanation given by these authors for the difference between C_3 and C_4 plants is that C_4 plants have a high diffusive resistance across bundle sheath cells and an elevated CO_2/O_2 ratio due to the nature of the C_4 pathway.

Amaranthus edulis, subjected to varying concentrations of CO_2, did not show any change in O_2 uptake (Canvin et al., 1980). The uptake of O_2 was only 20% of that shown by C_3 species. The authors concluded that some of the O_2 may be due to photorespiration but favored the contention that O_2 uptake was partially due to a Mehler reaction.

The above reports suggest that at low or normal O_2 concentrations oxygen inhibition of carbon assimilation is minimal. Only at high O_2 concentrations and after prolonged exposure is net photosynthesis reduced in C_4 plants.

The second theory for the absence of photorespiratory CO_2 release in C_4 plants suggests that any CO_2 released from bundle sheath cells is refixed by the mesophyll cells. This idea is supported by the work of Rathnam (1979). Leaf slices of the C_4 grasses *Panicum maximum* and *P. milaceum* were fed glycollate-1-^{14}C in the light and the dark. In the light no $^{14}CO_2$ was evolved. In the dark $^{14}CO_2$ evolution was detected but with added PEP this CO_2 evolution was eliminated. In experiments using maleate, an inhibitor of PEP carboxylase, even with added PEP and illumination, $^{14}CO_2$ was detected. This data supports the contention that PEP carboxylase mediates the refixation of photorespiratory CO_2. The fact that this C_4 plant is able to metabolize glycollate-1-^{14}C is evidence for a glycollate pathway. Work by Rathnam and Chollet (1979) on *Panicum milioides,* a C_3-C_4 intermediate species with Kranz-like anatomy, suggests that PEP carboxylase activity accounts for the CO_2 compensation point being intermediate between C_3 and C_4 plants. A paper by Kennedy and co-workers (1977) using tissue cultures of a C_4 plant in which Kranz anatomy had not developed but the response to O_2 was similar to C_4 metabolism, also suggests that PEP carboxylase is important in maintaining a low CO_2 compensation point.

This second theory suggests that there is photorespiratory CO_2 arising in C_4 plants. Evidence for a functional glycollate pathway in C_4 plants can be found in the literature.

Elevated O_2 levels clearly inhibit photosynthesis in isolated bundle sheath cells of corn (Chollet and Ogren, 1972). Also in this species, Volk and Jackson (1972) using $^{18}O_2$ showed a considerable O_2 uptake during active photosynthesis. These authors used excised leaves in a closed environment and flushed the chamber with 96 atom % ^{13}C at a concentration of 1.64% CO_2 and 8.2% O_2. In the first 15 minutes they observed a $^{12}CO_2$ evolution from the corn indicative of photorespiration. Uniformly labelled ribose-5-phosphate, fed to isolated bundle sheath cells of corn with added ADP and *a*-HPMS, an inhibitor of glycollate oxidase, in the presence of oxygen resulted in an accumulation of labelled glycollate in the dark (Chollet, 1974). Glycollate accumulation was reduced by decreasing the O_2 or by increasing the CO_2 concentrations. Added glycollate-1-^{14}C was metabolized to release $^{14}CO_2$ and this was dependent on oxygen. Oxygen dependency is consistent with an involvement of glycollate oxidase. In addition to this finding, *a*-HPMS also prevented $^{14}CO_2$ evolution. Similar results were found with isolated bundle sheath cells of *Digitaria sanguinalis*, supplied glycollate-1-^{14}C and glycine-1-^{14}C (Chollet, 1973).

Usuda and Edwards (1980) isolated bundle sheath cells of the C_4 *Panicum capillare* and demonstrated that glycerate was a product of $^{14}CO_2$ fixation and that it arose via the glycollate and glycerate pathways. Using inhibitors of the glycollate pathway they were able to prevent glycerate labelling. Low bicarbonate concentrations resulted in a large amount of label in glycine and serine as well as glycerate. High bicarbonate concentrations resulted in much less label in glycine and serine and the metabolites of the glycollate pathway. This indicates a CO_2/O_2 competition for RuBP carboxylase/oxygenase.

From the papers reported on isolated bundle sheath cells it is seen that C_4 plants possess the enzymes of the glycollate pathway localized within their bundle sheath cells and that the pathway operates in these isolated tissues in a manner resembling that reported for C_3 plants.

A study by Dimon and colleagues (1977) with the $^{18}O_2$ isotope and whole leaves of corn in the absence of CO_2 demonstrated that $^{18}O_2$ was quickly incorporated into the carboxyl group of glycine and serine. Such labelling is consistent with RuBP carboxylase/oxygenase giving rise to glycollate and other metabolites of the glycollate pathway.

Pulse chase experiments with intact corn leaves revealed that $^{14}CO_2$ was incorporated into glycine and serine (Morot-Gaudry et al., 1980). At 1% O_2 compared to 21% O_2 the amount of label in glycine was reduced by 90% and that in serine by 70%. In short (3 sec) chase experiments C-1 and C-2 of glycine were nearly equally labelled. During the same chase period C-1 of serine was more heavily labelled than C-2 or C-3. This pattern of labelling suggests that serine arises in reactions that are distinct from the glycine to serine conversion. In agreement with this, isonicotinyl hydrazide (INH), an inhibitor of the glycollate pathway, did not completely block serine labelling. The authors suggested that serine may arise from 3-phosphoglycerate. The carboxyl carbon of 3-PGA is also heavily labelled after 3 seconds of the chase period. These workers concluded that a glycollate pathway, sensitive to O_2, operates in corn but the glycine to serine conversion is not stoichiometric and serine may have other precursors. The O_2 sensitivity of glycine and serine synthesis in corn was also illustrated by Hickman and Keys (1972). In an atmosphere of nitrogen the label recovered in glycine and serine was 0.4% but in 76% O_2 the total in the two amino acids was 4.3%. Results similar to those of Morot-Gaudry and colleagues were obtained by Mahon et al. (1974) using corn leaf disks. At 21% O_2 and a 15 min $^{14}CO_2$ labelling the levels of glycine and serine were much higher than at 1% O_2. From $^{12}CO_2$ chase experiments, where the glycine and serine label declined at different rates, the authors concluded that the glycine and serine pools were not closely linked.

As illustrated in Fig. 3. the glycine decarboxylation reaction of the glycollate pathway is located in the mitochondria. Woo and Osmond (1977) isolated mitochondria from mesophyll cells and bundle sheath cells of several C_4 species. They found that glycine decarboxylation was much higher in the mitochondria from bundle sheath cells than from mesophyll cells in *Atriplex spongiosa* and *Panicum miliaceum*. They could not however detect glycine decarboxylation in either mesophyll or bundle sheath mitochondria of *Zea mays*.

A 1977 paper by Neuburger and Douce gives evidence linking the oxidation of glycine in mitochondria from the C_3 species *Spinacia oleracea* with the electron transport chain. There are three phosphorylation sites, each susceptible to inhibitors of the electron transport system. Mitochondria isolated from dark-grown plants were not capable of

oxidizing glycine. It was also shown that mitochondria isolated from corn did not support a glycine-stimulated O_2 uptake but they did display good ADP/O ratios when malate and NADH served as electron sources.

Corn leaf disks in the presence of *a*-HPMS, a glycollate oxidase inhibitor, accumulated glycollate (Zelitch, 1973). The glycollate was measured colorimetrically. The glycollate had a low specific activity when fed $^{14}CO_2$. Tobacco under the same conditions had a high specific activity. The label from pyruvate-3-^{14}C is incorporated into carbon-2 of glycollate in both corn and tobacco but the specific activity was much greater in corn. Added PEP stimulated glycollate formation in corn but, in conjunction with $^{14}CO_2$ labelling, it decreased the specific activity. PEP had an inhibitory affect on glycollate synthesis in tobacco. The inhibitor INH reduced glycollate synthesis in tobacco but not corn. Zelitch suggested from these observations that glycollate arises in corn through pyruvate rather than from the oxygenase activity of RuBP carboxylase/oxygenase as would be the case in tobacco.

The reports of the lack of glycine to serine conversion in whole leaf and mitochondria of bundle sheath cells and the work of Zelitch suggesting that glycollate does not arise due to RuBP carboxylase/oxygenase activity indicate that at least in corn the glycollate pathway may not be identical to that documented for C_3 plants.

The Present Study

As noted in the preceeding section, there is considerable evidence that a glycollate pathway operates in the bundle sheath cells of C_4 plants. Much of the data for *Zea mays* suggests that this C_4 plant has a glycollate pathway capable of metabolizing exogenously added glycollate. The reports by Woo and Osmond (1976) and Neuburger and Douce (1977) suggest that corn mitochondria are not capable of glycine decarboxylation and there is contradictory evidence that the glycollate pathway functions in the manner established for C_3 plants. Reports that glycine and serine are not stoichiometrically related when pulse-chase experiments are performed with *Zea mays* indicate that the glycollate pathway is not entirely responsible for synthesizing these two amino acids (Morot-Gaudrey et al., 1980 and Mahon et al., 1974). These papers also imply that the photorespiratory pathway does not supply sufficient serine for this C_4 plant as it must in

C_3 plants where an excess is used in the glycerate pathway. In contrast there has been little work on isolated bundle sheath cells to follow the complete metabolic fate of glycollate. Early studies were mainly concerned with demonstrating the ability of C_4 plants to decarboxylate glycollate to produce photorespiratory CO_2.

In order to examine the role of this pathway in C_4 species, radio-labelling experiments were carried out on isolated bundle sheath cells and whole leaves of *Zea mays* L. and *Sorghum bicolor* L.. A recently described method (Chapman et. al., 1980) for isolating the bundle sheath cells was employed in this work. Unlike earlier methods that relied on mechanical means for separating cell types this technique combines mechanical disruption with cellulase treatment and allows a more efficient preparation of bundle sheath cell strands. Glycollate-2-^{14}C and $NaH^{14}CO_3$ were fed to the isolated tissue. An inhibitor of glycine decarboxylase and serine hydroxymethyl transferase was used in conjunction with these feeding experiments. The inhibitor was aminoacetonitrile (AAN), an analogue of glycine,reported to be a more specific inhibitor than the pyridoxal phosphate inhibiting chemical, INH, used extensively in the literature (Usuda et. al., 1980; Creach and Stewart, 1982 and Yun et. al., 1979). Whole leaf experiments were also performed using AAN and $^{14}CO_2$.

In all experiments labelling of the known metabolites of the glycollate pathway was examined, including the controversial product formate. Unlike glycollate-1-^{14}C experiments, glycollate-2-^{14}C feedings allowed the tracing of carbon retained in the glycollate pathway.

II. Materials and Methods

Plants

Zea mays L. var. Gills Early Market, *Sorghum bicolor* L. var. P130 and *Hordeum vulgare* L. var. Galt were grown in soil in a controlled environment chamber at 25 °C under a 12 hr. day (200 uE m^{-2} s^{-1}, produced by fluorescent and incandescent lights). Seedlings were watered six days per week with 1/2 strength modified Hoagland's solution (Epstein, 1972).

Special Chemicals and Radionuclides

Onozuka R-10 cellulase was obtained from Yakult Biochemicals Co. Ltd., Japan. Aminoacetonitrile was supplied by Aldrich Chemical Company. The radionuclides used were obtained as follows: $NaH^{14}CO_3$ and $Na_2{}^{14}CO_3$ were obtained from Amersham. Glycolic acid-2-^{14}C was purchased from ICN. Other chemicals were either purchased from Sigma Chemical Company, St. Louis, Mo. USA or Fisher Scientific Company, Edmonton, Alberta.

Isolation of Bundle Sheath Cells

Bundle sheath cells were isolated from 14 to 21 day old corn and sorghum leaves using a method similar to that of Chapman *et. al.* (1980). For each experiment approximately 2 g (f.wt.) of leaf tissue of corn (midrib removed) or sorghum (intact) were cut transversely into 2 mm segments using a sharp razor blade. The leaf segments were then placed in 50 ml of cutting medium (see below) and blended for 20 seconds in a Virtis "45" homogenizer with a rheostat setting of 30. The tissue was then collected on a "80 mesh" nylon screen and transfered to 50 ml of cellulase medium (see below) and illuminated (15 uE m^{-2} s^{-1}) at 27 °C for 40 min. (corn) or 50 min. (sorghum). After incubation the cells were again collected on the nylon screen and washed with 50 ml of cutting medium to remove released mesophyll protoplasts and excess cellulase. The plant material was resuspended in 50 ml of cutting medium and blended for a further 40 seconds in the Virtis blender. The resulting cell suspension was placed on an 800 um metal sieve with the 80 mesh nylon screen below and washed 5 to 7 times with 50 to 60 ml of cutting medium. Large incompletely digested pieces of tissue, trapped on the metal sieve, and the broken

cells and mesophyll protoplasts passing through the nylon mesh were discarded. The resulting bundle sheath strands, collected on the nylon screen, were washed twice with 25 ml of reaction mixture (see below) and resuspended in the same mixture to a final volume of 25 ml.

The various media mentioned above consisted of:

Cutting medium: 20 mM HEPES–KOH, 0.35 M sorbitol, 4 mM $MgCl_2$, 2 mM potassium phosphate, and 10 mM isoascorbate. The final pH was 6.4 at room temperature.

Enzyme medium: The above solution with 1.5% (w/v) cellulase, 1% (w/v) polyvinylpyrolidone–40, 1 mM Ribose–5–phosphate, and 1 mM glucose–6–phosphate.

Reaction medium: 7.5 mM Tricine–KOH, 0.26 M sorbitol, 3 mM $MgCl_2$, 1.5 mM potassium phosphate, 3.8 mM potassium sulfate, 0.5 mM AMP, 5 mM ribose–5–phosphate, and 10 mM malate. The final pH was 8.2 at room temperature.

Purity of Bundle Sheath Cell Preparations

The bundle sheath cell suspensions were routinely examined by light microscopy to determine possible contamination by mesophyll cells. The chlorophyll a/b ratios were also determined (Harborne, 1973) as well as the following carboxylase activities.

Assays of RuBP carboxylase and PEP carboxylase were carried out on whole leaf and isolated bundle sheath strands of corn and sorghum using the method of Kennedy (1976). Samples (3 g) of whole leaf were suspended in extraction buffer (8 ml) (see below) and ground with a small amount of sea sand at 4 °C. The homogenate (10 ml) was filtered through Miracloth and centrifuged at 4 °C for 20 minutes at 30,000 x g. Aliquots of the supernatant were used for subsequent carboxylase assays. The enzymes from the bundle sheath cells were prepared in a similar way. After 3 ml of the 25 ml bundle sheath cell suspension (in cutting medium, see BSC isolation) was removed for chlorophyll determination, the remaining 22 ml was centrifuged at 18,500 x g. The resulting pellet was ground in a glass homogenizer at 4 °C in extraction buffer (see below). The 5 ml final volume was centrifuged as in the whole leaf extraction and aliquots of the supernatant were used for the enzyme assays.

Phosphoenolpyruvate and ribulose–1,5–bisphosphate carboxylase assays were carried out in 1.5 ml Eppendorff centrifuge tubes at 27 °C (Kennedy, 1976). Aliquots

(25–100 ul) were assayed in triplicate for 2 or 3 minutes. To the preceeding volumes, 165 ul of either RuBP carboxylase reaction mixture or PEP carboxylase reaction mixture were added. The reactions were initiated with 10 ul of RuBP (0.4 umol) or 10 ul of PEP (1.0 umol) respectively and terminated by the addition of 200 ul of 25% acetic acid (v/v). Reaction systems lacking substrate and extract respectively were included as controls. Protein was determined by the Folin method (Lowry *et.al.*, (1951).

The media used in these assays were as follows:

Extraction medium: Contained 20 mM tricine–KOH, 10 mM $MgCl_2$, 20 mM 2–mercaptoethanol, 0.2 mM EDTA, and 1.6 g/100 ml of polyvinylpyrolidone–40. Final pH 8.3 at room temperature.

RuBP carboxylase medium: Each reaction system contained 30 umol tricine–KOH, 10 umol $MgCl_2$, 8 umol 2–mercaptoethanol, 2.0 umol $NaH^{14}CO_3$ containing ca. 250,000 dpm. Final pH 8.0 at room temperature.

PEP carboxylase medium: The reaction systems contined 30 umol tricine–KOH, 10 umol $MgCl_2$, 8 umol 2–mercaptoethanol, 1 umol sodium glutamate and 2 umol $NaH^{14}CO_3$. Final pH 8.0 at room temperature.

Aliquots of acetic acid–treated systems were air–dried in liquid scintillation vials to remove excess carbonate followed by resuspension in 200 ul of water in preparation for liquid scintillation counting.

The Metabolism of Labelled Substrates

Bundle Sheath Cells

The following general procedure was used for all labelling studies of bundle sheath cells. The 25 ml suspension of bundle sheath cells was subdivided into two, 3 ml aliquots for chlorophyll determinations, and six, 3 ml aliquots for feeding studies. For inhibitor studies three of the aliquots were placed in Warburg flasks to which were added 100 ul of reaction mixture containing aminoacetonitrile (final concentration, 20 mM). To the three control flasks an extra 100 ul of reaction mixture was added. A 15 minute pre–incubation period was allowed before addition of 10 ul of labelled compound. The Warburg flasks were sealed and illuminated (380 uE m^{-2} $^{-1}$s) from below at 28 °C for 10 or 30 minutes. The tissue was killed by adding 5 ml of boiling water directly into the flasks and placing the

flasks in a boiling water bath for a minimum of 5 minutes. Samples were stored frozen overnight. The amounts of radionuclides added were as follows.

Glycollate-2-^{14}C; 0.5 uCi; 0.01 umol.

$NaH^{14}CO_3$; 20 uCi; 0.35 umol.

Whole Leaf Experiments

The general procedure for supplying $^{14}CO_2$ to whole leaves was as follows. Approximately 0.5 g of 14 to 21 day old leaves of corn, sorghum or barley were cut under water and placed in 50 ml beakers containg 25 ml of water or 20 mM aminoacetonitrile. After a 4 hour pre-incubation in the growth chamber, duplicates of the control and inhibitor-treated leaves were transferred to a feeding chamber and allowed to equilibrate for 15 minutes under 470 uE m^{-2} s^{-1} illumination from a mercury vapor lamp. The chamber was flushed with N_2 gas for 20 sec and the tissue was then placed in boiling water (15 ml) for rapid killing. Tissues were stored frozen overnight before grinding and processing.

Column Chromatography and Separation of Labelled Fractions

The amino acids, organic acids, and sugars were separated using Dowex 1 x 8 H^+ form (100-200 mesh) and Dowex 1 x 10 formate form (200-400 mesh) resins. The resins were prepared and used according to Cooper (1977).

The whole leaf tissue or the bundle sheath cell pellet was ground in a glass homogenizer followed by centrifugation for 10 minutes at 18,500 x g. The supernatant was combined with expended feeding solution. The pellet was further washed with 2 to 3 ml of water and again centrifuged. The combined supernatants (10-15 ml) were applied to a 1 x 6 cm H^+ form column and washed with 40 ml of water. The water wash contained the organic acid and sugar fractions. The amino acids were eluted with 40 ml of 6M HCl. The HCl fraction was placed in a boiling water bath for 2 hours to hydrolyze asparagine and glutamine to aspartate and glutamate respectively.

The amino acids were evaporated to dryness at 40°C under vacuum in a flash evaporator and resuspended in 3 ml of 0.2 M citrate buffer (pH 2.2) in preparation for analysis on a Beckman Automatic Amino Acid Analyzer, model 121. PA28 spherical resin was used (Clandinin and Cossins, 1972). Fractions of 30 drops, 1.82 ml, were collected

using a model LKB fraction collector. Aliquots of these fractions were taken for liquid scintillation counting. Controls of selected experiments were also run on the amino acid analyzer to determine pool sizes using a ninhydrin reaction.

The organic acids and sugars were separated by placing a 10 ml subsample on a column (1 x 11 cm) of Dowex formate resin. The sugars were eluted with 40 ml of water. The organic acids were eluted using a gradient of formic acid (Palmer, 1955; Thauer *et. al.* 1970). An upper reservoir containing 200 ml of 8N formic acid was allowed to drip into a 470 ml reservoir which in turn flowed onto the formate column. The effluent was collected in 24 drop, 1.42 ml, fractions using an LKB fraction collector. Sugar-phosphates and strongly bound organic acids were eluted with 40 ml of 8N formic acid (see appendix C for a list of retention times).

Identification of Labelled Compounds

Amino Acids

The elution order of amino acids on the analyzer is well known and reproducible. To verify the identity of individual compounds, pooled peaks from the analyzer were examined by thin layer chromatography. Plates of 0.1 mm cellulose MN-300 were used and run in various solvent systems reported in the CRC Handbook of Chromatography. Labelled standards of glycine-2-^{14}C and serine-3-^{14}C were detected by autoradiography; all other amino acid standards were detected by spraying with 0.1% ninhydrin in 80% acetone.

Organic Acids

The identities of labelled organic acids were determined by several methods. Glycollate was identified by the colorimetric method of Calkins (1943) and by co-chromatography with authentic glycollate on Dowex formate columns. Labelled formate was verified by oxidation to $^{14}CO_2$ as described by Yang (1969). This method involves reacting 0.25 g of $HgCl_2$ with the formate-^{14}C sample in a serum stoppered Warburg flask for 40 minutes at 85 °C. After cooling on ice, 0.2 ml of 1N NaOH was injected onto a folded filter paper wick in the center well. After one hour, the filter paper was removed and placed in Bray's (1960) solution for liquid scintillation counting.

High performance liquid chromatograhpy using an Aminex HPX-87 organic acid analysis column (Bio-Rad Laboratories) and a Varian model 5000 HPLC system was also beneficial in confirming the identity of labelled organic acid compounds. A 20 minute program using 0.01N H_2SO_4 isocratic elution was sufficient to separate the majority of Calvin cycle and Krebs cycle organic acids (see Appendix A for more information). Standards of the various organic acids (20 ul of 0.1 mg/ml) were run separately to determine the individual elution times and then collectively to confirm adequate resolution. The acids were detected by absorbance at 210 nm using a Varian model U.V. 50 spectrophotometer equipped with a flow cell. Four ^{14}C-labelled organic acids were available as standards. These were glycollate-2-^{14}C, formate-^{14}C, glyoxylate-U-^{14}C, and malate-U-^{14}C. These were run individually on the HPLC column and 4 drop (approx. 0.25 ml) fractions were collected in prepartion for liquid scintillation counting.

In other separations, 20 ml of selected organic acid and sugar fractions were adjusted to pH 7.5 to 8.0 with NH_4OH, evaporated to dryness, and resuspended in 0.5 ml HPLC grade water. A 20 ul sample containing at least 10,000 dpm was injected onto the column and fractionated as noted above for the radioactive standards. The elution profile of ^{14}C was compared to that of the ^{14}C-standards. Further confirmation of organic acid identities was based on the chromatographic behavior of standards (1 mg/ml) fractioned on the formate gradient. Aliquots of these fractions were subjected to HPLC analysis to determine the order of elution from the formate column. Thin layer chromatography and autoradiography were also used to identify some of the organic acids labelled in the various experiments. The detection system used for unlabelled standards was that of Chaefetz and Penner (1970). Equal voumes of 8% KI, 1% KIO_3, and 1% amylose were freshly mixed and sprayed on the TLC plates. A dark blue or brown spot appeared where the organic acids were present. Autoradiography was carried out using Kodak XAR-5 x-ray film exposed to the TLC plate for 1 to 2 weeks and developing for 2 minutes in GBX developer followed by a water rinse and 3 minutes in GBX fixer.

Sugars and Sugar-phosphates

Neither the sugars nor the 8N formic acid wash from the Dowex 1 x 10 resin were separated into individual components. However HPLC revealed that 3-PGA and pyruvate were present in the 8N fomic acid wash.

Liquid Scintillation Counting

Radioactive samples, in a dioxane based fluor (Bray, 1960), were counted on a Tracor Analytical Mark III 6881 Liquid Scintillation System.

Chlorophyll Determinations

Chlorophylls a and b were determined by the standard method of Arnon as reported in Harborne (1973). See Appendix B.

III. Results

Whole Leaf Studies

The glycine analogue aminoacetonitrile (AAN) has been demonstrated to be an inhibitor of photorespiration in C_3 plants (Yun et. al., 1979 and Usuda et. al., 1980). It is reported to inhibit glycine oxidation in isolated mitochondria and to inhibit glycine decarboxylation in isolated protoplasts of wheat (Usuda et.al., 1980). As an inhibitor of the glycollate pathway, AAN is reported to be more specific than isonicotinyl hydrazide (INH) (Usuda et. al., 1980). Unlike INH, AAN did not effect CO_2 assimilation in isolated chloroplasts.

For the reasons reported above AAN was used in the present studies of the glycollate pathway in C_4 plants. In order to learn more about the effect of this inhibitor on the distribution of metabolites from $^{14}CO_2$, preliminary experiments were undertaken using barley as a representative C_3 plant. As reported by Yun et. al. (1979) rice plants are able to take up AAN through the cut ends of leaves. This procedure was adapted to the present work. The results of the barley experiments are summarized in Table 1. The AAN concentration was 20mM, double that used by Yun et. al. (1979). The leaves were preincubated for 4.5 hours before supplying $^{14}CO_2$ for 2 minutes. As expected an accumulation of glycine-^{14}C in the inhibitor treated plants occurred and this corresponded with a decrease in the label found in serine. This is consistent with the operation of the glycollate pathway with glycine decarboxylase and serine hydroxymethyl transferase catalyzing the reactions for serine formation. In a recent paper by Gardenstrom *et. al.* (1981) it was reported that low concentrations of AAN (1-10mM) inhibit glycine oxidation in spinach mitochondria and that high concentrations of AAN (10 to 50mM) additionally inhibit serine hydroxymethyl transferase. Thus in the barley experiments both enzymes may be affected by the 20mM AAN used.

Also of note in these barley experiments (Table 1. Fig. 4.) is that the amount of total label incorporated was reduced in the inhibitor-treated leaves. Although Usuda et. al. (1980) reported that AAN (up to 50mM) did not inhibit net CO_2 uptake in isolated wheat chloroplasts the amount of label incorporated by isolated protoplasts was significantly reduced. They suggested that carbon which would normally be recycled through the

Table 1. Products of $^{14}CO_2$ fixation in barley leaves. *
2 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Aspartate	230	7.3	170	6.2
Serine	540	17.3	190	6.9
Glutamate	57	1.8	67	2.4
Glycine	100	3.2	1100	39.8
Alanine	690	22.0	220	8.0
Total	1620	51.8	1760	63.8
Organic acids				
Glyceric	65	2.1	19	0.69
Glycollate	210	6.7	65	2.4
Hydroxypyruvate	220	7.0	380	13.8
Total	510	16.3	460	16.7
Formic acid wash (8N)	180	5.8	130	4.7
Neutrals	820	26.2	410	14.9
Total incorporated	3130	100	2760	100

* Cut ends of whole leaves placed in 20 mM AAN 4.5 hours prior to feeding labelled $^{14}CO_2$.

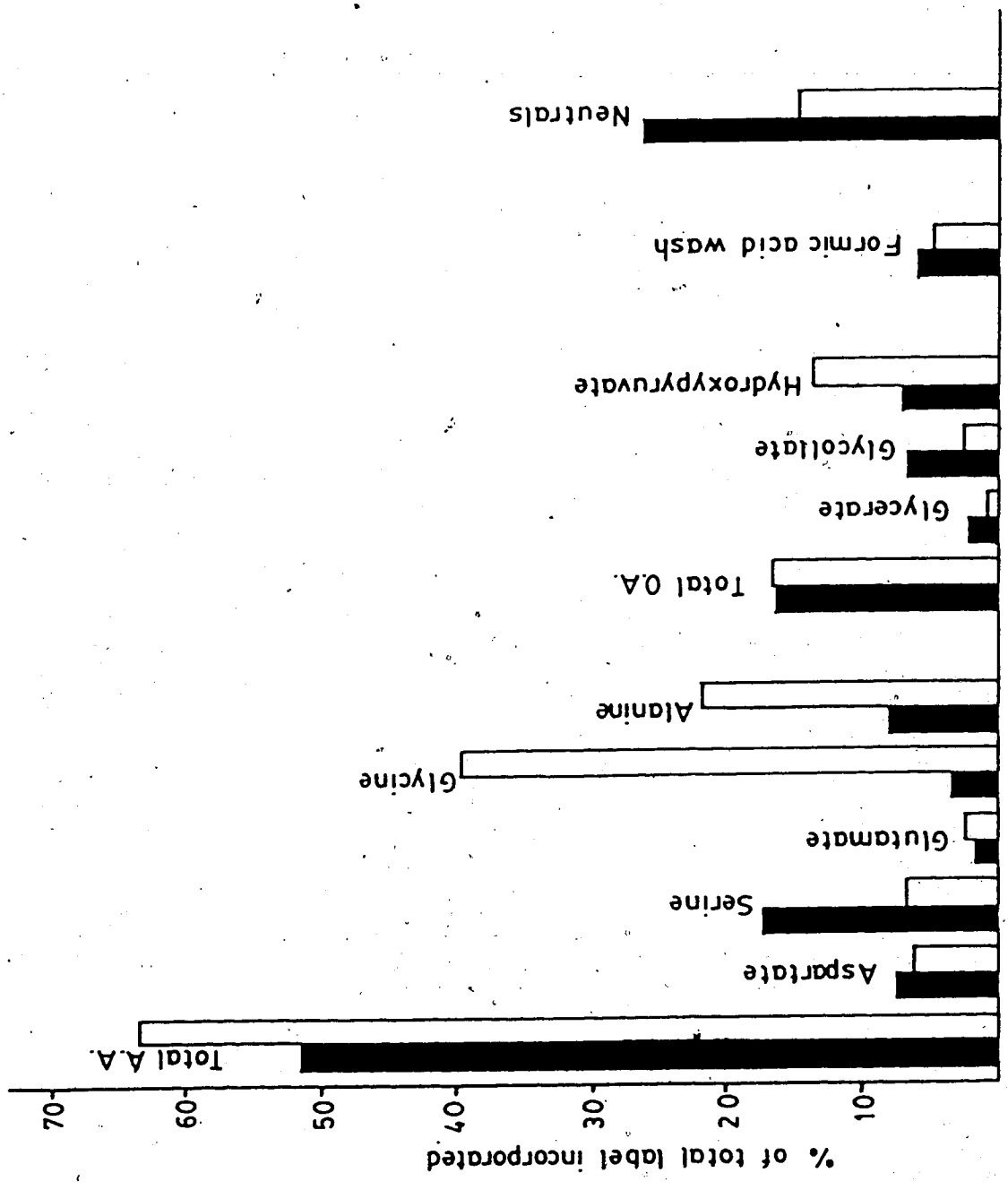


Fig.4 Products of $^{14}CO_2$ fixation in barley leaves.
2 minute labelling, effect of aminoacetonitrile.

glycerate pathway would no longer be available and that intermediates of the Calvin cycle would be reduced, including ribulose-1,5-bisphosphate required for CO_2 fixation. The barley data presented in Table 1. supports this view. For example the label in the neutral fraction was reduced suggesting that less sugar was synthesized. Another piece of evidence supporting less labelling of Calvin cycle intermediates is the reduction of label found in alanine. The precursor for alanine is pyruvate which is normally derived from phosphoenol pyruvate via 3-phosphoglycerate in photosynthetic tissues (Bryan, 1976).

An anomaly found in the organic acid fraction is the amount of ^{14}C incorporated into glycollate. There was less glycollate-^{14}C in the inhibitor-treated leaves than in the controls. Since glycine was accumulating it might be argued that the glycollate pool was in some way regulated.

The data in Figure 4. are expressed as percentages of total label incorporated in the barley leaf experiments. Expressing the data in this manner rather than as dpm incorporated distinguishes between potential effects of the inhibitor on uptake and effects of the inhibitor on metabolism. For example if the incorporation of CO_2 had been decreased solely due to AAN causing partial stomatal closure the distribution of ^{14}C would not be significantly altered. This was however not the case since the distribution of label was changed.

Before feeding $^{14}CO_2$ to corn leaves an attempt to find an optimal inhibitory concentration of AAN was undertaken. The cut ends of leaves were allowed to take up 0, 10, 20, or 40mM AAN for 4.5 hours prior to killing the tissue. As described in the Methods the amino acid fraction was separated and autoclaved for 2 hours in 6N HCl to ensure amide hydrolysis. The results (umol/mg chl.) obtained for serine and glycine were as follows.

	Control	10 mM AAN	20 mM AAN	40 mM AAN
Serine	1.17±0.09	1.21±0.33	0.97±0.11	1.71±0.44
Glycine	1.21±0.09	10.85±2.12	30.0±6.3	29.5±5.0

These data suggest that the serine pool was not decreased in response to AAN concentrations whereas the glycine pool increased considerably. When AAN was run on the amino acid analyzer it eluted as a ninhydrin positive peak well before glycine or any other of the amino acids labelled in the barley experiments. However it was later found

that the autoclaving procedure converted AAN to glycine. This phenomenon was confirmed by thin layer chromatography. Glycine and AAN migrated separately in n-butanol:acetone:diethylamine:water, 70:70:14:35 by volume, but acid-hydrolyzed AAN ran as two spots, one of which co-migrated with authentic glycine.

In Table 2. the results of a $^{14}CO_2$ feeding to whole leaves of corn, pretreated with 20mM AAN, are presented. In this experiment, any AAN which may have been converted to glycine during hydrolysis would not contribute to the labelling of the glycine pool. Furthermore, the dpm/ug chl presented for the glycine fraction would have accumulated prior to the killing and analytical procedures.

In barley $^{14}CO_2$ fixation yielded over 50% of the label in the amino acids with glycine and serine comprising half of this value (Fig. 4.). In corn (Table 2., Fig. 5.) only 30% of the total label was found in the amino acid fraction with glycine and serine only representing a third of the amount. Despite this difference, as in the barley, glycine increased and serine decreased when AAN was supplied. Alanine was also reduced by the inhibitor. Overall net incorporation was also reduced by 40% of the control with particularly large decreases in the neutral fraction. The proportion of malate was greater in the treated tissue but the net label incorporated was nearly the same. This was also true for glycerate and the formic acid wash. The latter contains 3-PGA, pyruvate and other acidic compounds (see Appendix C).

In the controls only a small amount of ^{14}C was detected in glycollate (0.76% of the total). In contrast, the inhibitor-treated tissue contained 5 times that of the control and accounted for 6.4% of the total incorporated. This result and the observation that glycine accumulates label due to AAN treatment suggests that a relatively small amount of the ^{14}C incorporated was passing through a glycollate pathway in corn.

Bundle Sheath Cell Bicarbonate Labelling Studies

Based on the whole leaf findings and the reports in the literature that the enzymes of the glycollate pathway are located within the bundle sheath cells it was decided to examine the nature of the glycollate pathway using isolated bundle sheath cells of corn

Table 2. Products of $^{14}CO_2$ fixation in corn leaves.*
2 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Aspartate	830	10.1	450	9.0
Serine	330	4.0	40	0.8
Glutamate	59	0.72	45	0.90
Glycine	390	4.8	500	10.0
Alanine	830	10.1	300	6.0
Total	2450	29.9	1340	26.9
Organic acids				
Glyceric	180	2.2	170	3.4
Glycollate	62	0.76	320	6.4
	43	0.52	31	0.62
Malate	610	7.4	690	13.8
Total	900	11.0	1210	24.3
Formic acid wash (8N)	1020	12.4	1010	20.3
Neutrals	3840	46.8	1410	28.3
Total incorporated	8200	100	4980	100

* Cut ends of whole leaves placed in 20 mM AAN 4.5 hours prior to feeding labelled $^{14}CO_2$.

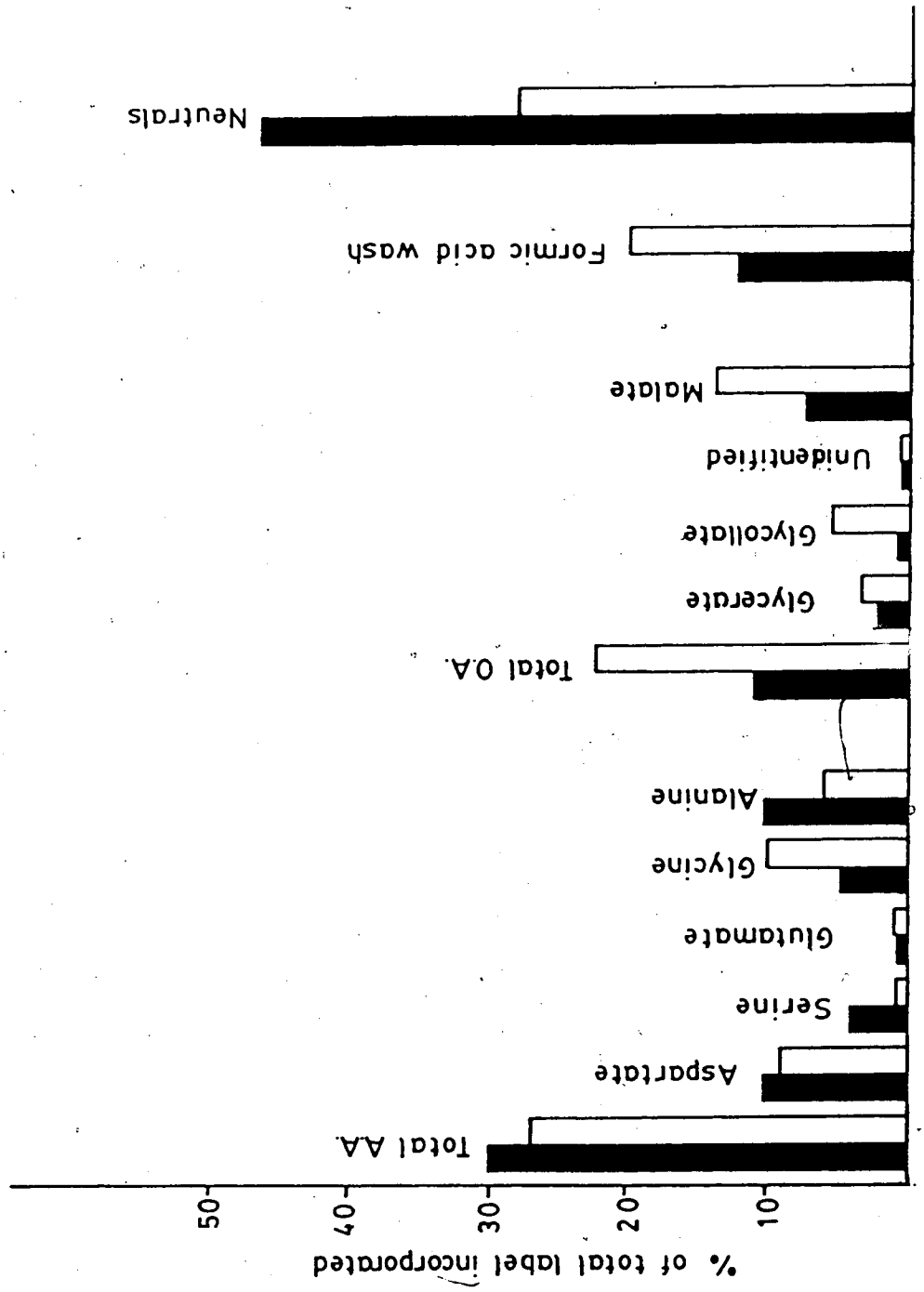


Fig. 5 Products of $^{14}CO_2$ fixation in corn leaves.
2 minute labelling; effect of aminoacetonitrile.

and sorghum. These experiments involved studies of bicarbonate and glycollate metabolism.

The method for isolation of bundle sheath cells was that of Chapman et. al. (1980). These authors determined optimal conditions which provide good bundle sheath preparations from corn as well as establishing conditions favorable to good CO_2 fixation. To check the purity of the isolated bundle sheath cells, prior to metabolic studies, cell suspensions were routinely examined by light microscopy. Chlorophyll a/b ratios were also determined, since it is reported that bundle sheath cells have a higher a/b ratio then mesophyll cells (Woo and Osmond, 1971). Assays of RuBP carboxylase and PEP carboxylase were also undertaken on bundle sheath cell extracts. As reported in the literature, PEP carboxylase is located in the mesophyll cells and the RuBP carboxylase in the bundle sheath cells (Chen et. al., 1974) Table 3. summarizes the chlorophyll a/b ratios (Also see appendix B) and the carboxylase data for both species. The specific activity of PEP carboxylase in corn bundle sheath cells was 8.5% that of the whole leaf; in sorghum the corresponding value was 11.1%. In sorghum the RuBP carboxylase activity was higher in the bundle sheath cell than in the whole leaf. After being satisfied that good bundle sheath cell preparations with low PEP carboxylase activity could be isolated, feeding experiments were undertaken. This involved the study of the effects of AAN on $NaH^{14}CO_3$ fixation by corn and sorghum bundle sheath cells. These results are presented in Tables 4 and 5 and in Figures 6 and 7. In the experimental design, the tissues were killed by adding boiling water directly to the sample flasks thus excess AAN was not removed from the suspensions and became part of the aqueous extract as the samples were prepared. As a result the unlabelled glycine pool was artificially increased. During chromatography this caused the glycine and alanine fractions, which normally elute closely together, to be poorly resolved. The counts for glycine and alanine were therefore pooled. It is assumed, based on whole leaf results, that the amount of label found in alanine would be no larger than the control. When the glycine and alanine peaks were run on thin layer chromatography and subjected to autoradiography only the glycine spot contained enough radioactivity to cause the film to develop in a two week exposure. Overspotting with unlabelled glycine and alanine ensured that the compounds were separating as determined by the ninhydrin spray reaction. The concentration of AAN used was 20mM, which was the

Table 3. Chlorophyll ^{a}/b ratios and carboxylase assays. *

Chlorophyll ^{a}/b ratios.

	Whole leaf	Bundle sheath cells
Zea mays L.	3.13±0.36 n=8	4.85±0.34 n=9
Sorghum bicolor L.	3.73±0.71 n=2	4.80±0.49 n=6

Carboxylase assays.

Zea mays L.

	Whole leaf	Bundle sheath cells
RuBP carboxylase	4.88	5.10
PEP carboxylase	4.69	0.40

Sorghum bicolor L.

RuBP carboxylase	8.40	363.8
PEP carboxylase	385.9	43.2

µmol $NaH^{14}CO_3$ fixed per mg protein per hour.

*Chlorophyll ^{a}/b ratios determined by method of Harborne (1973). Carboxylase crude enzyme extracts assayed by method of Kennedy (1976). ±std. dev.; n=number of determinations.

Table 4. Metabolism of $NaH^{14}CO_3$ in corn bundle sheath cells.* 10 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fractions 5-9	77.58±12.6	2.2	77.26±33.8	2.4
Aspartate	69.60±1.2	2.0	26.70±9.9	0.82
Serine	55.96±27.5	1.6	38.26±11.5	1.2
Glutamate	49.16±12.8	1.4	55.37±12.7	1.7
Glycine	289.3 ±16.5	8.2	671.0 ±178	20.6
Alanine	162.3 ±42.0	4.6	n.r.	
Total	703.9	20.0	868.6	26.7
Organic acids				
Glyceric	92.81±17.7	2.6	107.6±33.4	3.3
Glycollate	251.7 ±37.0	7.2	169.9±11.5	5.2
Malate	737.6 ±62.5	21.0	179.7±88.0	5.5
Total	1082	30.8	457.2	14.0
Formic acid wash (8N)	1411.7±531	40.1	1439.0±339	44.2
Neutrals	320.3±130	9.1	493.3±121	15.1
Total incorporated	3518	100	3258	100

* 0.35 µmol of $NaH^{14}CO_3$ (20 µCi) was fed to cell suspensions containing a total of 56.4 µg of chlorophyll. Values are given with ±standard deviation. n=3. n.r.= not resolvable; see text.

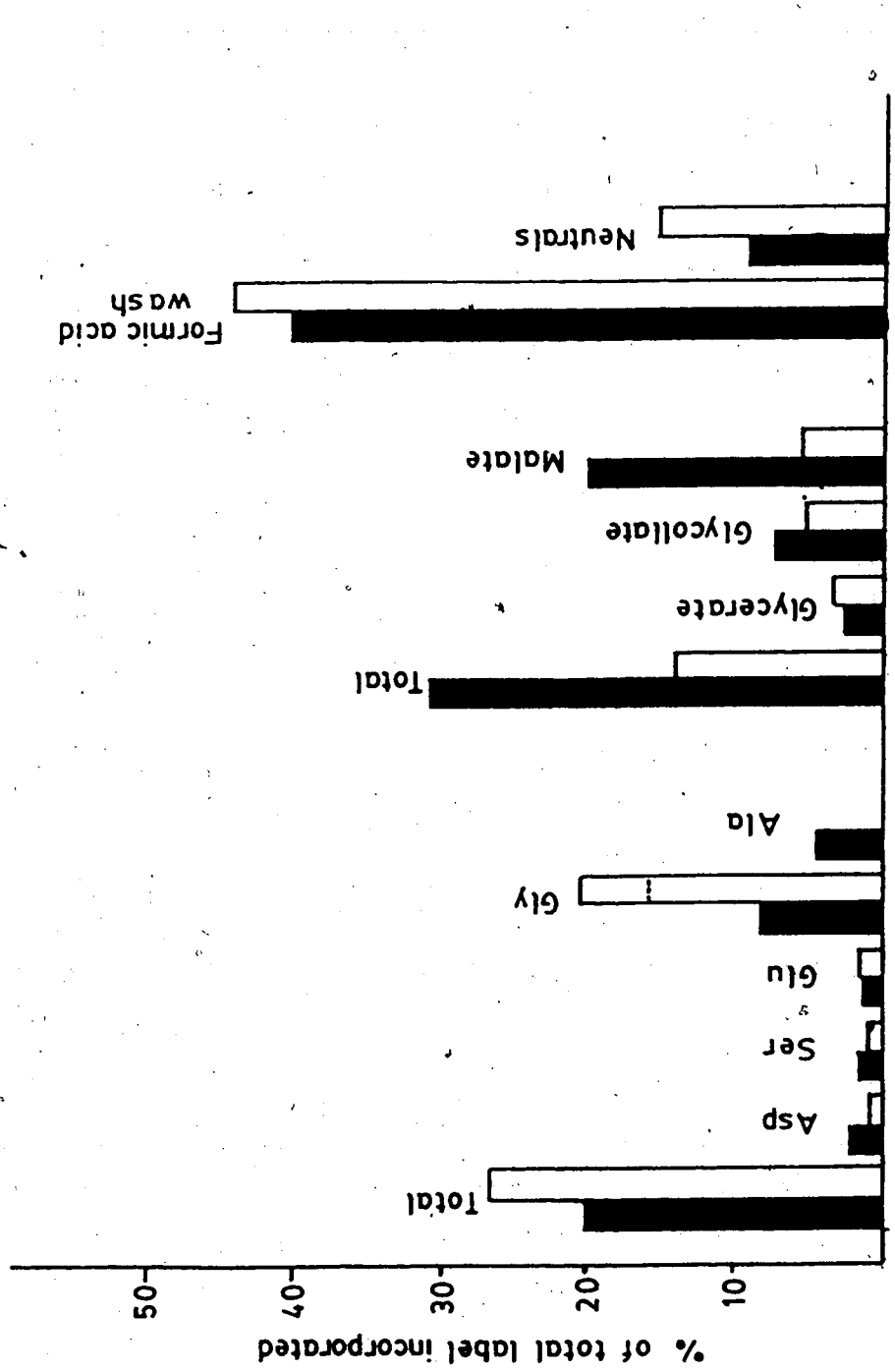


Fig6 Metabolism of NaH $^{14}CO_3$ in corn bundle sheath cells.
10 minute labelling; effect of aminoacetonitrile. ■ control □ 20 mM AAN

Table 5. Metabolism of $NaH^{14}CO_3$ in sorghum bundle sheath cells.* 10 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fractions 4-7	1444 ±200	6.2	118.9±19.4	1.4
Fractions 7-12	1979 ±2.8	8.5	272.6±122	3.3
Aspartate	481.5±8.4	2.1	n.d.	n.d.
Glutamate	45.9±2.6	0.20	217.3±42.7	2.6
Glycine	516.3±0.8	2.2	1371 ±156	16.6
Alanine	243.9±72.8	1.0	n.r.	n.r.
Total	4710	20.2	1980	23.9
Organic acids				
Glycerate	2334 ±962	10.0	208.0±69.2	2.5
Unidentified	233.3±72.8	1.0	312.9±58	3.8
Glycollate	8310 ±23.1	35.7	950 ±237	11.5
Malate	5193 ±494	22.3	729.3±161	8.8
Total	16071	69.0	2200	26.6
Formic acid wash (8N)	1680 ±350	7.2	1511 ±239	18.2
Neutrals	804 ±350	3.4	2589 ±118	31.3
Total incorporated	23265	100	8280	100

* 0.35 µmol of $NaH^{14}CO_3$ (20 µCi) was fed to cell suspensions containing a total of 65.1 µg of chlorophyll. Values given are with ± standard deviation. n=3. n.d.= not detectable, counts are not above background. n.r.= not resolvable; see text.

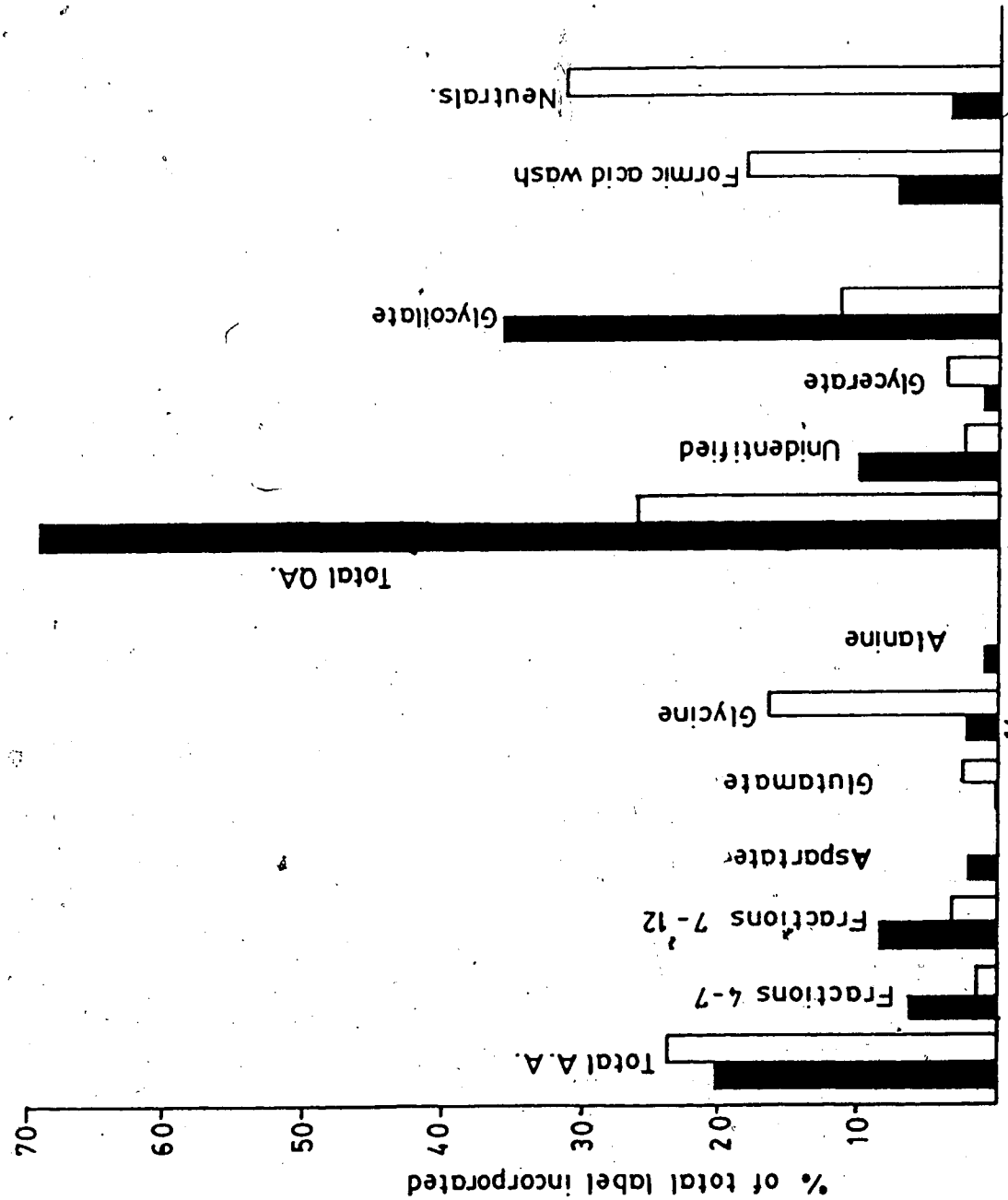


Fig. 7 Metabolism of $NaH^{14}CO_3$ in sorghum bundle sheath cells. 10 minute labelling; effect of aminoacetonitrile. ■ control □ 20 mMAAN

optimal concentration for inhibition found by Usuda *et. al.* (1980) for wheat protoplasts.

The labelled bicarbonate feeding to bundle sheath cells of corn demonstrated that the suspensions were photosynthetically active. Glycollate, glycine, and serine were products of the bicarbonate metabolism which suggests that there was an operating glycollate pathway. This is further supported by the effect of AAN in glycine. The decrease in the labelling of serine was however only marginal. Since glycine accumulated it is interesting that glycollate, which is a precursor of glycine, did not accumulate. The overall amount of label found in glycollate was clearly reduced as was the percentage of the total accounted for by glycollate. Malate was labelled in both the control and AAN-treated bundle sheath cells. As noted in Table 3., the preparations were not entirely lacking PEP carboxylase activity, an enzyme that might indirectly account for malate formation. It is notable that the inhibitor-treated suspensions had only 25% as much ^{14}C in malate as the controls. If the glycollate and glycerate pathways are operating in the bundle sheath cells as in C_3 plants this effect on malate may be due to the block in the glycine to serine conversion diminishing the amount of precursors regenerated in the Calvin cycle. Since 3-PGA is a precursor of PEP, the amount of substrate for PEP carboxylase would be reduced and thus the amount of malate would be reduced. This reasoning could also explain why the amount of ^{14}C in glycollate is also reduced by AAN. In this regard, Usuda et al. (1980) suggested that the supply of RuBP would be smaller and thus this would reduce the potential for O_2 competition in forming glycollate. The total amount of ^{14}C incorporated was only slightly reduced by AAN in these present experiments (Table 4.). It should be pointed out that malate and hydroxypyruvate were not separated by the formic acid gradient method. However, thin layer chromatography and autoradiography showed that activity was only present in the malate fraction.

The general observations for corn also apply to the data for sorghum bicarbonate labelling (Table 5.). The effect of AAN on total incorporation in sorghum was however even more pronounced. The amount of total incorporation in the sorghum control was almost 3 times that of the inhibitor treated tissue. The effects on glycine, glycollate, and malate are as they were in corn. The reduction in the organic acid totals was very pronounced. With the sorghum bicarbonate feeding there was however no serine labelling. This may indicate a small serine pool which rapidly turns over or that serine is not synthesized in the bundle

sheath cells but in the mesophyll cells.

In both corn and sorghum, glutamate-^{14}C was increased in inhibitor-treated tissue. This effect was more pronounced in sorghum than in corn. Glutamate may supply the necessary amino group to glyoxylate in synthesizing glycine in the peroxisome (Tolbert, 1980) and may itself be regenerated by accepting NH_3 arising from glycine decarboxylation in the mitochondria. Since the inhibitor prevents glycine decarboxylation the supply of NH_3 and the amount of glutamate would be diminished. The demand for amino groups would also decrease the amount of glutamate. However the increase in glutamate labelling is contradictory to this line of reasoning.

Serine can also provide the necessary amino group for the glyoxylate to glycine step of the pathway (Tolbert, 1980). If the demand for amino groups is high this could explain why no label is detected in serine since there would be a rapid turnover. This would then mean that label should be found in hydroxypyruvate and glycerate. As explained before hydroxypyruvate-^{14}C was not found but labelling of glycerate was apparent. In corn cells glycerate labelling was slightly higher in the inhibitor-treated suspension. This is contrary to a flow from glycine through serine and the glycerate pathway. However in the sorghum tissue there is a marked decrease in labelling due to the inhibitor.

There are two possible pools of serine, one mitochondrial, the other peroxisomal. It would be the mitochondrial pool which would be most directly linked to the glycine pool assuming serine arises by action of glycine decarboxylase and serine hydroxymethyl transferase. The phenomena observed with malate and the total incorporated label can be reasonably explained by a linked glycollate-glycerate pathway. However, the absence of serine labelling in sorghum and the small decrease observed in corn are clearly not consistent with the linked operation of such a pathway.

Bundle Sheath Cell Glycollate Labelling Studies

As noted above, the evidence supports an operating glycollate pathway up to the point where glycine is synthesized. With this in mind, experiments to further explore the glycollate pathway were undertaken using glycollate-2-^{14}C. As reported in the literature, bundle sheath cells were capable of metabolizing glycollate-1-^{14}C to release $^{14}CO_2$ (Rathnam, 1979). This would indicate that a decarboxylation was occurring. The

disadvantage of using glycollate-1-^{14}C glycollate is that the metabolic fate of the remaining carbon could not be followed and refixation of $^{14}CO_2$ would contribute label to pools not directly involved in the glycollate pathway. For these reasons glycollate-2-^{14}C was used. As outlined in the Introduction, $^{14}CO_2$ arising from glycollate-1-^{14}C could come indirectly from glycine decarboxylation or directly from a non-enzymatic decarboxylation of glyoxylate. For this reason all classes of metabolites were fractionated and counted.

Tables 6-9 report the results of glycollate-2-^{14}C feeding to corn and sorghum bundle sheath preparations and the effect of added AAN. Again, as with the bicarbonate feeding studies, the entire contents of the bundle sheath sheath suspensions were processed because the tissue was killed without removal of the unmetabolized glycollate. The percent of glycollate metabolized was determined by reference to the amount of glycollate remaining after the feeding period.

In all of the corn controls (Tables 6,7,and 11) over 60 % of the total glycollate metabolized was found in just 3 compounds. These compounds were glyoxylate, formate, and glycine. Glyoxylate, the immediate product of metabolism in the glycollate pathway was an expected product. The production of formate-^{14}C implies that either glyoxylate was undergoing direct decarboxylation in the peroxisome or that possibly methylene-tetrahydrofolate was converted to this product in the mitochondria. Of particular note is the absence of detectable label in serine in any of the bundle sheath cell controls.

Corn bundle sheath cells pretreated with 20 mM AAN for 15 minutes and fed glycollate-2-^{14}C metabolized less glycollate than the controls. After a 10 minute labelling (Table 6, Fig. 8) the amount of glycine-^{14}C in the inhibitor-treated tissue was less than half the amount detected in the controls but the percent of the total was the same. Labelling of formate and glyoxylate was also reduced but with these compounds, the percent of total was also reduced. The 20 minute labelling experiment (Table 7) also revealed a reduction in the amount of glycollate metabolized due to AAN. The difference from the 10 minute labelling was that glycine was reduced in both the amount of label and the percentage of the total. Glyoxylate also behaved differently in that the amount of label was the same for

Table 6. Metabolism of glycollate-2-^{14}C in corn bundle sheath cells.* 10 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fractions 5-9	87.7±30.4	2.0	80.3±23	4.4
Glycine	341.9±62	8.0	146.4±66	8.1
Total	431	10.1	263	14.5
Organic acids				
Glycollate metabolized	42 %		28 %	
Formate	1889 ±1089	44.2	660.5±147	36.4
Glyoxylate	998.8±450	23.4	64.4±45.7	3.5
Total	2888	67.6	724.9	39.9
Formic acid wash (8N)	308.3±171	7.2	300.5±27.7	16.6
Neutrals	641.5±169	15.0	526.8±197	29.0
Total incorporated	4269	100	1815	100

* 0.01 µmol of glycollate-2-^{14}C (0.5 µCi) was fed to cell suspensions containing a total of 65.6 µg of chlorophyll. Values given are ± standard deviation. n=3.

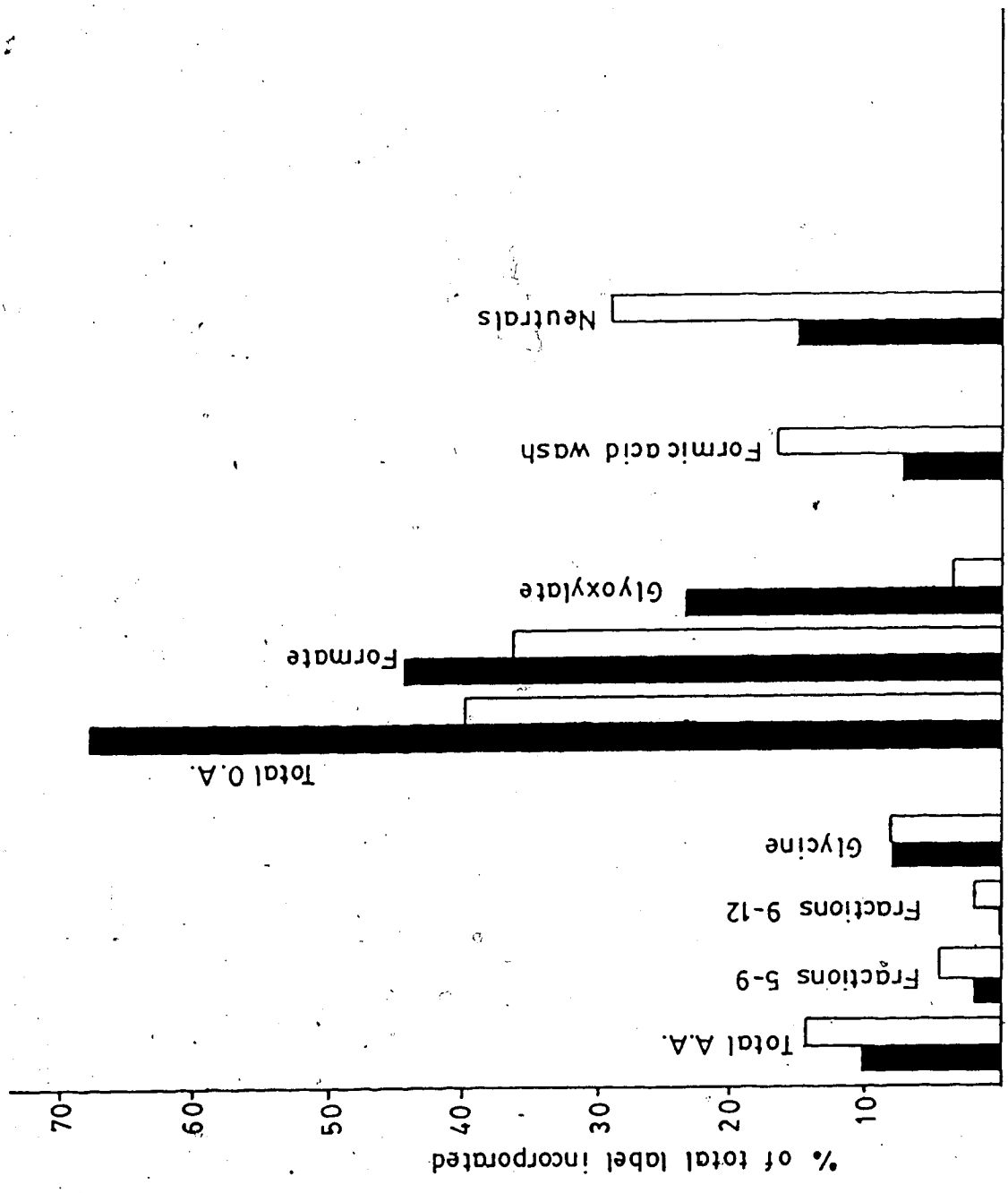


Fig. 8 Metabolism of glycollate-2-^{14}C in corn bundle sheath cells. 10 minute labelling; effect of aminoacetonitrile. ■ control □ 20mM AAN.

Table 7. Metabolism of glycollate-2-^{14}C in corn bundle sheath cells.* 20 minute labelling; effect of aminoacetonitrile.

	Control		20 mM AAN	
Product	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fractions 5-9	759.8±31.8	7.9	387.5±23.6	6.6
Fractions 9-12	397.3±68.4	4.1	413.5±23.6	7.0
Aspartate	17.3±7.9	0.17	67.9±89.3	1.2
Glycine	1076±141	11.2	106.0±61.4	1.8
Total	2251	23.4	975	16.5
Organic acids				
Glycollate metabolized	46%		20%	
Formate	4046±686	42.0	1528±206	26.0
Glyoxylate	1052±437	11.0	1151±156	26.0
Total	5099	53.0	2679	45.6
Formic acid wash (8N)	221.6±116	2.3	4213±232	7.2
Neutrals	2036±378	21.2	1804±310	30.7
Total incorporated	9607	100	5879	100

*0.01 µmol of glycollate-2-^{14}C (1.1×10^6 dpm) was fed to cell suspensions containing a total of 47.0 µg of chlorophyll. Values given are with ± standard deviation. n=3

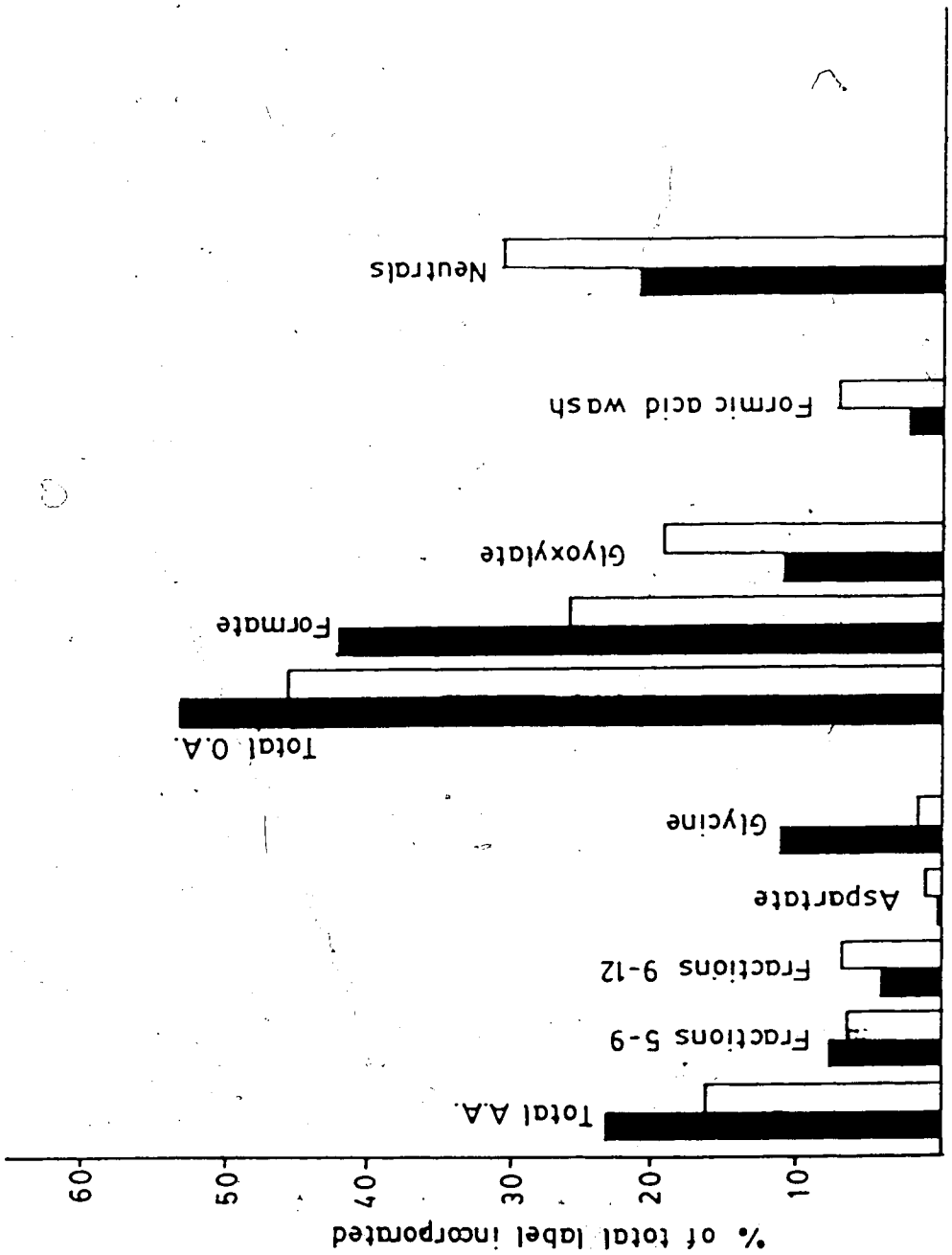


Fig.9 Metabolism of glycollate-2-^{14}C in corn bundle sheath cells
20 minute labelling; effect of aminoacetonitrile. ■ control □ 20 mM AAN

Table 8. Metabolism of glycollate-2-^{14}C in sorghum bundle sheath cells.* 10 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fraction 5-9	19.95±3.5	0.45	19.05±5.7	0.69
Unidentified	16.27±1.3	0.37	3.01±1.7	0.11
Glycine	2761 ±161	62.1	274.6 ±43	9.97
Total	2797	63.4	296.3	10.8
Organic acids				
Glycollate metabolized	58 %		36 %	
Formate	535.0 ±108	12.1	1200 ±95.2	43.6
Glyoxylate	508.7 ±159	11.5	205.6 ±76.2	7.5
Total	1043.7	23.6	1405.4	51.0
Formic acid wash (8N)	103.5 ±20.2	2.3	139.5 ±62.0	5.1
Neutrals	468.7 ±23.8	10.6	912.9 ±143	33.1
Total incorporated	4413	100	2754	100

* 0.01 µmol of glycollate-2-^{14}C (0.5 µCi) was fed to cell suspensions containing a total of 145.3 µg of chlorophyll. Values given are with ± standard deviation. n=3.

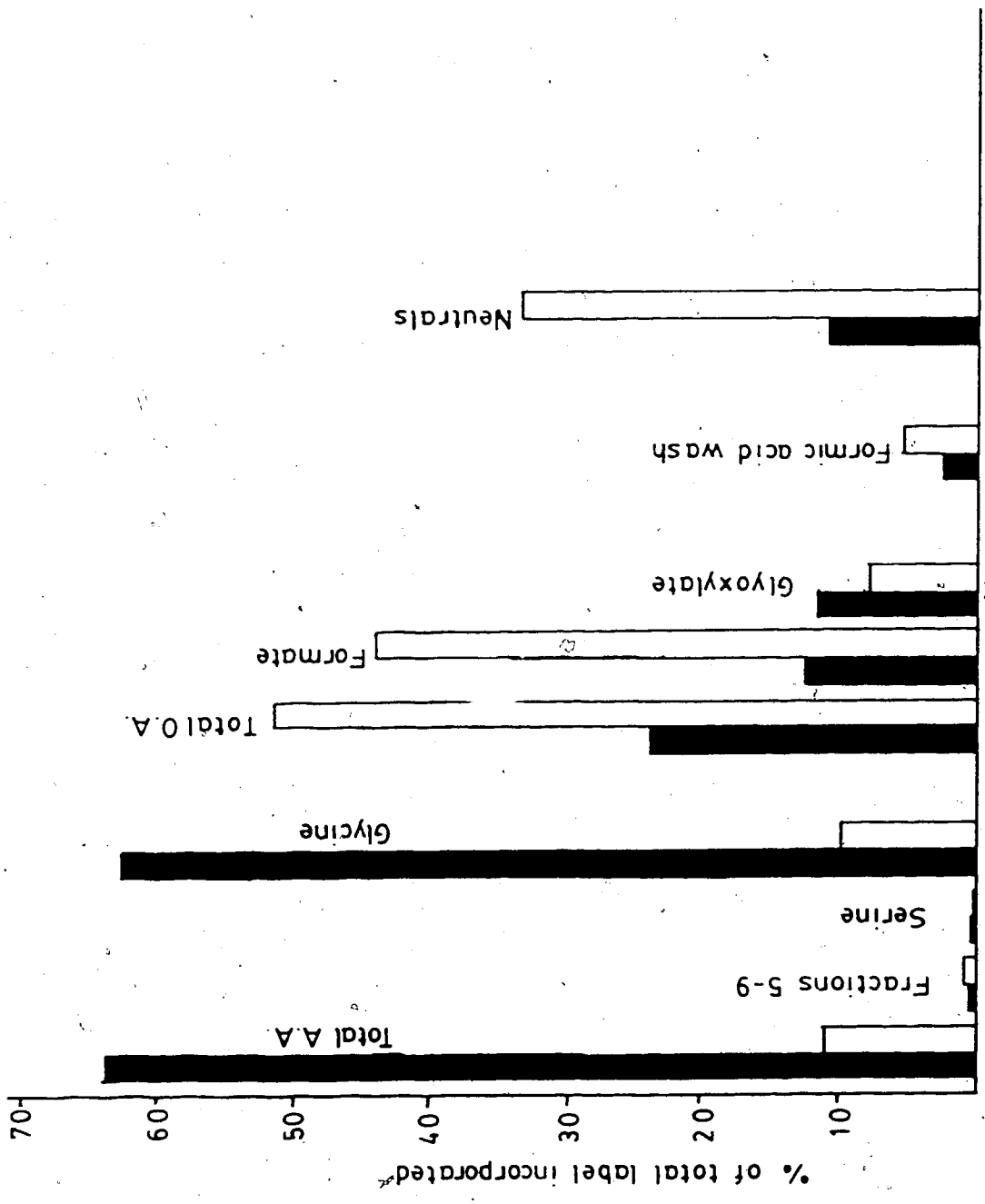


Fig.10 Metabolism of glycollate-2-^{14}C in sorghum bundle sheath cells. 10 minute labelling; effect of aminoacetonitrile. ■ control □ 20mM AAN

Table 9. Metabolism of glycollate-2-^{14}C in sorghum bundle sheath cells.* 30 minute labelling; effect of aminoacetonitrile.

Product	Control		20 mM AAN	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fraction 5-9	20.31±4.5	0.37	21.57±5.9	0.52
Glycine	1158 ±85.6	21.2	242.7 ±37.3	5.8
Total	1178	21.6	264.2	6.3
Organic acids				
Glycollate metabolized	60 %		41 %	
Formate	1628 ±63.2	29.8	1894 ±334	45.5
Glyoxylate	1570 ±300	28.8	736.1 ±119	17.7
Total	3198	58.6	2630	63.2
Formic acid wash (8N)	229.1 ±60.2	4.2	618.4 ±296	14.9
Neutrals	850.5 167	15.5	647.9 ±236	15.6
Total incorporated	5456	100	4160	100

* 0.01 µmol of glycollate-2-^{14}C (0.5 µCi) was fed to cell suspensions containing 86.49 µg of chlorophyll. Values given are with ± standard deviation. n=3.

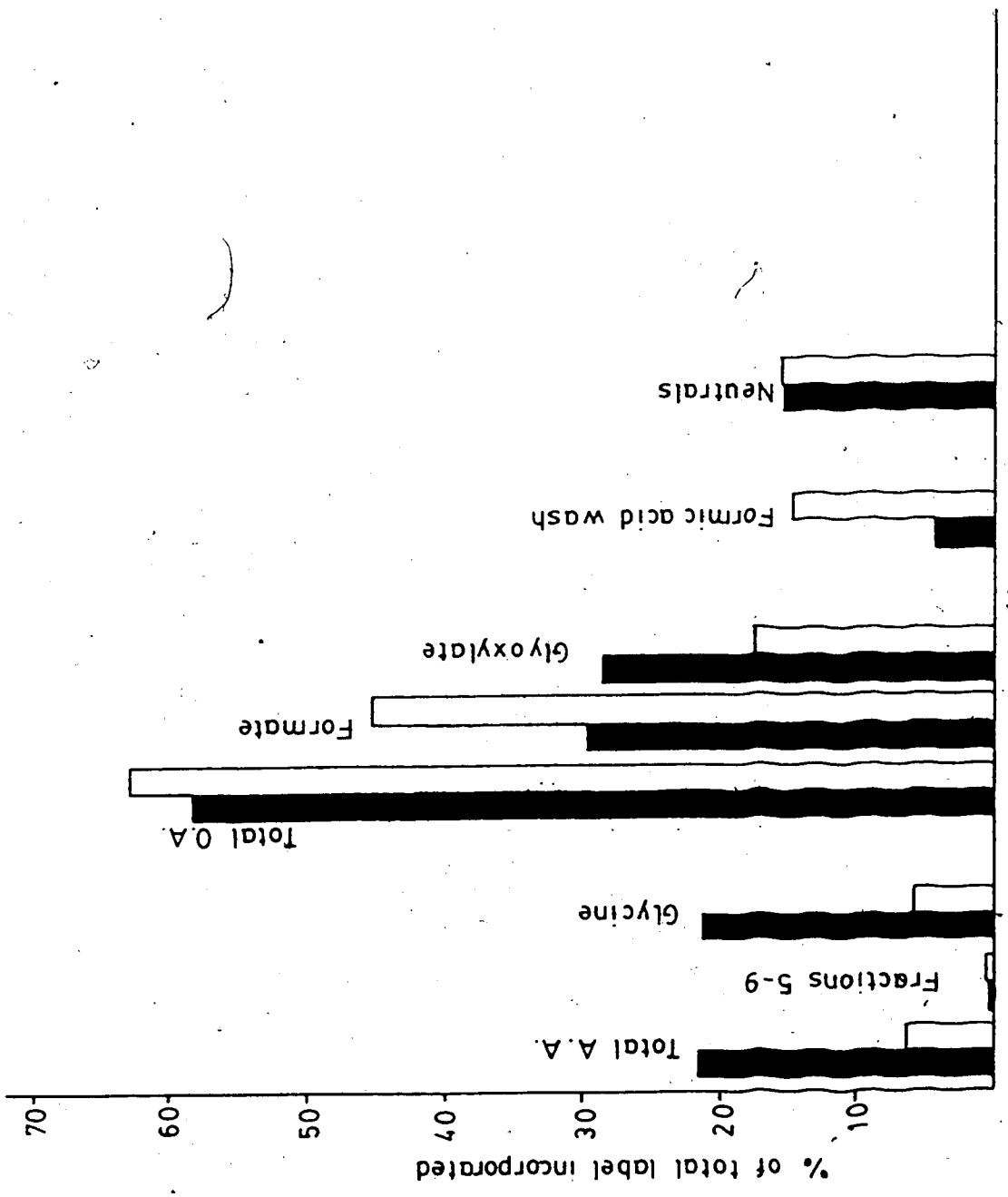


Fig 11 Metabolism of glycollate-2-^{14}C in sorghum bundle sheath cells. 30 minute labelling; effect of aminoacetonitrile. ■ control □ 20 mM AAN

both control and treatment but the percentage of the total was higher in the inhibitor-treated tissue. The effect on formate was shown for both the 10 and 20 minute labelling experiments.

The effect of AAN on glycine production was contrary to that expected. Since this compound prevents glycine decarboxylation it would be expected that AAN treatment would enhance glycine accumulation. As noted earlier it is possible that the amino group necessary for glycine formation was depleted by the AAN treatment. In this regard, this inhibitor potentially causes a reduction in serine and NH_3 which are involved in glycine synthesis.

Since less ^{14}C-formate was produced in the inhibitor-treated tissue it can be inferred that that this product arose from more than the direct decarboxylation of glyoxylate. Direct decarboxylation should be most favorable when the glyoxylate pool is greatest (Walton 1982). When the flow of carbon into glycine is reduced (as seen in Tables 5 and 6) there should be an increase in the amount of glyoxylate available for direct decarboxylation. Such an increase is seen in the 20 minute labelling after inhibitor treatment yet the amount of labelled formate is less than in the control. AAN treated tissue would have less methylene-THFA. This would suggest that some of the formate in corn is arising via this alternate route.

The results of glycollate-2-^{14}C feeding to sorghum bundle sheath cells (Tables 8 and 9) are similar to the results obtained for corn. Again the incorporation of ^{14}C into glycine was suppressed by AAN. Even in the controls, serine labelling was not detected. As in the corn experiments, the glyoxylate labelling was reduced by AAN treatment. The major difference in the two species was the effect of AAN on the formate labelling. Unlike corn bundle sheath cells, formate labelling was increased with AAN treatment.

In attempting to explain some of the effects of AAN on glycine labelling in the glycollate-2-^{14}C feeding experiments it was suggested that shortage of amino groups may affect the extent of glycollate metabolism. To examine this hypothesis, glycollate-2-^{14}C was supplied to serine-supplemented bundle sheath cells. Serine was chosen for two reasons. In the peroxisome serine can provide the necessary amino group required in converting glyoxylate to glycine (Tolbert, 1981) and in the mitochondria it would enlarge the normal pool size thus slowing the turnover of serine-^{14}C which may be

arising through the glycollate pathway from glycine. Serine was also reported to be preferred over glutamate as an amino donor in spinach beet chloroplasts by Walton and Butt (1981). The results of these experiments are presented in Tables 10 and 11.

As in the previous 10-minute experiments the inhibitor decreased the amount of label detected in glycine. The 30 minute preincubation with added serine resulted in a 2.75 fold increase in the amount of label recovered in glycine. The combination of serine and AAN yielded a glycine value intermediate between the control and the 20 mM AAN treatment. The serine pretreatment did not result in trapping of ^{14}C in this amino acid pool.

Table 11 gives the results of a 30 minute glycollate-2-^{14}C feeding experiment to corn bundle sheath cells with and without added serine. Cells supplemented with serine contained serine-^{14}C as a labelled product. This product however only accounted for a small percentage of the total label metabolized (0.5%). Label recovered in the glycine fraction of serine pretreated cells was substantially higher than in the contol. Both the formate and the glyoxylate labelling were also decreased by this treatment. The total ^{14}C incorporated was increased by the serine pretreatment. These results suggest that the supply of amino groups might limit the flow of carbon from glycollate through to glycine.

If the pool size of serine in the bundle sheath cells was much smaller than that of glycine it is conceivable that serine labelling would be minimal in all the glycollate-2-^{14}C labelling experiments except those involving serine pretreated cells. To examine this possibility, bundle sheath cells from the controls of both corn and sorghum preparations were analyzed for endogenous amino acids. The values obtained for sorghum were 0.40 umol/mg chl and 0.41 umol/mg chl for glycine and for serine respectively. For corn the averages of four determinations were 1.16±0.10 umol/mg chl and 1.29±0.17 umol/mg chl respectively. These values suggest that the total amounts of these amino acids are similar, although it should be emphasized that the peroxisomal and mitochondrial pools could well have been dissimilar. Other assays of whole leaf extracts of corn also indicated that the total amount of free glycine and serine were similar.

Table 10. Metabolism of glycollate-2-^{14}C in sorghum bundle sheath cells.* 10 min. labelling; effect of aminoacetonitrile and added serine.

Product	dpm/µg chl. Control	20 mM AAN	1 mM Serine	Serine; AAN
Amino acids				
Fractions 5-9	229.0±14.2	n.d.	115.7±30.8	n.d.
Fractions 11-15	87.8±6.3	21.5±10.3	70.8±34.1	9.7±3.5
Glycine	393.4±105	40.2±17.8	1082 ±14.9	229.9±12.4
Total	710.2±115	61.7±17.2	1268 ±72.3	239.6±12.6
Organic acids				
Glycollate metabolized†	(24%)	(18%)	(24%)	(–)
Formate	1265 ±130	603.5±154	832.7±85.3	437.0±66.1
Glyoxylate	32.7±17.1	156.5±85.3	28.2±26	55.6±12.4
Formic acid wash (8N)	239.2±68.2	254.7±120	243.5±190	273.6±146
Neutrals**	(503)	(1068)	(377.5)	(–)
Total incorporated**	(2750)	(2144)	(2750)	(–)

* 0.01 µmol of glycollate-2-^{14}C (0.5 µCi) was fed to cell suspensions containing 94.7 µg of chlorophyll. † Minimum estimate for amount of glycollate metabolized. ** Based on a single determination for neutrals. All but one each of the control, 20 mM AAN, and 1 mM serine were stored frozen at -20 C for 40 days before separation of the organic acids and neutrals was undertaken. Due to the instability of glycollate-2-^{14}C while frozen an inordinate amount of label in the neutral fraction, with a corresponding loss in the organic acid fraction, was detected. Consequently only a single value was obtained for the neutrals and the amount of glycollate metabolized. Values are given with ±standard deviation. n=3

Table 11. Metabolism of glycollate-2-^{14}C in corn bundle sheath cells.* 30 min labelling; effect of added serine.

Product	Control		1 mM Serine	
	dpm/µg chl.	%	dpm/µg chl.	%
Amino acids				
Fractions 5-9	531.2±112	4.2	409.2±97.2	2.6
Fractions 9-12	93.1±16.6	0.74	83.5±11.6	0.53
Serine	n.d.	n.d.	79.6±8.9	0.51
Glycine	371.4±63.8	3.0	6431 ±272	40.8
Total	996 ±155	7.9	7003 ±366	44.4
Organic acids				
Glycollate metabolized	59.9%		64.6%	
Formate	7289 ±1151	58.2	3988 ±589	25.3
Glyoxylate	233.1±74.9	1.9	158 ±7.3	1.0
Total	7522	60.1	4146	26.3
Formic acid wash (8N)	801 ±398	6.4	1034 ±678	6.6
Neutrals	3203 ±1852	25.6	3570 ±98.9	22.7
Total incorporated	12522	100	15753	100

* 0.01 mol of glycollate-2-^{14}C (0.5 µCi) was fed to cell suspensions containing a total of 37.2 µg of chlorophyll. Values are given with ± standard deviation. n=3.

n.d. = not detectable, counts are not above background.

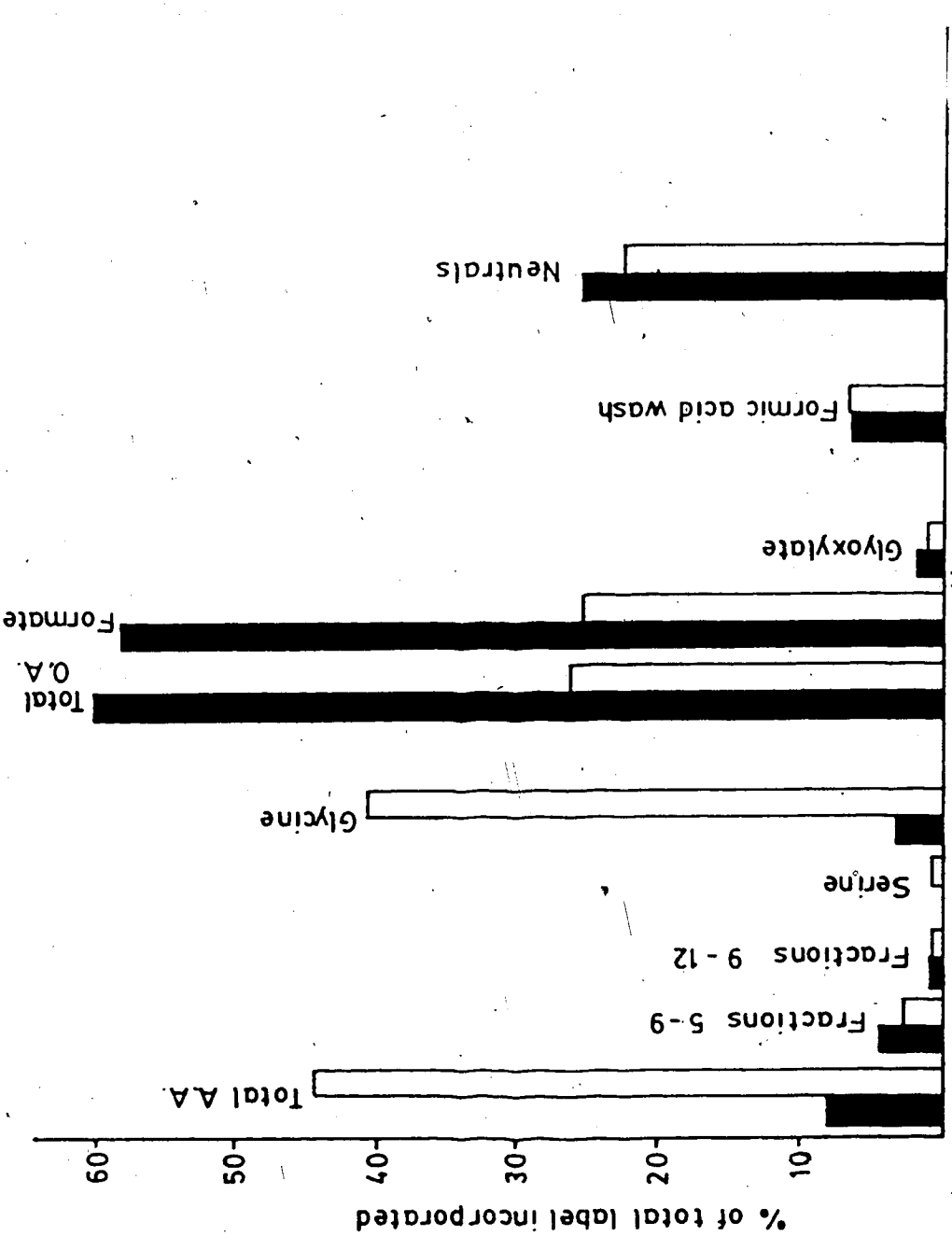


Fig. 12 Metabolism of glycollate-2-^{14}C in corn bundle sheath cells.
30 minute labelling; effect of 1mM serine. ■ control. □ 1mM serine.

The data reported in the tables contain several unidentified, ^{14}C labelled compounds. In most cases they account for less than 5% of the total ^{14}C metabolized. The most commonly found were recovered in the amino acid fraction and are indicated as fractions 5–9 and fractions 9–12. Attempts to identify these peaks were not successful. Despite this it was clear that these compounds did not include methionine sulphoxide, methionine sulphone, phosphoserine nor any of the 20 common protein amino acids for which the the elution times were known. The fractions 5–9 correspond to compounds that are not retained by the PA28 resin used for the amino acid analyzer. Fractions 9–12 are only slightly retained which suggests that the compound(s) at pH 2.2 is neutral or negatively charged. Yet at pH 7.0 these compounds were retained by the hydrogen column and did not elute until washed with 6N HCL.

IV. Discussion

Assimilation of $^{14}CO_2$ by barley and corn leaves and the metabolism of $NaH^{14}CO_3$ by bundle sheath protoplasts of corn and sorghum were inhibited by aminoacetonitrile (Tables 1,2,4,5). Usuda et. al. (1980) reported the same type of effect on assimilation in wheat protoplasts but not in isolated chloroplasts. They proposed that this phenomenon could be explained on the basis that AAN prevented flow of carbon through the glycerate pathway and thus reduced the levels of Calvin cycle intermediates, particularly RuBP. Carbon would then accumulate in glycine instead of being cycled. There is however a recent report in the literature that tends to contradict the above conjecture. Creach and Stewart, (1982) exposed soybean leaf discs to oxygen concentrations ranging from 2 to 60%. They found that AAN had no effect on RuBP levels at low O_2 levels but at high O_2 concentrations the amount of RuBP increased. The level of glycine also increased. These observations, as suggested by the authors, imply that the supply of RuBP does not limit CO_2 fixation but rather a direct inhibition of RuBP carboxylase must occur. They also suggested that an intermediate of the glycollate pathway may cause this inhibition. A report by Cook and Tolbert (1982) demonstrating that glyoxylate (10mM) inhibited isolated spinach RuBP carboxylase/oxygenase suggested to Creach and Stewart that this could be the cause of reduced assimilation. However a recent paper by Mulligan *et. al.*, (1983) suggested that the effect brought about by the glyoxylate was due to changes in pH since salts of other weak acids caused a similar inhibition. It was also suggested by these authors that glyoxylate would not likely have a regulatory role since it is produced in the peroxisome and would have to be transported to the chloroplast to affect RuBP carboxylase. The absence of detectable glyoxylate-^{14}C labelling in either whole leaf $^{14}CO_2$ studies (Table 2) or bundle sheath cell bicarbonate studies (table 4 and 5) reported in this thesis indicated that the level of glyoxylate is low and thus this type of inhibition appears unlikely.

Usuda and Edwards (1980a) reported that at low ^{14}C-bicarbonate concentrations glycerate was a major product in studies of isolated bundle sheath cells of *Panicum capillare*. Aminoacetonitrile prevented glycerate accumulation. At high bicarbonate concentrations there was only a small amount of label in products of the glycollate pathway. In a subsequent report Usuda and Edwards (1980b) showed that glycerate kinase activity was not found in bundle sheath cells but was active in mesophyll cells. They

reasoned that the glycerate, formed in the bundle sheath cells, was not utilized via the reductive pentose pathway. *P. capillare* may be unique among C_4 plants in having a deficiency of glycerate kinase in the bundle sheath cells. On the other hand if this is a general property of C_4 plants it would be difficult to account for the reduction of glycerate labelling seen in the sorghum bundle sheath cells (Table 5) but not in corn, (Table 4) brought about by AAN since glycerate would not be contributing to the reductive pentose pathway in any case.

In the low ^{14}C–bicarbonate concentration (0.34mM) used by Usuda and Edwards, glycerate accounted for 20% of the label and glycine and serine together accounted for 45%. The concentration of bicarbonate used in the bundle sheath cell labelling in the present work was 0.11 mM. However, carrier malate was also added and this would increase the effective CO_2 concentration. Nevertheless glycine, serine, glycollate and glycerate were products of metabolism in the corn although not to the extent reported by Usuda and Edwards (1980) for *P. capillare*. Glycine did accumulate markedly in both corn and sorghum bundle sheath cells treated with AAN. Serine declined in corn but was not detected in sorghum. Glycerate behaved as reported for *P. capillare* in the sorghum tissue but was marginally increased in the corn. These data suggest that carbon is flowing through a glycollate pathway in the bundle sheath cells of both species. It remains to be demonstrated that such a carbon flow occurs in whole leaves of C_4 plants. In this regard, it is perhaps noteworthy that data for $^{14}CO_2$ fixation in corn leaves (Table 2) showed that glycollate was a minor product of fixation (0.75%) and that serine (4.0%) and glycine (4.8%) were also products. The AAN treatment increased the amount of glycollate to 6.4%, the amount of glycine to 10% and decreased the serine to 0.8%. These data indicate that a glycollate pathway operates in corn.

Experiments by Jenkins et. al. (1982) using corn leaves and the glycollate oxidase inhibitor 2–hydroxy–3–butynoic acid (HBA), showed that CO_2 assimilation was markedly decreased by this compound. After 30 minutes of $^{14}CO_2$ fixation, glycollate accumulated from less than 0.1% in the control to 14.1% of the total in the treated leaves. At the same time glycine declined from 1% to less than 0.1% and serine declined from 1.6 to 1.2%. These were 30 min $^{14}CO_2$ labelling experiments. In another paper (Jenkins *et. al.* 1983) the action of aminooxyacetate, an inhibitor of serine:glyoxylate aminotransferase was studied.

At a concentration of 10uM, corn assimilation was reduced by 42% and at 100uM incorporation fell by 72%. At both inhibitor concentrations glycollate increased from less than 0.1% of the total to 13.0 and 26.4% respectively. These changes were accompanied by decreases in glycine from 1% to less than 0.1%. Serine increased at the 10uM concentration rising from 1.6 to 2.5% but was reduced to 1.4% by the 100uM concentration.

It is clear that AAN used in the present work and HBA and aminooxyacetate used by Jenkins et al. (1982, 1983) produce effects that can be attributed to operation of a glycollate pathway. Other reports already mentioned in the introduction which showed that $^{18}O_2$ was incorporated into glycine and serine also provide evidence for an operating glycollate pathway in corn (Dimon *et al.* 1977). Thus the apparent absence of measurable photorespiration in C_4 plants would not result from an absence of an operable glycollate pathway. In agreement with this contention a recent report by Berger and Fock (1983) suggests that corn leaves have photorespiratory rates that are about 25% of those shown for C_3 plants. This estimate was based on data obtained by feeding ^{14}C, ^{15}N glycine to corn leaves with and without the presence of methionine sulfoximine (MSO) an irreversible inhibitor of glutamine synthetase. Under control conditions ^{15}N was found mainly in serine, ammonia, glutamate, and glutamine. This is consistent with the glycine metabolism being mediated by glycine decarboxylase and serine hydroxymethyl transferase. The inhibitor caused an accumulation of ^{15}N in NH_4 and reduced the ^{15}N levels of glutamine and glutamate. The photorespiratory rate for wheat leaves was 18% of true photosynthesis, for corn leaves the rate was only 4%. An earlier paper by Platt and Rand (1982) also revealed NH_4 accumulation in corn as well as sorghum during MSO treatment. They could not however identify the source of NH_4 in the C_4 tissue. From the Berger and Fock report it would appear that that source is glycine. The absence of measurable photorespiratory CO_2 in C_4 plants could be explained by the lowered rates of photorespiration calculated for C_4 plants and by assuming that the CO_2 released in photorespiration is refixed by PEP carboxylase as suggested by Rathnam (1979) (see Introduction).

There are many recent papers linking the flow of carbon through the glycollate pathway with the metabolism of NH_4 containing compounds within the plant (Keys *et. al.*, 1978). The synthesis of glycine in spinach beet was shown to be linked with

hydroxypyruvate production arising from serine or if glutamate were the amino donor the production of 2-oxoglutamate occurred (Walton and Butt, 1981a). The peroxisomes isolated from spinach beet were shown to use serine in preference to glutamate as an amino source (Walton and Butt, 1981b). However if high concentrations of glyoxylate were present both amino donors were used. The report by Somerville and Ogren (1981) that a photorespiratory mutant of *Arabidopsis thaliana* lacking both glycine decarboxylase and serine hydroxymethyl transferase required additional NH_3 in order to continue glycine production also suggests that amino group availablility is important to operation of the glycollate pathway. Such reports on the nitrogen requirements of normal glycollate pathway operation also have relevence to the present glycollate-2-^{14}C experiments (Tables 6-11). In both corn and sorghum bundle sheath cells, AAN had the unexpected effect of reducing the amount of labelled glycine both in terms of total radioactivity incorporated and as a percentage of the total ^{14}C metabolized. The preincubation period used in these AAN experiments was 15 minutes and could conceivably be long enough to begin depletion of serine and glutamate. The experiments in which added serine increased glycine labelling clearly support this view.

The absence of serine labelling in the glycollate-2-^{14}C metabolism studies in all but the corn bundle sheath cells pretreated with serine is more difficult to explain particularly since the whole leaf and bicarbonate studies as well as reports in the literature indicate that the glycollate pathway operates in C_4 plants. Furthermore recent studies by Cossins and Jaleel (unpublished data) have shown that glycine-2-^{14}C readily gives rise to labelled serine in isolated corn bundle sheath cells. If it is assumed that the mitochondrial pool of serine is small and subject to rapid turnover that is related to glycine production then it would be expected that hydroxypyruvate and/or glycerate would become labelled. However, this was not observed. It seems more likely that glycine, arising from glycollate-2-^{14}C in the peroxisome, is not rapidly transported to the mitochondria for conversion to serine.

The patterns of labelling after a 10 minute feeding of bicarbonate (Tables 4 and 5) to corn and sorghum and that observed after 6 minutes for *P. capillare* (Usuda and Edwards, 1980) in the presence of AAN suggest that 10 and 30 minute labelling periods of the glycollate experiments (Tables 6-11) are long enough to accomodate serine labelling. In fact, the only difference between the bicarbonate-^{14}C and glycollate-2-^{14}C

experiments lies in the type of substrates supplied. The possibility that excess glycollate may somehow prevent glycine flow from the peroxisome to the mitochondria seems unlikely but cannot be ruled out completely. In this regard pulse chase experiments might provide further insight into the flux of labelled carbon between the various intracellular sites of the glycollate pathway.

Glycollate-2-^{14}C also gave rise to labelled formate and glyoxylate (Tables 6-11). The latter product is the expected intermediate between glycollate and glycine (Tolbert, 1981). Formate is known to arise by the non-enzymatic action of H_2O_2 on glyoxylate (Zelitch, 1972; Oliver, 1979, 1981). In the peroxisome, H_2O_2 is a product of glycollate oxidase action on glycollate. Thus in the glycollate-2-^{14}C feeding experiments, the added glycollate would tend to increase the levels of H_2O_2 and glyoxylate. Walton (1982) indicated that CO_2 arising from such a direct decarboxylation of glyoxylate, would only represent a small proportion of photorespiratory CO_2 and this amount could be regulated by the addition of exogenous glyoxylate. His conjecture was that the level of glyoxylate needed for direct decarboxylation would not normally exist *in vivo*. Oliver (1981) demonstrated that added amino group donors would decrease the amount of decarboxylation in soybean leaf cells. This same phenomenon was observed for corn and sorghum bundle sheath cells with added serine (Tables 10 and 11). As the level of glycine labelling was increasing, formate labelling was decreasing.

An interesting observation on formate production in the two types of bundle sheath cells was the difference in response to added AAN. In sorghum, formate labelling was increased with AAN treatment (Table 10). This would be consistent with a decrease in the availability of amino groups thus making more glyoxylate susceptible to decarboxylation. The corn bundle sheath cells produced significantly less formate when treated with AAN (Tables 6 and 7). This implies that the formate at least partially arises by a different reaction. Rat liver mitochondria have the ability to produce formate from methylene tetrahydrofolate (Lewis *et. al.*, 1978). As this folate derivative is an established product of glycine decarboxylation (Tolbert, 1981) the possibility exists that some of the formate in corn bundle sheath cells may have such an origin. Since AAN blocks glycine decarboxylation the production of methylene-THFA would be reduced and less formate labelling should be observed. If glycine is being transported to the mitochondria to yield

formate this may account for the lack of serine labelling since methylene-THFA is also a precursor of serine.

It is also possible that the supply of co-factors, required in the glycine decarboxylase reaction, may limit the conversion of glycine to methylene-THFA, CO_2 and NH_3. Tetrahydrofolic acid, NAD, and pyridoxal phosphate are all required for the decarboxylase reaction (Woo and Osmond, 1976). In isolated spinach mitochondria, NAD was required to enhance the rates of glycine decarboxylation (Woo and Osmond, 1977). Conceivably, highly soluble co-factors could be lost during preparation of bundle sheath cells. In the present work, the suspending medium contained co-factors necessary for high rates of CO_2 fixation (Chapman *et. al.* 1980) but other metabolites could be needed for the reactions that are secondary to the carboxylation event. Corn and sorghum are both C_4 plants of the NADP-malic enzyme type (Edwards and Huber, 1981). Thus the utilization of malate should not deplete NAD levels. Since bicarbonate feeding data for corn (Table 4) suggests that glycine and serine metabolism are linked it would appear that the necessary metabolites are present within the bundle sheath cells. In sorghum, where bicarbonate feeding did not result in serine labelling (Table 5), the situation may be different. Thus it may be necessary to supply labelled glycollate-2-^{14}C to the isolated bundle sheath cells together with NAD and/or tetrahydrofolate to determine whether the glycine decarboxylation and serine labelling is limited in this way. It is interesting to note that Woo and Osmond (1977) could not detect any ^{14}C evolution from glycine-1-^{14}C even in the presence of ADP or OAA/NAD in their studies of mitochondria isolated from mesophyll and bundle sheath cells of corn. Under the same conditions, the bundle sheath cell mitochondria of two other C_4 plants, *Atriplex spongiosa* and *Panicum milaceum* showed higher rates of CO_2 evolution than mitochondria isolated from mesophyll cells. A paper by Kisaki et. al. (1972) demonstrated that whole leaf segments of corn could evolve $^{14}CO_2$ when supplied glycine-1-^{14}C. Glycine metabolism was shown to be linked to the electron transport chain in mitochondria isolated from spinach but no glycine-dependent oxygen uptake was observed with corn mitochondria (Neuberger and Douce, 1977). These reports from the literature mentioned above, and the lack of serine labelling from glycine derived from glycollate-2-^{14}C reported in this thesis for corn and sorghum reveal that the situation for glycollate pathway metabolism for corn and sorghum appears to be different

from that found in C_3 plants.

Concluding Remarks

The ability of corn and sorghum bundle sheath cells to metabolize glycollate has been demonstrated (Tables 6-11). The major products were glyoxylate, glycine, and formate. The first two are known glycollate pathway intermediates. The absence of serine labelling from glycollate-2-^{14}C studies (Tables 6-10) implies that photorespiratory CO_2 may not arise in the manner generally accepted for C_3 plants. The production of formate however indirectly demonstrates that CO_2 arises from glyoxylate, either by direct decarboxylation or by glycine decarboxylase producing CO_2 and methylene-THFA which in turn gives rise to formate. In either case the production of formate demonstrates that the bundle sheath cells of these two C_4 plants are probably capable of producing photorespiratory CO_2. The labelling pattern from the whole leaf CO_2 study (Table 2) presented also provides evidence that this C_4 plant has a functional glycollate pathway. The amount of labelled glycollate produced under "normal" conditions was small and did not increase until the inhibitor was present. This type of effect was also seen by Jenkins *et. al.* (1982, 1983). The report that C_4 RuBP carboxylase has the same oxygenase activity associated with it as do C_3 plants (Bowes *et. al.*, 1971) supports the view that C_4 plants produce phosphoglycollate and require a metabolic pathway to detoxify this inhibitory product. The extent of oxygenase activity may depend on environmental stress (Lorimer and Andrews, 1978). Under drought conditions stomata would be closed, lowering the CO_2 availability and increasing the relative O_2 competition which would result in a greater flow through a glycollate pathway. The absence of detectable photorespiratory CO_2 in corn would thus be attributed to the reduction in flow through a glycollate pathway and efficient refixation by PEP carboxylase in mesophyll cells (Rathnam, 1979).

As well as the function of protecting the plant from the toxic phosphoglycollate the enzymes of the glycollate pathway may serve other purposes. In greening tissues of corn and barley, (Jenkins *et.al* 1982, 1983) it has been shown that inhibitors of glycollate oxidase and serine:glyoxylate aminotransferase prevent chlorophyll synthesis. Glycine and/or glutamate are required for this synthesis (Hendry and Stobart, 1977).

In C_3 plants the flow of carbon through the glycollate pathway is considerable (Chollet, 1977). For synthesis of glycine and serine it would appear that no other

mechanisms are necessary. However, in C_4 plants under normal growing conditions the synthesis of glycollate appears to be minimal. Thus in C_4 plants, unlike C_3 plants, the depletion of carbon from Calvin cycle intermediates and the subsequent regeneration through the glycerate pathway may not be as important. For these reasons it has been suggested that serine would arise from alternate pathways (Morot-Gaudry *et. al.*, 1980; Mahon *et. al.* 1974). The present study with corn and sorghum also suggest that serine is not readily formed from glycollate within the bundle sheath cells (Tables 6-10). Serine may arise by either a phosphorylated or non-phosphorylated pathway from Calvin cycle intermediates (Halliwell, 1978). The possible involvement of mesophyll cells in serine synthesis needs to be explored.

The effect of AAN in corn bundle sheath cells supplied glycollate-2-^{14}C indicates that some formate arises from glycine decarboxylation through methylene-THFA. Folate metabolism and the supply of C-1 units are important in many synthetic reactions (Cossins, 1980). Glycine labelling from formate-^{14}C has been demonstrated in barley (Gifford, 1980). Perhaps formate arising in bundle sheath cells is transported to the mesophyll cells for involvement in C-1 metabolism or the synthesis of glycine. More work is required in these areas.

The unexpected effect of AAN in decreasing glycine labelling from glycollate-2-^{14}C indicates that inhibition by this compound is more complex than reported in the literature. A comparison of specific activities of the glycine pools in control and inhibitor treated bundle sheath cells would be useful in assessing this. If the inhibitor-treated cells had a low specific activity it would imply that the AAN pretreatment had resulted in amino groups accumulating in the unlabelled pool. The addition of serine (Tables 10 and 11) would support such an accumulation since greater amounts of glycine labelling occured. Serine hydroxymethyl transferase and glycine decarboxylase may play an important role in regulating the nitrogen availability (Walton and Butt, 1981a; Berger and Fock, 1983; Keys *et. al.*, 1978.) Inhibition of these enzymes could account for a reduction in available amino groups and thus the observed effect on glycine.

The enzymes associated with the glycollate pathway would all be essential to both C_3 and C_4 species. A conventional C_3-type glycollate pathway may not be complete in C_4 plants, at least not in bundle sheath cells alone. Attempts at regulating photorespiration in

C_3 plants which would not regulate O_2 competition with CO_2 at the carboxylation site of RuBP carboxylase/oxygenase would thus be counter-productive.

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V. Appendix

A. Organic acid separation by high performance liquid chromatography

The HPLC system used, purchased from Varian Associates, Inc, was a model 5000 ternary pump system equipped with a Vista 401 data handling microprossesor. Organic acids were detected at 210 nm using a Varian model U.V. 50 variable wavelength spectrophotmeter. An Aminex HPX-87 organic acid analysis column was used. This column is packed with a strong cation exchange resin with 8% cross-linkage and an average particle size of 9 um. The solvent, 0.1N H_2SO_4 prepared using deionized Millipore-filtered water, was pumped at 0.6 ml/min at an average pressure of 76 atm. The column temperature was set to 25 °C (approximately ambient). The separation of organic acids is due to ion exclusion and partition chromatography (Turkleson and Richards, 1978; Bio-rad Industries ,1979). Samples were injected onto the column through a Rheodyne model 7125 fixed volume loop injector (20 ul). Since the elution was isocratic, no regeneration of the column was required between sample injections. All of the organic acids were eluted within 20 minutes. A sample elution is given in Fig 13. The Vista data handling system takes the analogue signal from the spectrophotometer and plots the output as a relative absorbance. The signal attenuation determines the size of the peak plotted. The range of attenuations available, in multiples of 2, is from 1 to 1024 with 1 being the most sensitive. To calibrate the Vista the following procedure was adapted. Individual standards were run to determine the retention time using the machine's analysis mode. This was followed by running the desired group of standards all in one injection with the Vista system in the "learn" mode which allowed the retention times to be stored in the memory. A calibration mode was then run using the same standard mixture. The quantity of each standard was entered into the memory prior to the calibration run so that a calibration factor, relative to peak area, could be computed . Three successive runs were made to give an average calibration factor. After this procedure was followed the HPLC used on analysis mode would both identify and quantify samples. Due to the nature of the absorbances of the different organic acids, peak heights could not be compared directly without determination of a calibration factor.

The amounts of standard organic acids injected in a volume of 20 µl was approximately 10 to 15 ug (100-200 nmoles). The following list gives the retention times of common organic acids using the above described method. The elution order is generally related to the pKa of the individual compounds with acids having low pKa values being retained for shorter times (Turkleson and Richards 1978).

Compound	Retention Time (min.)
Unretained fraction	6.08
Oxaloacetate	7.90
Citrate	8.15
Hydroxypyruvate	8.26
a-ketoglutarate	8.56
Pyruvate	9.30
Glyoxylate	9.37
L-malate	9.77
Glycerate	10.82
Glycollate	12.36
Formate	14.07
Acetic acid	15.41
Fumaric acid	16.78

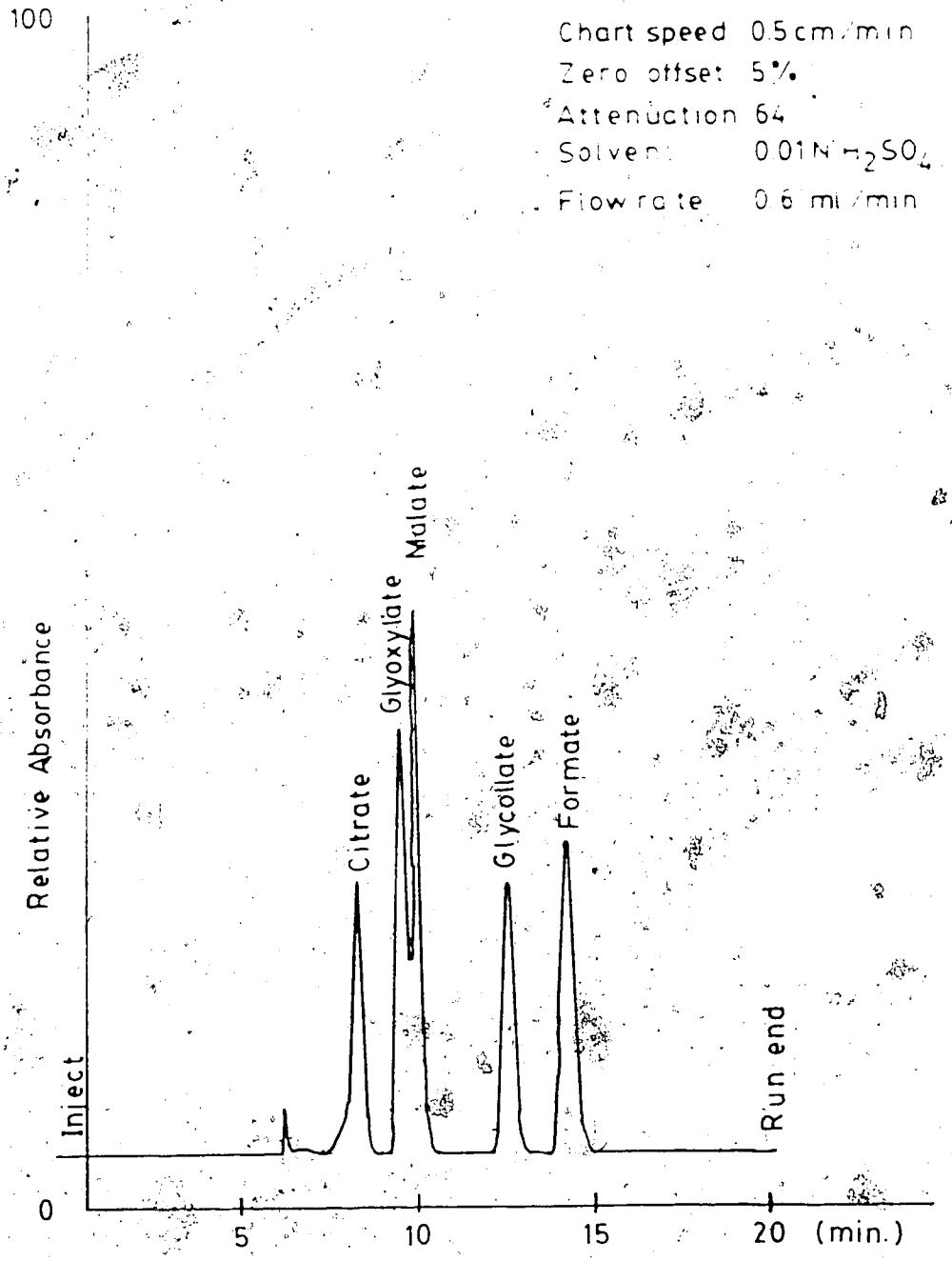

Fig. 13 HPLC analysis of organic acids.

B. Chlorophyll Determinations

Chlorophyll was extracted from whole leaves and bundle sheath cells using 80% acetone. Two equations for determining chlorophyll contents of these extracts can be found in the literature. Both determine the amount of chlorophylls a and b using different extinction coefficients. The formulae of Strain *et al.* (1971) use the absorbance values obtained at 665 and 649 nm. The method of Harborne (1973) uses absorbance values at 663 and 645 nm. The two sets of equations are given below.

Chl a (ug ml^{-1}) = $13.63A_{665} - 2.39A_{649}$

Chl b (ug ml^{-1}) = $20.11A_{649} - 5.18A_{665}$ **Strain et al.**

Chl a (ug ml^{-1}) = $12.7(A_{663} - 2.69A_{645}$

Chl b (ug ml^{-1}) = $22.9(A_{645} - 4.68A_{663}$ **Harborne**

The equations of Strain *et al.* were used for determining total chlorophyll in the present work. The chl a/b ratios determined using this method were however lower than those generally reported in the literature. For example Woo *et al.* (1971) using 4 varieties of corn reported average a/b ratios for the bundle sheath cells of 5.31. The extinction coefficients of Strain *et al.* yielded an average a/b ratio of 3.43 for the corn bundle sheath cells used in this thesis. Since high a/b ratios are an indicator of uncontaminated bundle sheath cells this anomaly was of some concern. In certain experiments the absorbances required for both equations were recorded. In those cases, both sets of calculations were done. The a/b ratio for the same tissue using the Harborne formulae was found to be 4.85. This is in much better agreement with the literature values. The total chlorophyll determined from both sets of equations was in better agreement. The average percentage difference in a/b ratios from six different determinations was calculated to be 30.2%. The difference in total chlorophyll was only 5.0%. Thus it is seen that the Harborne method only marginally exceeds the total chlorophyll value of the Strain et.al. method, but the difference in the a/b ratio is much higher.

The table on the following page contains the results of the chlorophyll determinations in the present study as well as published values.

Table 12. Chlorophyll a/b ratios; comparison to published values.

Present study

Corn bundle sheath cells		Sorghum bundle sheath cells	
Strain et al.	Harborne	Strain et al.	Harborne
3.43 ± 0.25	4.85 ± 0.34	3.28 ± 0.49	4.80 ± 0.49
n = 9	n = 9	n = 6	n = 6

± standard deviation

Published values

	Corn bundle sheath cells	Sorghum bundle sheath cells
Andersen et al. (1971)	5.39	
Chollet and Ogren (1973)	6.11	
Farineau (1975)	5.3	
Woo and Osmond (1971)	5.31	5.62

C. Elution of organic acids from a column of Dowex formate resin.

The standards were placed on the column in 10 ml of water and fractionated as described in the Methods section. Labelled standards were first run individually. A mixture of glycollate, glyoxylate, 3-PGA, malate, and formate-^{14}C as well as a mixture of glycollate-2-^{14}C, glyoxylate-^{14}C, and formate-^{14}C were chromatographed obtaining the retention times reported at the right. For liquid scintillation or HPLC, aliquots were taken from the fractions collected. The entire fraction was used for colorimetric determination of glycollate (Calkins, 1943).

Appendix C. Organic acids on Dowex formate resin column.

Acid	Amount	Range (tube number)	Peak (tube number)	Detection method
Glyceric	10 mg	25-31	27	HPLC
Glycolic	10 mg	30-36	34	HPLC
Glycolic	100 mg	33-41	36	Colorimetric (Calkins)
Glycollate-2-^{14}C	0.15 µg (0.1 µCi)	28-40	33-35	Liquid scintillation
Glyoxylic	10 mg	51-57	53	HPLC
Glyoxylate-^{14}C	0.06 µCi	52-61	55	Liquid scintillation
Formate-^{14}C	0.12 µg (0.1 µCi)	42-53	47	Liquid scintillation
Malate-4-^{14}C	0.01 µCi	70-90	76-77	Liquid scintillation
Malic	10 mg	70-85	75-76	HPLC
Hydroxypyruvate	20 mg	61-89	74-76	HPLC
3-PGA	10 mg	+100 (8N wash)		HPLC
Pyruvic	10 mg	+100		HPLC
Citric	10 mg	+100		HPLC