ABSTRACT

A STUDY OF THE ENZYMES IN LEAF PEROXISOMES

by Russell K. Yamazaki

Peroxisomes have been isolated from leaf homogenates by isopycnic centrifugation. An analysis of the enzymic content of the leaf peroxisomes has revealed the presence of a serine-pyruvate aminotransferase and isozymes of NAD-malate dehydrogenase, NADP-isocitrate dehydrogenase, and aspartate-α-ketoglutarate aminotransferase in addition to catalase, glycolate oxidase, NAD-glycerate dehydrogenase and glutamate-glyoxylate aminotransferase which have been described in previous publications by our laboratory.

Characteristics of the NAD-malate dehydrogenase activities of spinach leaves were studied. The peroxisomal enzyme, which was assayed by following reduced pyridine nucleotide oxidation, had a broad pH optimum from 6.4 to 7.4 and was specific for NADH. The peroxisomal and mitochondrial forms of NAD-malate dehydrogenase were differentiated by their kinetic and electrophoretic behavior. The mitochondrial form had a Km (oxalacetate) of 5.7×10^{-6}M and was inhibited by oxalacetate concentrations above 7×10^{-5}M. The peroxisomal form had a Km (oxalacetate) of 1.4×10^{-5}M and was inhibited by oxalacetate concentrations in

ex
as
th
tr
of
wi

lo
de

ro
of
su
ac
as

ch
ca
wi

is
an
af
fo
th

fr

excess of 2×10^{-4}M. The supernatant NAD-malate dehydrogenase showed kinetic characteristics intermediate to those of the peroxisomal and mitochondrial forms. Starch-gel electrophoresis of the supernatant fraction showed the presence of both the peroxisomal and mitochondrial forms together with another isozyme.

The serine-pyruvate aminotransferase was found to be localized specifically in the peroxisomes. No activity was detected in mitochondria or chloroplasts.

Low but significant levels of NADP-isocitrate dehydrogenase were found in peroxisomes and mitochondria. Most of the activity in leaf homogenates was associated with the supernatant fraction. Whether or not this supernatant activity was due to leakage from particles could not be assessed.

Aspartate aminotransferase activity was found in chloroplasts, mitochondria, and peroxisomes. In the last case, it is assumed that the enzyme operates in conjunction with the NAD-malate dehydrogenase also present.

Other enzymes of the tricarboxylic acid cycle (NAD-isocitrate dehydrogenase, fumarase, citrate synthetase, and aconitase) were localized in the mitochondrial fraction after separation of cellular organelles. Malic enzyme and formate dehydrogenase were also found to be localized in the mitochondrial fraction.

The diversity of enzymic content of the microbodies from various sources is suggested to be an indication of

dive
thi
lat
act
of
str
hav
gly
fix

som
kno
to
of
tro

diversity of function. In the case of the leaf peroxisomes, this function is suggested to be the conversion of glycolate to glycerate, *i.e.*, the glycolate pathway. All enzyme activities necessary for this pathway, with the exception of the conversion of glycine to serine, have been demonstrated in the peroxisome. These peroxisomal activities have been shown to be sufficient to convert glycolate to glycerate, in the intact leaf, assuming 50% of the carbon fixed during photosynthesis passes through glycolate.

A unified scheme for the function of leaf peroxisomes has been suggested. This scheme, which utilizes known peroxisomal enzymes, assumes the peroxisomal membrane to be impermeable to pyridine nucleotide. The consequences of such a scheme are discussed in terms of metabolic control in the plant.

A STUDY OF THE ENZYMES IN
LEAF PEROXISOMES

By

Russell K. Yamazaki

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1969

ACKNOWLEDGMENTS

I thank Dr. N. E. Tolbert for his guidance, encouragement, and support during the course of this work. Support from the National Institutes of Health is also gratefully acknowledged. I especially thank Miss Angelika Oeser whose technical assistance has been invaluable to this thesis. I also thank my fellow graduate students for stimulating discussions which have contributed to much of this work. Finally I think my wife, Jane, whose contribution in help and encouragement have made much of this possible.

INT

LIT

MAT

TABLE OF CONTENTS

LIST OF TABLES

Fig

LIST OF FIGURES

LIST OF ABBREVIATIONS

bicine	N,N-bis(2-hydroxyethyl)glycine
CoA	Coenzyme A
CoA-SH	Coenzyme A, free reduced form
DCPIP	2,6-dichlorophenolindophenol
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	(Ethylenedinitrilo)tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
α-KG	α-ketoglutarate
MES	2-(N-morpholino)ethanesulfonic acid
α-NPO	α-naphthylphenyloxazole
OAA	oxalacetate
PEP	phosphoenolpyruvate
PHMS	2-pyridine-hydroxymethanesulfonate
PPO	2,5-diphenyloxazole
TCA cycle	tricarboxylic acid cycle
TES	N-tris(hydroxymethyl)methylamino-ethanesulfonic acid
tricine	N-tris(hydroxymethyl)methylglycine

en
yea
re
de
ex
ne
re
of
tha
Cy
se
a
ac
ca
st
el
it
ti
ch
or
tha
sh

INTRODUCTION

The study of the intracellular localization of enzymes has seen much progress within the last twenty years. Before this period, it was felt necessary to remove particulate material from extracts in order to demonstrate enzyme action - thus the term "cell-free extract." This removal effectively silenced the proponents of vitalism who maintained that the biochemical reactions observed in extracts were due to the presence of whole cells. In the early 1950's, it became evident that the enzyme activities of the Tricarboxylic Acid Cycle were localized in particulate material. Work from several laboratories established that the mitochondrion, a distinct cellular organelle, was the site of TCA Cycle activity and energy production. Later, photosynthetic carbon dioxide fixation and ATP formation were demonstrated to take place in another distinct cellular organelle, the chloroplast of green plants. All enzyme activities necessary for photosynthetic carbon dioxide fixation have since been demonstrated to be present in the chloroplast. Since many of the above activities were originally thought to be "soluble," it became evident that intracellular localization of enzyme activities should be studied.

for
and
the
it
hyd
tec
ena
org
oxi
lis
Per
tis

cel
Thi
gly
pat
loc
App
ti
co
lo
th
Wi
te

Use of the differential centrifugation techniques for cell fractionation as developed by Claude, Hogeboom, and Schneider led to the discovery of another organelle, the lysosome. Through the work of de Duve and coworkers, it was demonstrated that the lysosomes were the site of hydrolase activity in liver cells. Application of the technique of isopycnic centrifugation to rat liver homogenates revealed the presence in the cell of still another organelle. Because of the presence of flavoprotein oxidases and catalase and the implied role in H_2O_2 metabolism, these particles were termed "peroxisomes" by de Duve. Peroxisomes were found to be limited to liver and kidney tissue in the animal.

In the case of plant tissue, the issue of the intracellular localization of glycolate oxidase has been confused. This flavoprotein oxidase, which catalyzes the oxidation of glycolate to glyoxylate as the first step of the glycolate pathway, was first assumed to be soluble. Later reports located activity in chloroplasts and in mitochondria. Application of isopycnic centrifugation to particulate fractions as done in our laboratory has now revealed that glycolate oxidase, NAD-glyoxylate reductase, and catalase are localized in microbodies in leaf tissue (97). Because of the similarity in enzyme content of the plant particles with liver peroxisomes (24), these particles have been termed "leaf peroxisomes." Further work has demonstrated the presence of a glutamate-glyoxylate aminotransferase activity in the leaf peroxisomes (47).

Concurrent work on microbodies from various sources indicate that, although all the microbodies characterized to date contain an α-hydroxy acid oxidase and catalase, diversity with respect to other enzyme activities exists. The glyoxysomes as isolated from germinating castor bean endosperm contain all the enzymes of the glyoxylate cycle and β-oxidation (10, 18). Peroxisomes from rat liver contain D-amino acid oxidase, urate oxidase, and NADP-isocitrate dehydrogenase (23). Peroxisomes from Tetrahymena contain D-amino acid oxidase, isocitrate lyase, malate synthetase, and NADP-isocitrate dehydrogenase (67).

The purpose of this thesis was to study the enzymic composition of the leaf peroxisomes. Large parts of this thesis have already been published (97, 98, 103) and will only be summarized at this time. Comparisons with the microbodies from other sources have been drawn where possible. A unified scheme for the function of leaf peroxisomes has been proposed on the basis of demonstrated enzyme activities.

Acid
plan
most
what
to h
anim
synt
phat
aco
ani
app

qua
com
aci
poi
tio
wit
tio
exi

LITERATURE REVIEW

General Comments About Enzymes of the Tricarboxylic Acid Cycle

It is now generally assumed that the Tricarboxylic Acid Cycle (TCA Cycle) is operative in the mitochondria of plant cells just as it is in animal mitochondria where most of the component enzymes have been studied in somewhat greater detail. A few of the enzymatic steps appear to have different specificity than the corresponding animal enzyme (21). For instance, the plant succinyl-CoA synthetase utilizes ADP rather than IDP or GDP as the phosphate acceptor. Suggestions have been made that the plant aconitase dehydrates citric acid differently than the animal enzyme. But the functioning of the total TCA cycle appears to be the same in plants and animals.

Plants have long been known to accumulate large quantities of acids (Ref. 79). Some plants are utilized commercially for production of acids; in many cases these acids are acids of the TCA cycle. As Ranson (79) has pointed out, the TCA cycle cannot be used for the accumulation of any of its intermediates or metabolites thereof without arresting the action of the cycle. But, accumulation of any of the cycle acids may be accomplished if there exists an enzymic mechanism for the synthesis of one of the

aci
cit
rea
con
of
est
dri

ass
en
po
75
ch
in
ca
of

cy
of
th
st
se
ge
pl
ac
ro

acids independent of the TCA cycle. PEP Carboxylase may be cited as one such example. Following the same line of reasoning, we might also expect to find enzymes involved in conversion of the cycle intermediates operating independently of the TCA cycle. Putting this another way, we now have established a rationale for the existence of extramitochondrial forms of TCA cycle enzymes.

In animal systems which have been studied, it is assumed that the extramitochondrial forms of TCA cycle enzymes exist to provide for the transporting of reducing power into and out of the mitochondria (for example, see Ref. 78). Several roles can be postulated for the extramitochondrial TCA cycle enzymes in plants. They may be involved in the storage of organic acids for reducing power and/or carbon skeletons. Or they may be involved in the formation of amino acids.

The existence of extramitochondrial forms of the TCA cycle enzymes has been inferred in plants from the presence of multiple isozymes of various enzymes (e.g., Ref. 84), but the locations and properties of these isozymes have not been studied extensively because of the problems involved in the separation of plant cell organelles. Thus malate dehydrogenase activity has been described as residing in chloroplast fractions from differential centrifugation (109), but actual proof of the existence of a chloroplast malate dehydrogenase could not be obtained because of the presence of mit

It

as

tha

thu

NAD

pri

ref

ger

abo

the

is

at

bu

di

pr

be

or

tw

ho

en

ut

de

mitochondria and peroxisomes in these chloroplast fractions. It is only through the application of other techniques such as nonaqueous isolation or density gradient centrifugation that satisfactory separation of organelles can be attained, thus allowing further study.

NAD-Malate Dehydrogenase

In the following review, discussion will be limited primarily to the enzymes as studied in plant sources. With reference to specific enzymes, the case of malate dehydrogenase stands out in confusion. Reference has been made above to a few reports as to the intracellular location of the extramitochondrial forms. A somewhat more complete list is given in Reference 103. Many of these references attribute malate dehydrogenase activity to the chloroplast, but, as will be discussed later, in all of these reports, differential centrifugation was used for the chloroplast preparations. Use of differential centrifugation alone has been shown to be inadequate for separation of cellular organelles (98).

By use of DEAE chromatography, Davies (20) separated two malate dehydrogenase activities from pea epicotyl homogenates. One activity was attributed to mitochondrial enzyme and the other, to a supernatant enzyme. Yue (104), utilizing polyacrylamide gel electrophoresis, was able to demonstrate the existence of three malate dehydrogenase iso

and

Cit

stu

Vel

the

ion

re

es

Ac

by

ac

ac

t

t

c

1

a

(

isozymes from barley. Two of these were termed supernatant, and the third was termed the mitochondrial activity.

Citrate Synthetase

Citrate synthetase or condensing enzyme has been studied in homogenates of peanut cotyledons by Marcus and Velasco (61). The enzyme was found to be associated with the particulate fractions and was inhibited somewhat by Mg ions. The enzyme was further shown to be specific with respect to the CoA ester, reacting with only acetyl-CoA ester, at a significant rate.

Aconitase

Aconitase localization has been studied in tobacco by Pierpoint (74) who concluded that although most of the activity was found in supernatant fractions, some of this activity could be attributed to leaching of the enzyme from the mitochondria. The particulate activity was found in the mitochondrial fraction.

NAD- and NADP-Isocitrate Dehydrogenases

The cases of the plant NAD- and NADP-linked isocitrate dehydrogenases appear to be similar to those found in animal tissues. The NAD-specific isocitrate dehydrogenase activity is associated exclusively with the mitochondria (21). The NADP-linked activity has been found both in the mit

par

In

pla

act

fou

the

ity

ani

of

deh

act

α-

wi

ch

wo

<u>F</u>

wa

to

dr

ca

ch

hi

mitochondria and in supernatant fractions, with the major part of the activity residing in the supernatant (21, 61). In two cases (56, 71) it has been suggested that chloroplasts contain an NADP-specific isocitrate dehydrogenase activity.

The NAD-linked isocitrate dehydrogenase has been found to be irreversible (21), a situation also found with the animal NAD-linked activity. As to NADP-specific activity, reversibility can be achieved in both the plant and animal enzymes. Cleland (16) has suggested on the basis of kinetic evidence that the NADP-specific isocitrate dehydrogenase of the cytoplasm in animal tissues may be acting in the direction of the reductive carboxylation of α-ketoglutarate. Such studies have not been carried out with the enzyme from plant sources, but comparative biochemistry might lead one to suggest that the plant enzyme works in a similar manner.

Fumarase

The distribution of fumarase in tobacco homogenates was studied by Pierpoint (73) who found that 90% of the total fumarase activity was associated with the mitochondria. Pierpoint, however, was not able to state unequivocally that all of the cellular fumarase activity was mitochondrial because of the presence of nuclear material in his particulate fractions. He thought it possible that an enzyme such as fumarase might become bound to the nucleic acids upon homogenization, thus producing an artifactual distribution of the activity.

Enzyme Activities Related to the Tricarboxylic Acid Cycle

Malic Enzyme

In 1949, Conn, Vennesland, and Kraemer (17) studied the occurrence of malic enzyme and found this activity to be widespread among higher plants. More recently the intracellular distribution of malic enzyme has been studied in *Opuntia* phylloclades by Mukerji and Ting (65) and in maize leaves by Slack and Hatch (87). In both cases the particulate activity was found to be localized in the chloroplasts, suggesting that malic enzyme in these plants is active in the photosynthetic fixation of carbon dioxide. The intracellular localization of malic enzyme was also studied in corn root tips by Danner and Ting (19) who concluded that the activity was nonparticulate, *i.e.*, nonmitochondrial. In later work by Mukerji and Ting (66), three isozymes of malic enzyme were found in *Opuntia* stem tissue. These were designated as mitochondrial, chloroplastic, and soluble and were separated by DEAE cellulose chromatography and electrophoresis. The three isozymes were found to differ with respect to kinetic and physical properties. No conclusions could be drawn, however, concerning the metabolic functions of

<u>Asp</u>

tou

rev

th

th

As

py

ar

ar

br

th

sh

Be

ta

wi

ar

in

wi

of each of the isozymes.

Aspartate: α-Ketoglutarate Aminotransferase

Aspartate aminotransferase is an apparently ubiquitous enzyme (for example, Ref. 8) which catalyzes the reversible reaction shown in Equation I. The enzyme is

$$\text{L-aspartate}^{-} + \alpha\text{-ketoglutarate}^{-} \rightleftharpoons \text{L-glutamate}^{-} + \text{oxalacetate}^{-} \qquad \text{(I)}$$

thought to function in transporting of oxalacetate and in the biosynthetic formation of aspartate from oxalacetate. As isolated from all sources to date, the enzyme contains pyridoxal phosphate and can be resolved in an apoenzyme and the pyridoxal phosphate.

Aspartate aminotransferase activity as isolated in animal systems has been found in both mitochondrial membranes and in the cytosol (53). These two isozymes are thought to mediate transport of the oxalacetate carbon skeleton from inside the mitochondria to the cytosol. Because of the high reactivity of the α-keto acid oxalacetate, aspartate is assumed to be a transport form along with malic acid. Thus it might be expected that aspartate aminotransferase activity would be associated with systems involved in oxalacetate metabolism. This may be the case with peroxisomes, mitochondria, and chloroplasts.

Aspartate aminotransferase and malate dehydrogenase

are
dur
put
wit
the
aci

fe
lat
glu
the
ind
act
mat
fer
pr
(4

Fo

in
To
is
pr
po
ti

are often found to be associated during purification procedures (*e.g.*, Ref. 20), and in fact the suggestion has been put forth that in *Neurospora* these activities are associated with the same protein (68). This suggestion was based upon their physical properties, immunochemical behavior, amino acid composition, tryptic maps, and genetic behavior.

Kisaki and Tolbert (47) found aspartate aminotransferase activity in leaf mitochondria and peroxisomes. The latter activity was attributed to nonspecificity of the glutamate-glyoxylate aminotransferase. Investigation of the amino group donor and acceptor specificity, however, indicates that there is more aspartate aminotransferase activity present than can be accounted for by the glutamate-glyoxylate aminotransferase. Aspartate aminotransferase activity has also been reported in chloroplast preparations from *Triticum vulgare* and *Vicia faba* by Heber (40) and from *Opuntia* by Mukerji and Ting (65).

Formic Acid and Formate Dehydrogenase

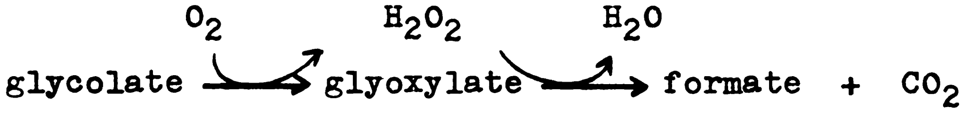
The roles for formic acid and formate dehydrogenase in plant metabolism are not completely understood. Tolbert (94) has shown that when ^{14}C-labeled formic acid is fed to barley leaves, the distribution of labeled products indicate entry of formic acid into both the C_1 pool and intermediates of the Photosynthetic Carbon Reduction Cycle. The entry of formic acid into the C_1 pool is probably mediated by the formate-activating enzyme, thus leading to ^{14}C-label in tetrahydrofolate derivatives. This label in turn spreads into products of the glycolate pathway via the β-carbon of serine through serine transhydroxymethylase action.

Radioactive formic acid was also found to label malic, aspartic and glutamic acids in the light but not in the dark. This difference may be explained if both formate dehydrogenase and one or more of the β-carboxylating enzymes are present in the mitochondria. Labeled CO_2 released by formate dehydrogenase might thus be preferentially utilized by the β-carboxylating enzyme(s) of the mitochondria rather than being fixed via the Photosynthetic Carbon Reduction Cycle. The light-dependence may simply be a reflection of the need of the β-carboxylating enzymes for PEP or pyruvate which presumably would be in greater abundance during photosynthesis. It has been suggested that formic acid is an intermediate in the reductive fixation of carbon dioxide, but the above observations tend to rule out such theories.

Early work on plant formate dehydrogenase was limited to seedlings. The enzyme was shown to be present in pea (22) and bean (101) seedlings but was found to disappear after the seventh day of germination. The pea epicotyl enzyme was shown by Davies (21) to be localized in the mitochondrial fraction. Mazelis (63) later demonstrated the presence of NAD-formate dehydrogenase in leaf

tissue from several plants including spinach. In all the tissues studied, the activity was associated primarily with the cytoplasmic particles, *i.e.*, mitochondria. No suggestion has been brought forth as to the function of the enzyme, but studies of the reaction show that the equilibrium lies far toward the direction of the oxidation of formate. This suggests the function probably does not reside in formate formation.

The source in the plant of formic acid, if indeed there is one, is unknown at present. *In vitro* studies of glycolate oxidase (46) demonstrated that formate could be produced by nonenzymatic oxidation of glyoxylate by hydrogen peroxide (Equation II), but the *in vivo* significance of



$$\text{glycolate} \xrightarrow{O_2 \searrow\nearrow H_2O_2} \text{glyoxylate} \xrightarrow{H_2O_2 \searrow\nearrow H_2O} \text{formate} + CO_2 \qquad \text{(II)}$$

this observation must be questioned in light of the finding of Kisaki and Tolbert (47) that labeled CO_2 is not released during the peroxisomal oxidation of 1-^{14}C-glycolate. Formate might arise from aldehyde oxidase or dehydrogenase action upon formaldehyde. Little knowledge is available concerning the metabolism of formaldehyde in plants.

Phosphoenolpyruvate Carboxylase

The enzyme PEP carboxylase was first described by Bandurski and Greiner (6) in 1953 in spinach leaf homogen-

ate
in

of
of
th
the
as
PE
ca
ca

th
ri
(8
le
it
po

in
en
no

ates. The enzyme irreversibly catalyzes the reaction shown in Equation III. If ^{14}C-labeled bicarbonate is used, all

$$PEP + HCO_3^- \xrightarrow{Mg^{++}} oxalacetate + P_i \qquad (III)$$

of the label incorporated is found in the β-carboxyl group of the oxalacetate. The enzyme is probably responsible for the major part of the dark fixation in plants possessing the Photosynthetic Carbon Reduction Cycle. In plants such as sugarcane which possess the C_4-dicarboxylic acid pathway, PEP carboxylase is apparently the major photosynthetic carbon-dioxide-fixing enzyme (87). In such plants the PEP carboxylase activity is associated with the chloroplasts.

The location of PEP carboxylase in plants utilizing the Photosynthetic Carbon Reduction Cycle has never been rigorously determined. Rosenberg, Capindale and Whatley (82) found activity in chloroplast extracts of spinach leaves. Mukerji and Ting (65) found PEP carboxylase activity in chloroplasts of Opuntia, a plant which probably possesses Crassulacean Acid Metabolism.

PEP Carboxykinase activity has also been reported in spinach leaf preparations, but anomalies with two different assay procedures have cast some doubt as to whether or not carboxykinase activity is present (64).

Glyoxysomes and Enzymes of the Glyoxylate Cycle

The glyoxylate cycle by which acetate is converted to ca

ar

st

is

ex

c

sy

to

la

ly

we

in

th

in

ar

th

pe

ch

en

a

fr

(6

ac

(3

carbohydrate was first elucidated in bacteria by Kornberg and Krebs (50). In 1957 Kornberg and Beevers (49) demonstrated the presence in castor bean endosperm extracts of isocitrate lyase and malate synthase, two enzyme activities exclusive to the glyoxylate cycle, as well as NADP-isocitrate dehydrogenase, NADP-glyoxylate reductase, citrate synthetase, and malate dehydrogenase. They were also able to demonstrate the net conversion of exogenously-supplied labeled acetate to labeled sucrose in the endosperm.

Later studies of the distribution of isocitrate lyase and malate synthase showed that these key enzymes were present only in those plant tissues actively converting lipid storage material into carbohydrate (7). Thus these enzyme activities were found only in those germinating seeds which contain a high proportion of lipid. Marcus and Velasco (61) studied the intracellular localization of the glyoxylate cycle enzyme activities in germinating peanuts and castor beans and suggested that they were mitochondrial.

In 1963, Hogg and Kornberg (42) concluded that the enzymes of the glyoxylate cycle in Tetrahymena pyriformis, a protozoan, were localized in a specialized mitochondrial fraction. More recent work of Müller, Hogg and de Duve (67) identified this special mitochondrial fraction as actually being a peroxisomal fraction. Harrop and Kornberg (36) have also demonstrated the presence of isocitrate lyase in a dense particulate fraction from Chlorella with a functional glyoxylate cycle.

In the case of higher plants, the association of the glyoxylate cycle activity with a subcellular particle distinct from the mitochondrion was first shown by Breidenbach and Beevers (10). These particles, because of the presence of the glyoxylate cycle, were termed glyoxysomes and were shown to contain isocitrate lyase, malate synthetase, citrate synthetase, and malate dehydrogenase. They were devoid of cytochromes, fumarase, NADH oxidase, and succinic dehydrogenase which were localized in the mitochondrial fraction. Further characterization of the glyoxysomes by this group (11) indicated that glycolate oxidase and catalase were also present in the particles. By using sulfhydryl-protecting reagents in the gradients, the authors were also able to demonstrate the presence of aconitase in the glyoxysomes, thus completing the sequence of enzymatic activities required in the glyoxylate cycle.

The problem of the source of the acetyl-CoA utilized in the glyoxylate cycle has been clarified with the finding of the presence of the complete β-oxidation system in the glyoxysomes (18). More than 80% of the particulate β-oxidation activity was found to be localized in the glyoxysomes. Addition of palmityl-CoA to the glyoxysomes were shown to produce oxygen uptake, NADH accumulation and acetyl-CoA production in a $\frac{1}{2}$:1:1 stoichiometry. The addition of cyanide doubled the oxygen uptake, but had no effect on the NADH accumulation. These data were interpreted as indicating the presence of an oxygen-requiring acyl-CoA dehydrogenase which yields hydrogen peroxide which in turn is decomposed by catalase. One of the problems remaining to be solved is the fate of the NADH generated during operation of the β-oxidation pathway and the action of the malate dehydrogenase of the glyoxylate cycle. It may be that NADH generated internally must pass outside of the glyoxysome to be oxidized. Nothing is known at the present concerning the permeability of the glyoxysomal membrane with respect to organic acids, pyridine nucleotides and cofactors. The presence of the β-oxidation system in these dense particles from castor bean endosperm has been confirmed by Hutton and Stumpf (44). These authors found ricinoleate to be oxidized most rapidly by the particles from the maturing seeds whereas the particles from the germinating seed were found to utilize palmitate and linoleate most rapidly.

Possible control mechanisms with respect to the glyoxylate cycle have been demonstrated by several workers. One of the problems concerned with the operation of the glyoxylate cycle is how is it that the isocitrate in the tissue is metabolized primarily by the isocitrate lyase rather than being used by the mitochondrial isocitrate oxidizing system. Tanner and Beevers (92) studied this competition and found that the isocitrate lyase activity was three-fold higher than the isocitrate oxidizing system, thus providing a possible explanation for the low TCA cycle oxidation activity in castor bean endosperm.

Hock and Beevers (41) studied the development and decline of the glyoxylate cycle enzymes and the effect of Dactinomycin and cycloheximide in watermelon seedlings. They concluded that the increase in activity of isocitrate lyase and malate synthetase was due to reutilization of a relatively stable m-RNA which is produced only during the first day of germination. The later decline in activity of the enzymes was attributed to the limited half-life (2-3 days) of the enzymes.

Control at the feedback inhibition level has been suggested by Nagamachi, Fujii, and Honda (69) who found that the isocitrate lyase of the germinating castor bean endosperm was uncompetitively inhibited by glucose-6-phosphate. Competitive inhibition has been demonstrated by Kornberg (48) in the case of isocitrate lyase from <u>Escherichia</u> <u>coli</u> upon the addition of PEP. Similarly John and Syrett (45) have shown that oxalacetate and pyruvate inhibited competitively the isocitrate lyase of <u>Chlorella</u> <u>pyrenoidosa</u> grown on acetate.

Control of the glyoxylate cycle activity at the hormonal level has been suggested by Penner and Ashton (72) who found that benzyladenine and cytokinin promoted

isocitr
They al
the ac
_de_ _nov_
of gib
hazel

bean
repor
taken
the
simil
biosy
the
leve
by o

Cher
have
mala
ing
of t
were
tion
tei
synt

isocitrate lyase activity in squash and peanut cotyledons. They also found that puromycin inhibited the formation of the activity and so suggested that the activity arose from de novo synthesis. Pinfield (75) found that the addition of gibberellin increased the isocitrate lyase activity in hazel cotyledons.

Repression of isocitrate lyase synthesis in castor bean seeds germinated in the presence of glucose has been reported by Lado, Schwendimann and Marre (52). These data taken with the reports of hormonal effects indicate that the glyoxylate cycle activity is under metabolic control similar to that found in the case of bacterial pyrimidine biosynthesis. Feedback inhibition of an early enzyme in the system is seen. Further control at the nucleic acid level is evidenced in the hormonal responses and repression by one of the products of the pathway.

Recent studies by Longo (58) and Gientka-Rychter and Cherry (32) utilizing the technique of density labeling have indicated unequivocally that isocitrate lyase and malate synthetase are synthesized de novo in the germinating peanut cotyledon. The increases in buoyant densities of the proteins upon germination in either $H_2{}^{18}O$ or D_2O were larger than could be accounted for by the introduction of heavy isotopes due to hydrolysis of reserve protein. This suggests that the amino acids utilized in the synthesis of these enzymes are derived from other sources than reserve protein.

Investigations of the development of glyoxysomes in germinating peanut and castor bean have revealed that the glyoxysomes as a whole are under the same type of control as found in the cases of the individual enzymes of the cycle. Longo (59) was able to demonstrate that culturing of isolated peanut cotyledons in a glucose solution reduced both the isocitrate lyase activity and the total amount of glyoxysomes.

Gerhardt and Beevers (31) have reported that the density of the glyoxysomes from castor bean endosperm remains at 1.25 g/cm^3 at all stages of germination. They also reported the puzzling observation that, at the earlier stages of germination, only 20-30% of the isocitrate lyase activity was associated with the particulate fractions. After day 4 of germination, 70-80% of the isocitrate lyase was associated with the particulate fractions. They were able to rule out breakage of the glyoxysomes as the cause for the soluble isocitrate lyase by showing that other enzymes of the glyoxysome remained in the particulate fractions. The process by which the isocitrate lyase activity becomes particle-bound is not known at present. This situation of the anomalous behavior of isocitrate lyase is similar to that found by Harrop and Kornberg (36). They found the Brannon No. 1 strain of *Chlorella vulgaris* to be constitutive for isocitrate lyase activity but also found that the glyoxylate cycle was not functional as a whole unless the isocitrate lyase was incorporated into a dense particle.

The Glycolate Pathway

During photosynthesis with $^{14}CO_2$, glycolic acid becomes labeled at early times. Feeding experiments have established that glycolate may be metabolized in plants via glycine, serine and glycerate to give sucrose in the light. This series of reactions has been termed the glycolate pathway (95). The glycolate formed during photosynthesis is uniformly labeled and the same has been found to be true of the intermediates of the glycolate pathway, thus establishing evidence for operation of the pathway in the plant. Estimates of the magnitude of the pathway have indicated that 50% or more of the carbon fixed during photosynthesis may be passing through this route. The origin of the glycolate is unknown at present, but it has been suggested that it arises from the oxidation of sugar phosphates in the chloroplast. The initial product of this oxidation is presumed to be P-glycolate, which is then dephosphorylated by a specific phosphatase to give glycolate (81).

Glycolate oxidase, the first enzyme in the pathway starting from glycolate, was first described by Claggett, Tolbert and Burris (15). The enzyme as isolated from tobacco and barley was characterized as an α-hydroxy acid oxidase which was specific for the L-isomers. Glycolate was found to be the best substrate. Glycolate oxidase was later shown by Kenton and Mann (46) and Zelitch and Ochoa (113) to be a flavoprotein. The enzyme was crystallized from spinach by Frigerio and Harbury (30). These workers found that the enzyme had a minimum molecular weight of 70,000, but showed enzyme activity only with the components of molecular weights 140,000 and 270,000. It was also noted that the 270,000 molecular weight component did not require the addition of FMN for maximal activity whereas the 140,000 molecular weight component did. These findings suggest that the enzyme exists as a tetramer when flavin is firmly bound and as a dimer when the flavin is loosely bound or becomes free.

Tolbert and Cohan (96) found glycolate oxidase activity to be low in etiolated material and to increase upon greening. These observations and those of Kuczmak and Tolbert (51) established that the glycolate oxidase activity in etiolated wheat could not be detected unless the material was homogenized in the presence of either glycolate or FMN. This substrate-protected or alternate form of glycolate oxidase was isolated and purified by Baker and Tolbert (5) who found the spectrum of the enzyme to be different from typical flavoproteins. Similarities of the spectrum with and stimulation of the enzymic reaction by ferredoxin led the authors to sp

ir

te

ar

ca

be

g

f

e

t

b

n

r

l

c

a

r

f

i

l

a

i

l

c

i

speculate that the enzyme might have ferredoxin or non-heme iron bound to it.

NAD-glyoxylate reductase, first isolated and characterized by Zelitch and Ochoa (105), should be included among the enzymes of the glycolate pathway, but the significance of this activity is uncertain. The suggestion has been made by Zelitch (106) that the combination of NAD-glyoxylate reductase and glycolate oxidase in the plant may function as an NADH-oxidizing system, thus getting rid of excess reducing power. Such a system might work in conjunction with the chloroplast NADP-glyoxylate reductase described by Zelitch and Gotto (112) to reoxidize the pyridine nucleotides of the chloroplast during periods of over-reduction. The acidic pH optimum (6.2) of the NAD-glyoxylate reductase activity in comparison to that of glycolate oxidase ($pH_{opt} = 8.7$) make the possibilities of the two activities acting as an NADH-oxidizing system somewhat remote. A more probable function of this enzyme may be found in its D-glycerate dehydrogenase activity. Zelitch found the enzymic rate to be four times greater with hydroxypyruvate as substrate than with glyoxylate. Holzer and Holldorf (43) and Laudahn (55) have concluded that the same enzyme is responsible for both activities and have preferred the name NAD-D-glycerate dehydrogenase for the enzyme in view of the lower Km for hydroxypyruvate (ca. 10^{-4}M vs. 10^{-2}M) and the larger V_{Max} (4 times greater with hydroxypyruvate).

The enzymatic reaction involved in the conversion of glyoxylate to glycine has been elucidated by Kisaki and Tolbert (47). An aminotransferase activity utilizing glyoxylate as the amino acceptor and glutamate and alanine as the amino donors was shown to be located specifically in leaf peroxisomes. The high degree of substrate specificity and sharp pH optimum at pH 7.3 led these authors to conclude that a single enzyme was responsible for the conversion of glyoxylate to glycine.

The next step in the glycolate pathway involves the conversion of two molecules of glycine to one molecule each of serine, CO_2, and NH_3. Because Kisaki and Tolbert (47) could find no evidence of glycine decarboxylation or conversion to serine in the peroxisomes, it was concluded that these steps occur elsewhere in the cell. The site of this conversion appears to be the mitochondria (W. J. Bruin and N. E. Tolbert, unpublished results).

The conversion of L-serine to hydroxypyruvate is accomplished by means of an aminotransferase activity. Sallach (14) has described an L-alanine:hydroxypyruvate aminotransferase activity as being present in a number of plant tissues and has implicated this activity in the gluconeogenic flux of the glycolate pathway. This aminotransferase activity was found to have a distribution similar to that of the D-glycerate dehydrogenase (99).

Sa
a
t
h
t
w

t
S
h
(
s
a

Sallach was able to demonstrate the formation of D-glycerate and L-alanine from L-serine and pyruvate through the use of the isolated enzymes D-glycerate dehydrogenase and alanine: hydroxypyruvate aminotransferase. In this case the aminotransferase activity was reversed, *i.e.*, serine and pyruvate were used as substrates.

The last reaction unique to the glycolate pathway is the phosphorylation of D-glycerate to give 3-P-glycerate. Such an enzyme activity has been described recently in higher plant extracts by Cheung, Rosenblum, and Sallach (14) and Hatch and Slack (38). Both preparations were specific for D-glycerate and ATP and required Mg ions for activity.

The enzymes of the glycolate pathway as described above act as a system for the conversion of 2 molecules of glycolic acid to 1 molecule of the sugar precursor 3-P-glyceric acid. In this conversion one carbon is lost as CO_2. The system also requires the net input of amino groups in the form of L-glutamate, the net input of reducing power in the form of NADH, and one ATP is required for the conversion of D-glycerate to 3-P-glycerate.

Peroxisomes

Peroxisomes were first recognized as distinct cellular organelles through the work of de Duve and coworkers (24). During isolation of lysosomes from rat liver, it was

foun
simi
tase
pycn
oxid
some
cat
aci
Bec
cat
to

in
fr
ca
ur
gl
da
to
(6
ma
in
o

er
(9

found that the enzymes catalase and urate oxidase showed similar distributions to the lysosomal marker acid phosphatase upon differential centrifugation. Application of isopycnic centrifugation established that catalase and urate oxidase were present in a particle distinct from the lysosomes. Further characterization of these particles indicated that several flavoprotein oxidases such as D-amino acid oxidase and α-hydroxy acid oxidase were also present. Because of the presence of these H_2O_2-producing oxidases and catalase, these particles were given the name peroxisomes to indicate their role in H_2O_2 metabolism.

The association of flavoprotein oxidases and catalase in peroxisomes has been found to hold for liver and kidney from a number of sources, two protozoans (23), germinating castor bean endosperm (reviewed in the preceding section under the term glyoxysomes), and leaf tissue (97). The glyoxysomes have been found to contain the complete β-oxidation system and the complete glyoxylate cycle in addition to the oxidases and catalase. Müller, Hogg and de Duve (67) have demonstrated the presence of isocitrate lyase and malate synthetase as well as NADP-isocitrate dehydrogenase in the *Tetrahymena* peroxisomes but were unable to find the other enzymes of the glyoxylate cycle.

Leaf peroxisomes have been found to contain the enzymes glycolate oxidase, NAD-glyoxylate reductase, catalase (97), glutamate:glyoxylate aminotransferase (47), and NAD-malate dehydrogenase (103). A summary of the enzymes known to date to be present in peroxisomes and glyoxysomes is given in the Results and Discussion Section.

Morphologically the peroxisomes from the various sources and the glyoxysomes appear similar. They are all bound by a single membrane, show a dense granular matrix or stroma, and band in sucrose gradients at an equilibrium density of 1.24-1.25 g/cc. Electron micrographs indicate the peroxisomes to be roughly spherical in shape with a diameter of 0.5-1.0 μ. Ribosomes have been conspicuously absent in all preparations to date. In the case of leaf tissue, a close association of the peroxisomes with the chloroplasts has been noted by Frederick and Newcomb (29).

Because of the presence of the peroxisomes in those animal tissues which are active in gluconeogenesis, de Duve (24) has speculated that the peroxisomal function may be related to gluconeogenesis. No direct correlation has been shown to date, however, with the possible exception that the α-hydroxy acid oxidase of the liver peroxisomes can oxidize lactate to pyruvate, thus initiating gluconeogenesis from lactate. A definite gluconeogenic function has been shown for the glyoxysomes which can convert long-chain fatty acyl CoA esters to succinate through β-oxidation and the glyoxylate cycle (18). The succinate thus formed is then available for conversion to carbohydrates elsewhere in the cell. The leaf peroxisomes have also been shown to contain

carried

to the s

contain some of the enzymes of the glycolate pathway which carried out conversion of the two carbon compound glycolate to the sugar precursor 3-P-glycerate.

MATERIALS AND METHODS

Plants

Spinach (Spinacia oleracea L.) leaves were the prime material used in this study. The spinach was purchased locally or grown in a growth chamber on a 16-hour day, 8-hour night regime. Other plant material was grown in a greenhouse with double-strength Hoagland's medium applied once a week. Genus, species and variety, where known, are listed with the results.

Preparation of Fractions by Differential Centrifugation

In typical spinach preparations leaf tissue was washed, deribbed and cut into small strips. Subsequent operations were carried out at 0-4°, either in a cold room or in ice buckets. The tissue was homogenized in a Waring blendor at maximum speed for 10 sec with one volume by weight of grinding medium (0.5 M sucrose, 0.02 M potassium glycylglycine, pH 7.5). The resulting slurry was squeezed through 8 layers of cheesecloth, and the pH (approximately 7) was readjusted to 7.5 with KOH. The sap was then centrifuged at 100 g for 20 min, and the resulting pellet was resuspended in the grinding medium. This was designated the "Whole Chloroplast" fraction. The sap was further centrifuged at 6000 g for 20 minutes. This pellet after resuspension was designated the "Broken Chloroplast" fraction. A "Mitochondrial" fraction was prepared by centrifugation of the sap at 37000 g for 20 min and resuspension of the pellet in the grinding medium. The supernatant fluid after the last centrifugation was designated the "Supernatant" fraction. Preparations were made with other plants using substantially the same procedure, although in some cases the volume of the grinding medium and the length of blendor time were increased to facilitate homogenization.

Sucrose Density Gradient Centrifugation

In typical experiments a discontinuous sucrose density gradient was prepared in the cold by pipetting successively 4 ml of 2.5 M, 8 ml of 2.3 M, 10 ml of 1.8 M, 15 ml of 1.5 M, and 13.5 ml of 1.3 M sucrose into a cellulose nitrate tube designed for the Spinco SW 25.2 swinging-bucket rotor. All sucrose solutions were made up at room temperature in 0.02 M potassium glycylglycine, pH 7.5. For studies of the intracellular location of enzymes, a 4 ml portion of the "Peroxisome and Broken Chloroplast" fraction was layered on top of the gradient; and the sample was centrifuged in a Spinco Model L centrifuge at 25,000 rev/min for 3 hours at 4°. Fractions were then collected from the bottom of the tube after puncturing and were numbered in the order of collection. Fraction contents and volumes are given in the

Results and Discussion Section.

Assay Methods

Enzyme assays were run on a Gilford automatic recording spectrophotometer at 25° except where noted. A unit of activity is defined as that amount of enzyme catalyzing the disappearance of 1 µmole of substrate per min at 25°.

Malate Dehydrogenase
(L-Malate:NAD Oxidoreductase, EC 1.1.1.37)

Malate dehydrogenase was assayed spectrophotometrically by following the oxidation of NADH at 340 mµ (103). The assay mixture contained 0.67 ml of 0.1 M HEPES (pH 7.4), 0.03 ml of 0.5% Triton X-100, 0.04 ml of 0.01 M oxalacetic acid (neutralized to pH 7.4 with KOH), 0.02 ml of 2.8×10^{-3}M NADH, and enzyme plus water to give a total volume of 1 ml in the cuvette (d = 1 cm). The reaction was initiated by the addition of oxalacetate, after measurement of the rate of endogenous oxidation of NADH. The recorder chart speed was set at 1 in/min, and readings on each cuvette were for a duration of 1.5 seconds. Full scale (250 mm) on the recorder was 0.5A. Under these conditions 1 mm/min = 0.482 nmole/min at saturating oxalacetate concentrations.

Glycolate Oxidase
(Glycolate:O_2 Oxidoreductase, EC 1.1.3.1)

Glycolate oxidase was assayed anaerobically by following the rate of DCPIP reduction at 600 mμ (97). The assay mixture in a Thunberg tube contained 2 ml of 0.1 M pyrophosphate (pH 8.5) containing 1.5×10^{-4}M DCPIP, 0.05 ml of enzyme plus water, and 0.1 ml of 0.125 M potassium glycolate in the side arm. The total volume was 2.5 ml. The cuvette was evacuated with a water aspirator and then flushed with pre-purified nitrogen which had been bubbled successively through Fieser's solution to remove traces of O_2 and then saturated lead acetate to remove traces of H_2S formed through the breakdown of the dithionite in the Fieser's solution. Complete removal of O_2 was found to be essential for obtaining linear assays due to the presence of peroxidative reoxidation of the DCPIP (97). The reaction was initiated by tipping the substrate from the side arm into the main compartment. The recorder chart speed was 0.2 in/min, and full scale was set at 2.5 A (250 mm). Under these conditions 1 mm/5 min = 0.770 nmole/min at saturating substrate and DCPIP concentrations.

Cytochrome c Oxidase (Cytochrome c:O_2 Oxidoreductase, EC 1.9.3.1)

Cytochrome oxidase was used as a mitochondrial marker and was assayed by following the oxidation of reduced cytochrome c at 550 mμ (97). Enzyme (0.5-10 μl) was placed in the bottom corner of a microcuvette (vol = 0.5 ml) with a syringe and 5 μl of 4% digitonen added. After mixing and a

1

(p

ad

di

no

la

en

no

as

cu

(2

1

As
(A

li

ge

re

0.

de

0.

10

10

0.0

spe

Und

1 min incubation period, 200 µl of 0.1 M phosphate buffer (pH 7) was added. The reaction was then initiated by the addition of 50 µl of 1.5 mM cytochrome c reduced with dithionite. Care must be taken that the cytochrome c is not over-reduced by the addition of excess dithionite since lag periods in the assay would result. A sample without enzyme must be also used in order to measure the rate of nonenzymatic oxidation of reduced cytochrome c. For this assay a recorder chart speed of 1 in/min was used, each cuvette being read for a duration of 1.5 sec. Full scale (250 mm) on the recorder was 1.0 A. Under these conditions, 1 mm/min = 48.8 picomole/min.

Aspartate Aminotransferase (Aspartate:2-Oxoglutarate Aminotransferase, EC 2.6.1.1)

Aspartate aminotransferase activity was measured by linking oxalacetate formation with exogenous malate dehydrogenase, thus giving NADH oxidation followed at 340 mµ. The reaction mixture contained 0.5 ml of 0.1 M TES (pH 7.4), 0.03 ml of 0.5% Triton X-100, 0.05 ml of stabilized malate dehydrogenase (1,000 units), 0.30 ml of enzyme plus water, 0.03 ml of 0.1 M α-ketoglutarate (pH 7), 0.02 ml of 5 x 10^{-3}M pyridoxal phosphate (pH 7), and 0.02 ml of 3.8 x 10^{-3}M NADH. The reaction was initiated by the addition of 0.05 ml of 0.4 M L-aspartate (pH 7). A recorder chart speed of 0.2 in/min and full scale of 0.5 A were employed. Under these conditions 1 mm/5 min = 64.4 picomole/min.

Isocitrate Dehydrogenase, NAD and NADP (*threo*-D_s-Isocitrate:NAD Oxidoreductase (EC 1.1.1.41) and *threo*-D_s-Isocitrate:NADP Oxidoreductase (EC 1.1.1.42))

The assay for the isocitrate dehydrogenases was modified from that employed by Leighton *et al.* (57). The formation of reduced pyridine nucleotide is followed spectrophotometrically at 340 mμ. The assay mixture for the NADP-isocitrate dehydrogenase contained 0.67 ml of 0.1 M TES (pH 7.4), 0.03 ml of 0.5% Triton-X-100, 0.03 ml of 0.1 M $MgCl_2$, 0.04 ml of 2.5 x 10^{-3}M $NADP^+$, and 0.13 ml of enzyme plus water. The reaction was initiated by the addition of 0.1 ml of 0.2 M DL-isocitrate, tri-sodium salt, *allo*-free. For the assay of the NAD-isocitrate dehydrogenase, NAD^+ was used in place of $NADP^+$, and 1 mM citrate was included in the assay mixture (25). The recorder chart speed was 0.2 in/min, and full scale (250 mm) was set at 0.5 A. Under these conditions, 1 mm/10 min = 32.2 picomole/min.

Malic Enzyme (L-Malate:NADP Oxidoreductase (Decarboxylating) EC 1.1.1.40)

Malic enzyme was measured using a modification of the procedure of Ochoa (70). The reaction mixture contained 0.67 ml of 0.1 M TES (pH 7.4), 0.01 ml of 0.1 M $MnCl_2$, 0.03 ml of 0.5% Triton X-100, 0.04 ml of 2.5 x 10^{-3}M $NADP^+$, and 0.23 ml of enzyme plus water. The reaction was initiated by the addition of 0.02 ml of 1 M DL-malate (pH 7.4). Full scale was set at 0.5 A with a chart speed of 0.2 in/min.

Under these conditions 1 mm/10 min = 32.2 picomole/min.

PEP Carboxylase (EC 4.1.1.31)

PEP Carboxylase was measured using a modification of the method of Slack and Hatch (87). The reaction mixture contained 0.3 ml of 0.1 M TES (pH 7.4), 0.01 ml of 0.5% Triton X-100, 0.03 ml of 0.1 M $MgSO_4$, 0.01 ml of 0.01 M dithiothreitol (pH 7), 0.05 ml of 10^{-2}M L-glutamate (pH 7), 0.20 ml enzyme plus water, and 0.05 ml of 0.01 M PEP. The reaction was initiated by the addition of 1 μC (0.2 μmole) $NaH^{14}CO_3$ contained in a 0.02 ml volume. The assay mixtures were then mixed and incubated at 30° in stoppered test tubes. The reaction was stopped after either 15 or 20 min by the addition of 0.5 ml EtOH followed by 0.1 ml of glacial acetic acid. The tubes were then flushed with unlabeled CO_2 for 20 min, and 0.1 ml aliquots were removed and counted. Control tubes contained all ingredients listed above with the exception of PEP. A sample without enzyme was also run to determine the rate of nonenzymatic oxalacetate formation.

Radioactivity was determined with a TriCarb scintillation counter using 15 ml of counting solution. The counting solution contained 10 g PPO, 0.1 g α-NPO, 160 g naphthalene, 770 ml xylene, 770 ml p-dioxane, and 462 ml absolute ethanol.

Citrate Synthetase (EC 4.1.3.7)

Citrate synthetase was measured by a modification of the method of Srere, Brazil and Gonen (88). The buffer component (0.5 ml) in the assay was either 0.1 M potassium HEPES (pH 7.4) or 0.4 M potassium tricine (pH 8.1). Other components were 0.03 ml of 0.5% Triton X-100, 0.10 ml of 10^{-3}M of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), (pH 8), 0.05 ml of 10^{-3}M acetyl-CoA, and 0.27 ml of enzyme plus water. The reaction was initiated by the addition of 0.025 ml of 10^{-3}M potassium oxalacetate (pH 8); and the increase in absorbance at 412 mμ was measured. Full scale on the recorder was 0.5 A and the chart speed was 0.2 in/min. This assay makes use of the reaction of DTNB (Ellman's reagent) with the free sulfhydryl group of CoA, released by citrate synthetase action. This assay is much more sensitive than the old methods of using malate dehydrogenase or following disappearance of the thiol-ester bond at 232 mμ and is less subject to troubles caused by high background readings. Under these assay conditions, 1 mm/5 min = 30 picomole/min.

Malate Synthetase (EC 4.1.3.2)

The assay used for malate synthetase was modified from the assay for citrate synthetase. The basic reaction mixture contained 0.5 ml of 0.1 M potassium HEPES (pH 7.4), 0.03 ml of 0.5% Triton X-100, 0.10 ml of 10^{-3}M DTNB (pH 7), 0.05 ml of 10^{-3}M acetyl-CoA, 0.01 ml of 0.1 M $MgCl_2$, and 0.26 ml of enzyme plus water. The reaction was initiated by the addition of 0.025 ml of 0.1 M sodium glyoxylate.

The recorder chart speed was 0.2 in/min, and full scale (250 mm) was 0.25 A. Under these conditions 1 mm/5 min = 15 picomole/min.

Isocitrate Lyase (EC 4.1.3.1)

The assay method for isocitrate lyase was modified from that of Dixon and Kornberg (26). The reaction is followed through formation of the glyoxylate phenylhydrazone which absorbs at 324 mμ. The assay mixture contained 0.5 ml of 0.1 M HEPES (pH 7.4), 0.03 ml of 0.5% Triton X-100, 0.025 ml of 0.1 M $MgCl_2$, 0.05 ml of 4×10^{-2} M dithiothreitol, 0.03 ml of 0.1 M phenylhydrazine•HCl (neutralized to pH 7 just before use), and 0.32 ml enzyme plus water. The reaction was initiated by the addition of 0.05 ml of 0.2 M sodium DL-isocitrate (allo-free). An extinction coefficient of 1.7×10^4 M^{-1} cm^{-1} was used for the glyoxylate phenylhydrazone at 324 mμ.

Aconitase (EC 4.2.1.3) and Fumarase (EC 4.2.1.2)

Aconitase was determined by the method of Anderson (1), and fumarase was determined by substituting fumarate in the same assay system. The assay utilized absorption of 240 mμ light by the double bond in both cis-aconitate and fumarate, and disappearance of the double bond was measured. The reaction mixture contained 0.40 ml of 0.1 M TES (pH 7.5), 0.03 ml of 0.5% Triton X-100, 0.240 ml of 0.1 M

$(NH_4)_2SO_4$, 0.400 ml of enzyme plus water, and 0.030 ml of either 10^{-2} M potassium cis-aconitate or 10^{-2} M potassium fumarate (both pH 7.5), depending on which enzyme was being measured. The recorder chart speed was 0.2 in/min, and full scale was 1.0 A. Using an extinction coefficient for cis-aconitate at 240 mμ of 3.55×10^3 M^{-1} cm^{-1}, 1 mm/10 min = 0.135 nmole/min.

Formate Dehydrogenase (EC 1.2.1.2)

For the assay of formate dehydrogenase, the method of Quayle (77) was used wherein the formation of reduced NAD is followed at 340 mμ. The assay mixture contained 0.67 ml of 0.1 M potassium phosphate (pH 7.0), 0.03 ml of 0.5% Triton X-100, 0.10 ml of 10^{-2} M NAD^+, and 0.20 ml of enzyme plus water. The reaction was initiated by the addition of 0.025 ml of 0.2 M potassium formate (pH 7). The recorder chart speed was 0.2 in/min, and full scale was 1.0 A. Under these conditions, 1 mm/10 min = 64.4 picomole/min.

Serine-Pyruvate Aminotransferase (EC 2.6.1.-)

Serine-pyruvate aminotransferase activity in spinach preparations was assayed by linking hydroxypyruvate formation to D-glycerate dehydrogenase activity, thus giving NADH oxidation which was followed at 340 mμ in the Gilford spectrophotometer. The assay mixture for gradient fractions contained 0.5 ml of 0.1 M HEPES (pH 7.3), 0.03 ml of 0.5% Triton X-100, 0.02 ml of crystalline glyoxylate reductase (D-glycerate dehydrogenase), 0.35 ml of enzyme plus water, 0.02 ml of 5 mM pyridoxal phosphate, 0.03 ml of 0.1 M potassium pyruvate, and 0.02 ml of 3.8 mM NADH. The reaction was initiated by the addition of 0.05 ml of 0.4 M DL-serine (pH 7). No pyruvate-dependent oxidation of NADH was noted in the gradient fractions. This follows the observation of Zelitch that pyruvate does not serve as a substrate for plant D-glycerate dehydrogenase.

Starch Gel Electrophoresis

The procedure of Fine and Costello (27) was used for the separation of isozymes of malate dehydrogenase by starch gel electrophoresis. For preparation of the gel, 3.5 ml of 0.2 M citric acid, 21.5 ml of 0.2 M Na_2HPO_4 and 475 ml of distilled water were mixed together. This solution was then slowly added with stirring to 70 g of hydrolyzed starch. The resulting suspension was then heated with stirring until bubbles appeared (ca. 65°). The slurry was then quickly poured into a 2 l vacuum flask (heated in a water bath to the same temperature) and the mixture was boiled under reduced pressure from a water aspirator for 1 min. The starch was next poured into forms for electrophoresis, with filter-paper wicks being used for contact with the electrode solutions; and a slot former was placed in the gel to allow easy sample introduction. The gel was hydrated overnight before use.

The tank buffer used contained 30 ml of 0.2 M citric acid, 240 ml of 0.2 M Na_2HPO_4, and 1230 ml of distilled water. For the preparation of the enzyme samples, 0.3 ml fractions from the differential and sucrose gradient centrifugation of spinach leaf homogenates were incubated at ice temperature with 0.03 ml of 0.5% Triton X-100 in order to solubilize enzymatic activity. After 10 min, the samples were centrifuged for 15 min at 39,000 g and the resulting pellet was discarded. For the electrophoresis, 0.2 ml fractions were applied to the gel; these were subjected to 27 mA (210 V) for eight hours at 4°.

For localization of the malate dehydrogenase activity, the method of Fine and Costello (27) was used. The staining solution contained 35 ml of 0.1 M potassium bicine (pH 8.5), 2.25 ml of 2 M potassium L-malate, 0.9 ml of NAD^+ (30 mg/ml), 0.18 ml of phenazine methosulfate (5 mg/ml), and 1.5 ml of p-nitro blue tetrazolium (10 mg/ml). The gel was incubated in the dark for 30 min at 35° with this solution, activity of the malate dehydrogenase appearing as a purple-colored spot.

Protein and Chlorophyll Determinations

Protein was estimated by the method of Lowry, using powdered bovine serum albumin as the standard. Chlorophyll was estimated at 652 mμ by the method of Arnon.

RESULTS AND DISCUSSION

The detection, isolation, and partial characterization of leaf peroxisomes has been accomplished as a group effort in our laboratory during the course of research for this thesis. Some of these results have been published and constitute a portion of this thesis. Leaf peroxisomes were first isolated from spinach (97). Later survey work established that peroxisomes can be isolated from a variety of plants (98). In this survey work my major contribution was the study of the intracellular distribution of NAD-malate dehydrogenase. The NAD-malate dehydrogenase from spinach leaf peroxisomes was characterized in detail, and this work constitutes a major portion of this thesis (103). The work on NAD-malate dehydrogenase activity is summarized in this section. Studies of other activities are given in full.

NAD-Malate Dehydrogenase

My data on the distribution and properties of spinach leaf NAD-malate dehydrogenase have been published (Ref. 103). Consequently these findings will be presented in summary form in this section.

The same technique of organelle separation was used to determine particulate distribution for NAD-malate dehydregenase and all other enzymes described in later sections. Details are given in the Materials and Methods Section. The basic procedure involved homogenization of leaf tissue for short times in a Waring blendor. Buffered 0.5 M sucrose was used as the grinding medium. A fraction enriched in peroxisomes and mitochondria was then obtained by centrifugation at 100-6,000 g. This fraction was then layered on a sucrose density gradient ranging from 2.3 to 0.5 M sucrose. Isopycnic centrifugation allowed a separation of chloroplasts, mitochondria, and peroxisomes according to their respective densities. Chlorophyll, cytochrome oxidase, and glycolate oxidase, respectively, were used as markers for these organelles.

Figures 1 and 2 present the sucrose density gradient distribution of NAD-malate dehydrogenase activity. Clear peaks of activity can be seen to be associated with the peroxisomes and mitochondria. The <u>two</u> peaks of mitochondrial activity are probably an artifact of the non-continuous gradient used. The peroxisomal enzyme as assayed by following NADH oxidation exhibited a broad pH optimum from 6.4 to 7.4. The ratio of the rates with the two pyridine nucleotides ($\frac{\text{NADPH}}{\text{NADH}} = 0.006$) indicated that the activity was specific for NADH. The kinetic properties of the malate dehydrogenase activities from the gradient are summarized in Table 1. It can readily be seen that the mitochondrial isozyme is more sensitive to oxalacetate inhibition than is the peroxisomal form.

Figure 1

Distribution of NAD-Malate Dehydrogenase and Marker Activities After Sucrose Density Gradient Centrifugation of Spinach Broken Chloroplast Fraction.

NAD-Malate dehydrogenase, glycolate oxidase, and cytochrome oxidase units expressed as μmole substrate transformed per min per ml at 25°. ●——●, malate dehydrogenase; ■---■ , glycolate oxidase; ▲···▲ , cytochrome oxidase.

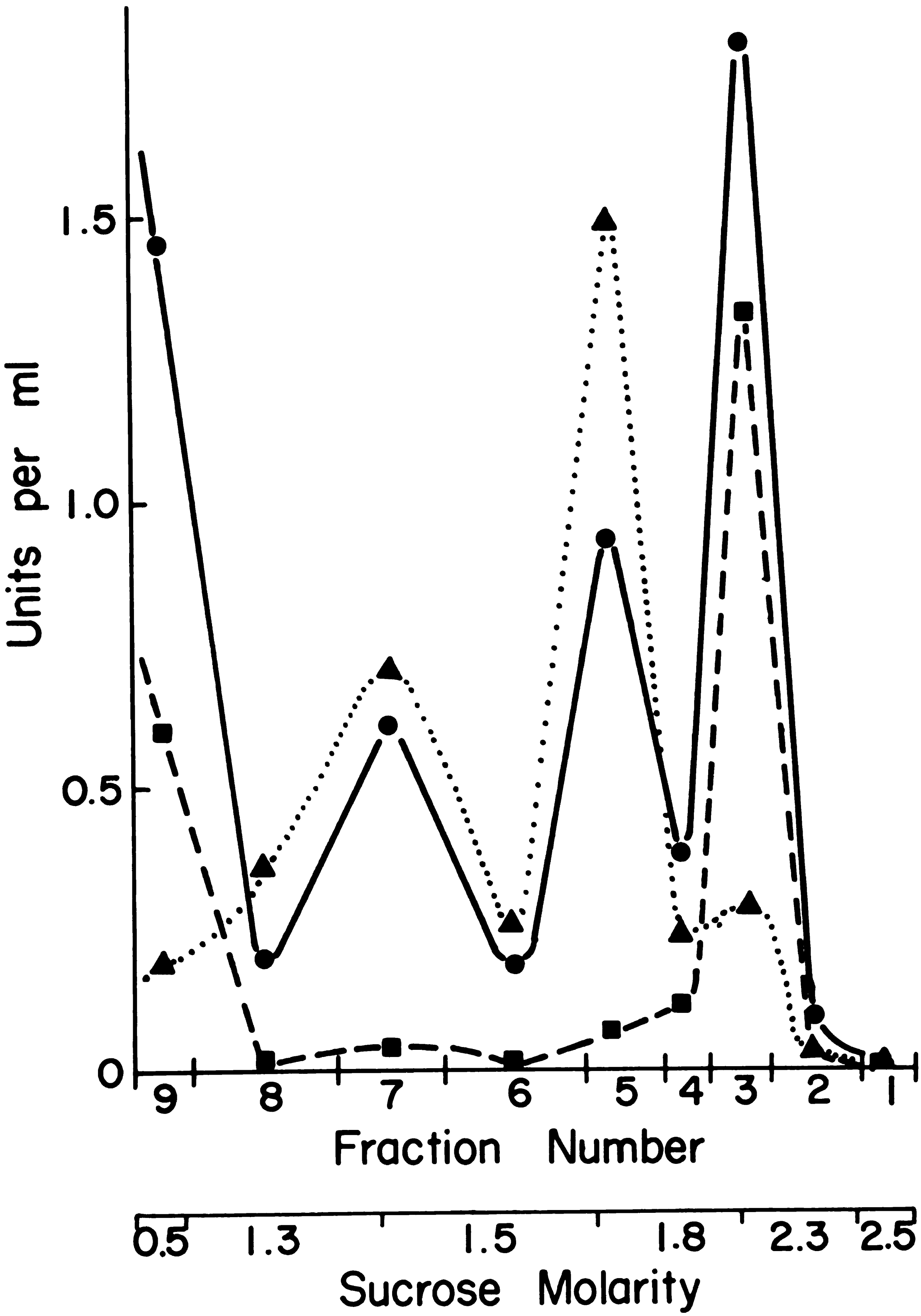
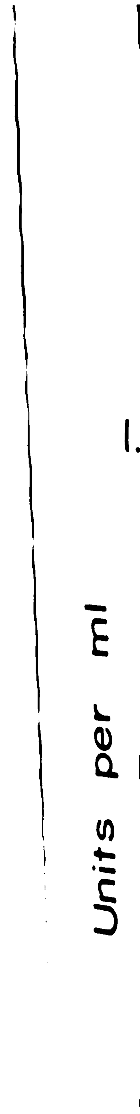

Units per ml
1.5
1.0
0.5
0
9
8
7
6
5
4
3
2
1
Fraction Number
0.5
1.3
1.5
1.8
2.3
2.5
Sucrose Molarity

Figure 2

Distribution of NAD-Malate Dehydrogenase and Marker Activities After Sucrose Density Gradient Centrifugation of Spinach Broken Chloroplast Fraction (Cont'd).

NAD-Malate dehydrogenase units expressed as μmole substrate transformed per min per ml at 25°. Protein and chlorophyll expressed as mg per ml. ●——●, malate dehydrogenase; ▲—••—▲ , protein (1/5 scale); ■—•—■ , chlorophyll.



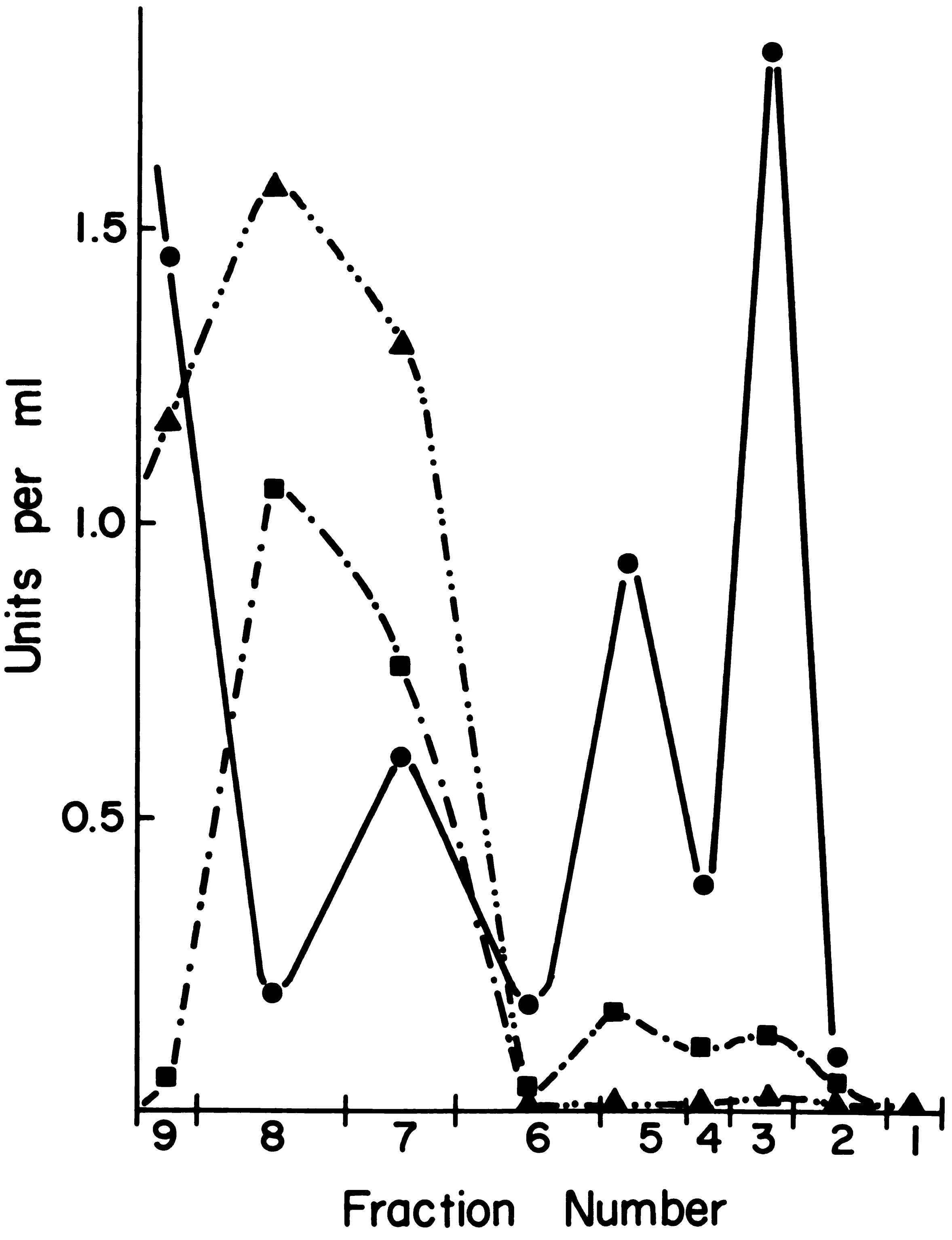

Units per ml
1.5
1.0
0.5
9
8
7
6
5
4
3
2
1
Fraction Number

TABLE 1

Kinetic Properties of Malate Dehydrogenase Forms

Peroxisomal and mitochondrial fractions taken from sucrose density gradients and used without further purification. The supernatant is from the differential centrifugation.

Location	K_m (OAA)	OAA Conc. That Begins to Inhibit	Inhibition at 2×10^{-3}M OAA
Peroxisomal	1.4×10^{-5}M	2×10^{-4}M	24%
Mitochondrial	5.7×10^{-6}M	7×10^{-5}M	50%
Supernatant	1.3×10^{-5}M	1×10^{-4}M	23%

The peroxisomal and mitochondrial forms of NAD-malate dehydrogenase were easily separated by starch gel electrophoresis. This is shown in Fig. 3. A third distinct activity was seen in the supernatant fraction, but this could not be investigated further because of the large amount of the mitochondrial and peroxisomal forms present in this fraction.

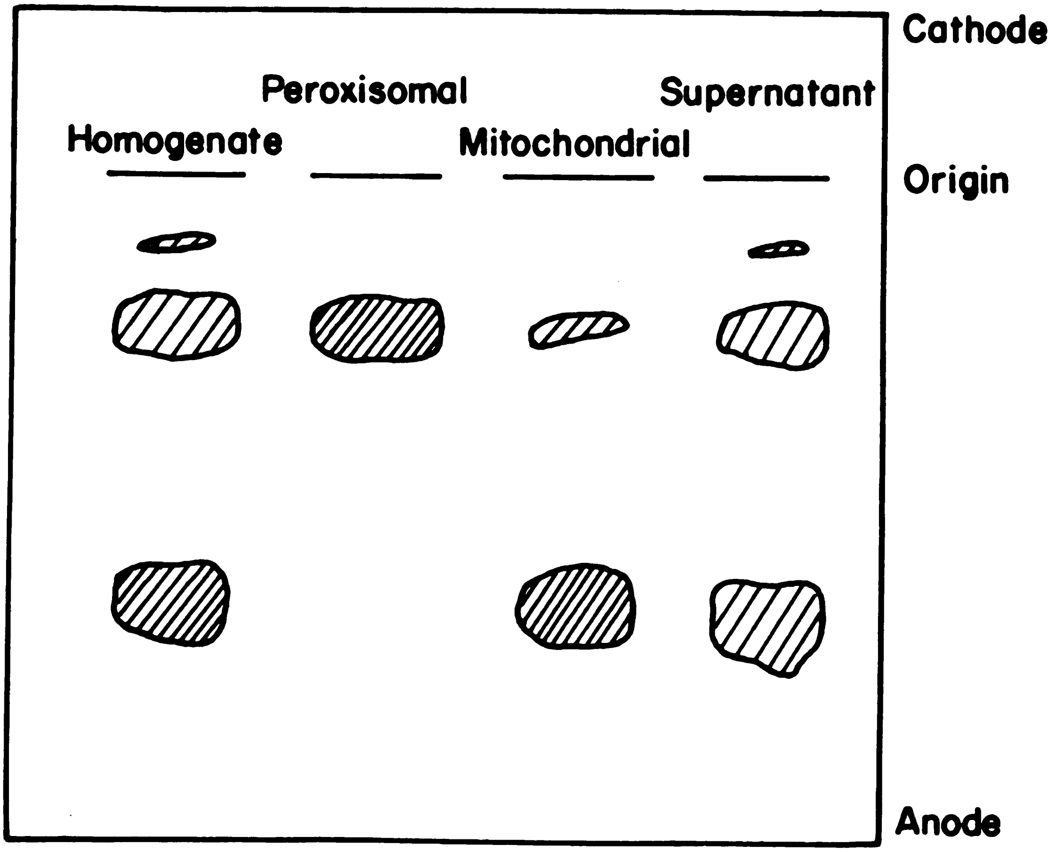
NAD-Malate dehydrogenase activity has been reported by several workers (see Ref. 103) to be present in chloroplasts, but these reports could not be confirmed in the case of spinach leaves. No evidence could be found for NAD-malate dehydrogenase activity in either whole chloroplasts or in chloroplast fragments. Hatch and Slack (38) have recently reported the presence of an NADP-malate dehydrogenase activity in maize chloroplasts. No such activity was detected in preliminary experiments with spinach chloroplast preparations, but a difference between maize and spinach chloroplasts could easily be possible.

In Ref. 103, it was stated that oxidation of L-malate could not be observed at pH 7.4, probably because of the unfavorable equilibrium of the reaction. Since this statement was made, an aspartate-α-ketoglutarate aminotransferase has been found in the peroxisomes. By the addition of L-glutamate to the assay mixture, it is now possible to demonstrate L-malate oxidation as measured by following NAD^+ reduction. In this case the unfavorable

Figure 3

Schematic Representation of Starch-Gel Electrophoresis of the Various NAD-Malate Dehydrogenases.

Separation and staining techniques are described in the Materials and Methods Section.


Cathode
Peroxisomal
Supernatant
Homogenate
Mitochondrial
Origin
Anode

equilibrium position is shifted toward malate oxidation through removal of the product, oxalacetate by transamination.

Because of a report by Munkres (68) that NAD-malate dehydrogenase and aspartate aminotransferase activities are apparently associated with the same protein in Neurospora, amino acids and pyridoxal phosphate were added to the standard NAD-malate dehydrogenase assay using leaf peroxisomes. No effect was observed with 4 mM L-aspartate, L-glutamate, DL-serine, or 0.1 mM pyridoxal phosphate. The fact that L-glutamate had no effect may seem surprising in view of the fact that aspartate-α-ketoglutarate aminotransferase activity is present in leaf peroxisomes (discussed in the next section), but Kisaki and Tolbert (47) have shown that this reverse (glutamate-oxalacetate) aminotransferase activity of peroxisomes is very low. One may also conclude from these data that serine-oxalacetate aminotransferase activity in the peroxisomes is low relative to the NAD-malate dehydrogenase activity.

Aminotransferase Activities

In preliminary experiments on other transaminase activity of the peroxisomal fractions, it was found that serine plus pyruvate exhibited a relatively high rate of activity. This transaminase was measured by linking hydroxypyruvate formation to the D-glycerate dehydrogenase activity present in these peroxisomal fractions. This can be done with peroxisomal fractions, utilizing L-serine, L-aspartate, or glycine as amino donor since dehydrogenase or reductase activity is present for each of the corresponding keto acids. In this study it was found that no NADH oxidation was observed with peroxisomal fractions in the presence of either pyruvate or α-ketoglutarate. Alanine and glutamic dehydrogenase activities likewise could not be detected in peroxisomal fractions. The number of possible combinations of amino donors and acceptors which can be studied with such a system is extremely limited (6 combinations exist), but several activities which are believed to be highly specific were detected.

In Table 2 are summarized the data on the six aminotransferase combinations which could be tested. Significant activity was detected only with the combinations serine + pyruvate and aspartate + α-ketoglutarate. Addition of glyoxylate, oxalacetate, and hydroxypyruvate (all at a final concentration of 10 mM) to separate control cuvettes established that the respective dehydrogenase and reductase activities were not limiting. The two aminotransferase activities appear to be quite specific with respect to substrates. This was also found to be the case with the glutamate-glyoxylate aminotransferase of the peroxisomes (47). Using a different assay procedure, Kisaki and Tolbert were able to test a number of amino donors and acceptors in reaching this conclusion.

TABLE 2

Aminotransferase Activity of Leaf Peroxisomes as Measured by Coupling to Endogenous Dehydrogenases.

The assay mixture contained 0.5 ml of 0.1 M HEPES (pH 7.3), 0.05 ml of Fraction 3 from a sucrose density gradient, 0.35 ml of distilled water, 0.03 ml of the potassium salt of α-keto acid, and 0.02 ml of 3.8 mM NADH. The reaction was initiated by the addition of 0.05 ml of 0.4 M amino acid (pH 7). No rate was observed in the presence of α-keto acids alone or amino acids alone. Dehydrogenase or reductase activities were in excess as measured by addition of appropriate α-keto acids. The rate of serine-pyruvate aminotransferase activity was set at 100.

Amino Acid	α-Keto Acid	Relative Rate
DL-Serine	Pyruvate	100
	α-Ketoglutarate	3
Glycine	Pyruvate	0
	α-Ketoglutarate	0
L-Aspartate	Pyruvate	18
	α-Ketoglutarate	179

The lack of reaction using glycine as the amino donor is not surprising when one takes into account the unfavorable amino-group-transfer potential, i.e., the highly unfavorable free energy change in converting glycine to glyoxylate. Kisaki and Tolbert (47) also found the glutamate-glyoxylate aminotransferase to be "irreversible" and have discussed this point in some detail.

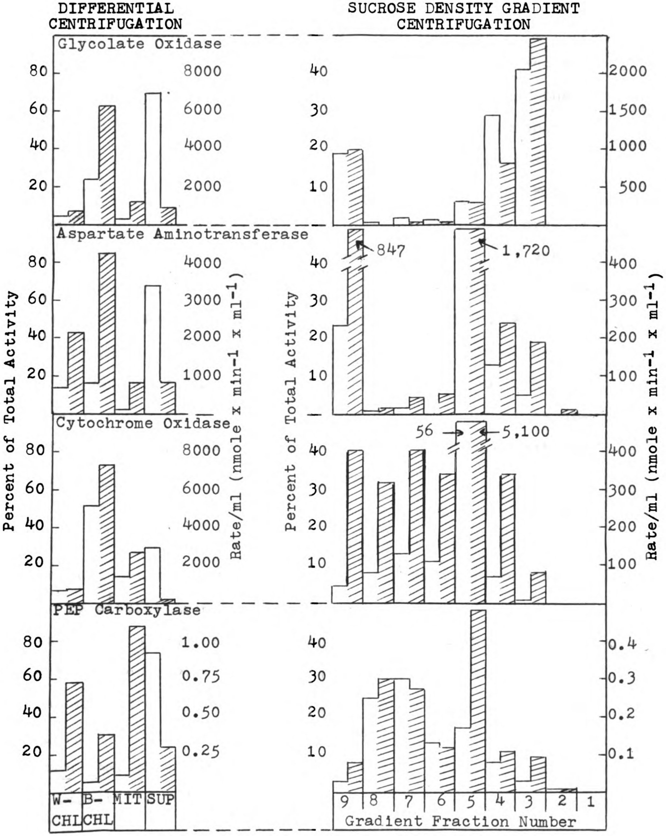
In order to determine whether the aspartate aminotransferase activity found in Fraction 3 could be attributed to peroxisomes, the distribution of this activity throughout differential and isopycnic centrifugation was studied (Fig. 4). Marker activities for the peroxisomes and mitochondria are included for comparison. PEP Carboxylase activity is also included since it appears that a component of this activity is located in the chloroplasts as is evidenced by the relatively high rate/ml in both the Whole Chloroplast fraction and Fractions 7 and 8 of the gradient (broken chloroplast fragments). Some of the activity in Fraction 5 may be attributed to chloroplasts since it has been observed that intact chloroplasts are present along with mitochondria in Fraction 5. The distribution of PEP carboxylase will be discussed further in a later section.

The aspartate aminotransferase activity exhibits a peak coinciding with the cytochrome oxidase peak, thus indicating mitochondria contain aspartate aminotransferase

Figure 4

Distribution of Aspartate Aminotransferase and PEP Carboxylase Activities Among Differential and Sucrose Density Gradient Centrifugation Fractions.

Abbreviations used: W-CHL, Whole Chloroplast Fraction; B-CHL, Broken Chloroplast Fraction; MIT, Mitochondrial Fraction; SUP, Supernatant Fraction as described in the Materials and Methods Section. ☐ , Percent of total activity; ▨, Rate/ml, expressed as nmole per min per mg protein.


DIFFERENTIAL CENTRIFUGATION
SUCROSE DENSITY GRADIENT CENTRIFUGATION
Glycolate Oxidase
Aspartate Aminotransferase
Cytochrome Oxidase
PEP Carboxylase
Percent of Total Activity
Rate/ml (nmole x min-1 x ml^{-1})
80
60
40
20
8000
6000
4000
2000
4000
3000
2000
1000
1.00
0.75
0.50
0.25
40
30
20
10
2000
1500
1000
500
400
300
200
100
0.4
0.3
0.2
0.1
847
1,720
56
5,100
W-CHL
B-CHL
MIT
SUP
9
8
7
6
5
4
3
2
1
Gradient Fraction Number

activity. A portion of the animal aspartate aminotransferase activity has been demonstrated by Lardy (53) to be mitochondrial. The relatively high rate/ml of the Whole Chloroplast Fraction is taken to be an indication of the presence of aspartate aminotransferase activity in chloroplasts. PEP Carboxylase can be seen to show a similar high relative rate in the Whole Spinach Chloroplast Fraction. Rosenberg, Capindale, and Whatley (82) have presented evidence for PEP carboxylase activity in spinach chloroplasts. The low percentage of both aspartate aminotransferase and PEP carboxylase activities in the Whole Chloroplast Fraction may be a reflection of solubilization of these enzymes upon homogenization. Heber (40) and Mukerji and Ting (65) have reported the presence of aspartate aminotransferase activity in chloroplast preparations from Vicia faba and Opuntia respectively.

In comparison of the rate/ml for cytochrome oxidase and aspartate aminotransferase, more aspartate aminotransferase activity is found in Fraction 3 than can be accounted for by mitochondrial activity. Kisaki and Tolbert (47) also found a large amount of aspartate aminotransferase activity coinciding with the peroxisomal fraction. Close examination of the substrate specificity of the glutamate-glyoxylate aminotransferase studied by these authors indicates that the aspartate-α-ketoglutarate activity cannot be attributed to substrate non-specificity of the glycineforming enzyme. Investigations of the specific activity of the aspartate aminotransferase activity as detailed in the Summary and Conclusions Section indicate this activity to be 40 times greater than the glutamate-glyoxylate aminotransferase activity. From all these considerations, it is concluded that peroxisomes, mitochondria, and chloroplasts all contain aspartate aminotransferase activity.

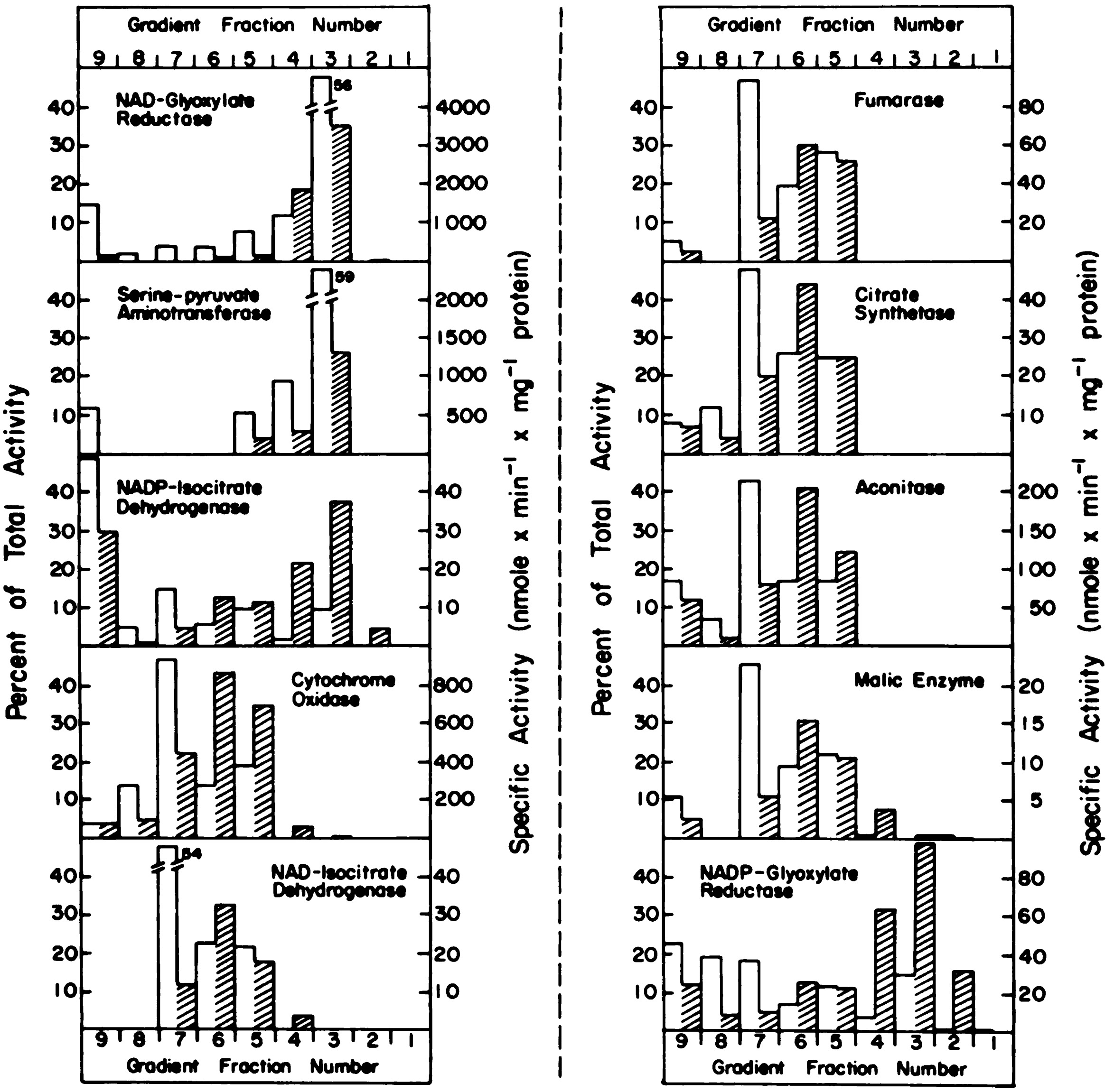
Aspartate is thought to be an intracellular transport form of oxalacetate (53). Thus it is not surprising to find aspartate aminotransferase activity in peroxisomes, mitochondria, and chloroplasts since all three organelles apparently contain enzymes involved in oxalacetate metabolism.

Data for the distribution of serine-pyruvate aminotransferase activity on a sucrose density gradient are presented in Fig. 5. Clear correspondence of the aminotransferase activity with that of the peroxisomal marker, NAD-glyoxylate reductase, indicates that the serine-pyruvate aminotransferase activity is peroxisomal. This aminotransferase activity has been previously described by Sallach (14) and implicated in the operation of the glycolate pathway, but the intracellular localization was not determined. With respect to various plant sources, Willis and Sallach (99) were able to demonstrate that the aminotransferase activity closely paralleled the activity of the D-glycerate dehydrogenase described by Stafford, Magaldi, and Vennesland (90). That is, plants with high glycerate dehydrogenase

Figure 5

Distribution of Serine-Pyruvate Aminotransferase, NADP-Isocitrate Dehydrogenase, NAD-Isocitrate Dehydrogenase, Fumarase, Citrate Synthetase, Aconitase, Malic Enzyme, and Marker Activities After Sucrose Density Gradient Centrifugation of Spinach Broken Chloroplast Fraction.

□, Percent of total activity; ▨, Specific activity, expressed as nmole per min per mg protein at 25°. Data for NAD- and NADP-glyoxylate reductase activities from A. Oeser and N. E. Tolbert.


Gradient Fraction Number
9 8 7 6 5 4 3 2 1
Percent of Total Activity
Specific Activity (nmole x min^{-1} x mg^{-1} protein)
NAD-Glyoxylate Reductase
56
40 30 20 10
4000 3000 2000 1000
Serine-pyruvate Aminotransferase
59
40 30 20 10
2000 1500 1000 500
NADP-Isocitrate Dehydrogenase
40 30 20 10
40 30 20 10
Cytochrome Oxidase
40 30 20 10
800 600 400 200
NAD-Isocitrate Dehydrogenase
54
40 30 20 10
40 30 20 10
9 8 7 6 5 4 3 2 1
Gradient Fraction Number
Gradient Fraction Number
9 8 7 6 5 4 3 2 1
Percent of Total Activity
Specific Activity (nmole x min^{-1} x mg^{-1} protein)
Fumarase
40 30 20 10
80 60 40 20
Citrate Synthetase
40 30 20 10
40 30 20 10
Aconitase
40 30 20 10
200 150 100 50
Malic Enzyme
40 30 20 10
20 15 10 5
NADP-Glyoxylate Reductase
40 30 20 10
80 60 40 20
9 8 7 6 5 4 3 2 1
Gradient Fraction Number

act

pla

ser

ism

act

pyr

deh

per

tio

of

pro

si

fer

exh

it

ph

tr

li

bo

inh

the

obs

red

per

ion

activity had high aminotransferase activity, and conversely plants with low glycerate dehydrogenase activity had low serine-pyruvate aminotransferase activity. This parallelism can now be interpreted in terms of high peroxisomal activity versus low peroxisomal activity.

This demonstration in the peroxisome of serine-pyruvate aminotransferase activity coupled to D-glycerate dehydrogenase activity establishes the ability of the peroxisome to convert serine to glycerate. Thus all reactions of the glycolate pathway leading to the conversion of glycolate to glycerate have been demonstrated to be present in the peroxisome with the exception of the conversion of glycine to serine.

The serine-pyruvate and aspartate-α-ketoaminotransferase activities of the peroxisome have been found to exhibit several common properties. Neither of the activities is stimulated by the inclusion of 0.1 mM pyridoxal phosphate in the assay mixture. This was also found to be true of the glutamate-glyoxylate aminotransferase (unpublished results of T. Kisaki and N. E. Tolbert). Likewise, both of the aminotransferase activities studied here were inhibited 10-20% by the addition of 0.01% Triton X-100 to the assay. This observation contrasts strikingly with the observations that the glycolate oxidase, NAD-glyoxylate reductase, and malate dehydrogenase activities of the peroxisome are stimulated 30% by the addition of the non-ionic detergent Triton X-100.

NADP

gena
were
roge
beer
seer
NADP
perc
part
the
seen
dehy
mito
citr
some

the
of t
age
dria
roge
leaf
was
centr
dehyd

NADP-Isocitrate Dehydrogenase

As was found in the case of the NAD-malate dehydrogenase, two isozymes with respect to particle localization were found. The distribution of the NADP-isocitrate dehydrogenase is given in Fig. 5. NAD-glyoxylate reductase has been used here as a peroxisomal marker. It can be clearly seen that the peak specific activity (Fraction 3) for the NADP-isocitrate dehydrogenase coincides with that of the peroxisomal marker in a sucrose density gradient of spinach particulate material. Relative to the specific activity of the NAD-glyoxylate-reductase, Fractions 5, 6 and 7 can be seen to have a high specific activity for NADP-isocitrate dehydrogenase. This activity parallels that found for the mitochondrial marker cytochrome oxidase. Thus NADP-isocitrate dehydrogenase activity is located in both peroxisomes and mitochondria.

The large amount (50%) and high specific activity of the NADP-isocitrate dehydrogenase found in the supernatant of the gradient (Fraction 9) may be an indication of leakage of this activity from the peroxisomes and/or mitochondria. Such was not found in the case of NAD-malate dehydrogenase, however. Upon fractionation of the original leaf homogenate, 90% of the NADP-isocitrate dehydrogenase was found in the supernatant fraction from a 39,000 g centrifugation (data not given). In contrast NAD-malate dehydrogenase which is also localized in peroxisomes and

and mitochondria showed only 40% of its activity in supernatant fractions. It is difficult to conceive of one enzyme (NAD-malate dehydrogenase) being rather tightly bound to cellular organelles while another is easily lost unless the second enzyme (NADP-isocitrate dehydrogenase) is located _on_ the membrane(s). No evidence to support this membrane localization exists to date. It is thus inviting to ascribe the supernatant NADP-isocitrate dehydrogenase activity to a cytoplasmic or soluble isozyme. Some of the gradient supernatant activity might be attributed to contamination from this cytoplasmic activity.

NADP-Isocitrate dehydrogenase has been found in all peroxisomes tested to date. This includes peroxisomes from rat liver, _Tetrahymena_, and spinach leaves. The specific function of this peroxisomal activity, however, is unknown. In the case of the spinach leaf peroxisomal NADP-isocitrate dehydrogenase, the relatively low specific activity may preclude its playing a major role in the peroxisomal metabolism. This point will be discussed further in later sections.

Müller, Hogg and de Duve (67) found a somewhat similar distribution for NADP-isocitrate dehydrogenase in _Tetrahymena_. They found about 50% of the activity in the peroxisomes, 5% in the mitochondria, and the rest was found to be soluble. The distribution of activity from spinach leaves agrees in localization, but the quantitative aspects differ considerably.

NAD-Isocitrate Dehydrogenase, Fumarase, Citrate Synthetase, and Aconitase

As is seen in Figure 5, NAD-isocitrate dehydrogenase, fumarase, citrate synthetase and aconitase activities parallel that of the mitochondrial marker cytochrome oxidase. This is true both of the specific activities and percentage distributions. It is thus concluded that these activities are exclusively mitochondrial with respect to organelle distribution. No conclusions as to occurrence of cytoplasmic isozymes can be drawn from the data.

The NAD-isocitrate dehydrogeanse activity was measured in the presence of 1 mM citrate. This was in keeping with the report that the enzyme from Brassica napis is stimulated by citrate (25). The enzyme from Brassica was reported to be unaffected by AMP. This is in contrast to reports from animal systems where the enzyme is activated by AMP. No effort was made in the present study to optimize assay conditions with respect to activators. Thus higher specific activities might be obtained by the addition of activator(s) other than citrate. Specific activity figures should be regarded as being minimal.

Fumarase was assayed in the presence of 20 mM ammonium sulfate. Once again no effort was made to optimize activator and assay conditions. These results must also be regarded as being minimal with respect to specific activity.

Citrate synthetase was assayed at pH 7.4, utilizing the disulfide interchange reaction between CoA-SH and Ellman's reagent. Early reports utilizing this assay had reported the disulfide interchange reaction to be base-catalyzed. A pH of 8.1 was used in these reports. In this study the rate of reaction in the citrate synthetase assay at pH 7.4 was found to be 1.44 times that at pH 8.1. If the interchange reaction is base-catalyzed, it must be that the increased enzymic activity at pH 7.4 more than compensates for the lower rate of disulfide interchange. Similar results must have been found by Hock and Beevers (41) who used a similar assay for malate synthetase. These authors used a pH of 7.1 in their assay procedure.

In assaying the sucrose density gradients for possible malate synthetase activity, it was found that the mitochondrial citrate synthetase exhibited a low rate when glyoxylate was used as substrate in place of oxalacetate. The rate with saturating glyoxylate was found to be about 5% of that with oxalacetate. These data are presented in Table 3. No reaction was observed with pyruvate as substrate. It can be seen that the combination of oxalacetate and glyoxylate resulted in 20% inhibition over the control. The addition of 10 μM palmityl-CoA was found to give 25% inhibition (Table 4). ATP and ADP to a lesser extent were also found to give inhibition. These findings are in agreement with those found by Hathaway and Atkinson (39)

TABLE 3

α-Keto Acid Specificity of Citrate Synthetase

Assay conditions as described in Methods and Materials for citrate synthetase except for the substrate used. All α-keto acids used at a final concentration of 2.5 mM.

α-Keto Acid	Rate (Arbitrary units)	Relative Rate (OAA = 100)
Oxalacetate	252	100
Glyoxylate	12	5
Pyruvate	0	0
Oxalacetate + glyoxylate	204	80

TABLE 4

Effects of Adenine Nucleotides and Palmityl-CoA on "Malate Synthetase" Activity of Citrate Synthetase

Assay conditions as described in Materials and Methods.

Additions	Relative Rate
Control 1	100
2	100
+ AMP, 1 mM	100
10 mM	81
+ ADP, 1 mM	75
10 mM	50
+ ATP, 1 mM	56
10 mM	47
+ Palmityl-CoA, 10 μM	75

in the case of yeast citrate synthetase. The differential effects produced by the degree of phosphorylation of the adenine nucleosides probably reflects control of citrate synthetase activity by the energy charge of the cell as advocated by Atkinson (4).

This activity of citrate synthetase with glyoxylate was found to be stimulated by the addition of Mg^{++}, with maximal stimulation (30%) occurring at a concentration of 1 mM Mg^{++}. To my knowledge no reports exist in the literature concerning either utilization of glyoxylate by citrate synthetase or stimulation of citrate synthetase by metal ions. The significance of this "malate synthetase" activity of citrate synthetase is unknown.

It should be noted that neither fumarase nor citrate synthetase activities were found in the peroxisome fractions. These two activities were checked because of the presence of NAD-malate dehydrogenase activity in peroxisomes. With respect to TCA enzymes involved in malate or oxalacetate metabolism, the peroxisomal NAD-malate dehydrogenase appears to be isolated.

The fact that aconitase activity was not found in the peroxisomes may indicate an isolated role for the NADP-isocitrate dehydrogenase. No attempt was made to determine if any α-keto acid dehydrogenase activity was present in peroxisomes, but this possibility seems remote in view of the mitochondrial localization in animal tissue.

Malic Enzyme

The distribution of the malic enzyme is given in Fig. 5. In the particulate fractions the malic enzyme activity is clearly associated with the mitochondria. To my knowledge a mitochondrial localization has not been reported previously from plant leaf tissue. Several reports have located malic enzyme activity in chloroplast fractions, but these reports have been limited to cactus (65) and maize (87). In the case of the bovine adrenal cortex, Simpson and Estabrook (86) have reported the occurrence of two forms of malic enzyme. The mitochondrial form is thought to function in the direction of malate oxidation, thus providing NADPH for the 11 β-hydroxylation system. The cytoplasmic form is thought to function in the direction of the reductive carboxylation of pyruvate to regenerate L-malate. The two enzymes thus may mediate the transport of NADPH reducing equivalents from the cytosol into the mitochondrion for use in steroid hydroxylation. Because malic enzyme activity has not been measured in spinach leaf homogenates, no conclusions can yet be drawn concerning the function of the mitochondrial malic enzyme activity, but it may be that the NADPH-shuttle mechanism proposed by Simpson and Estabrook for bovine adrenal cortex will find more general application.

PEP Carboxylase

DATA for the distribution of PEP carboxylase activity in a sucrose density gradient are given in Fig. 5. The enzyme activity does not closely parallel any of the marker activities. There does appear to be a chloroplast component of this activity as evidenced by the relatively high rates obtained in the Whole Chloroplast Fraction and Fractions 7 and 8 of the gradient. The significance of the high rate of the Mitochondrial Fraction is not clear at this time since the activity does not follow the distribution of the cytochrome oxidase. Consideration of this rate plus the high rate of Fraction 5 from the gradient have led to the tentative conclusion that mitochondria also contain PEP carboxylase activity.

Rosenberg, Capindale, and Whatley (82) reported the occurrence of PEP carboxylase activity in chloroplast extracts from spinach leaves but a rigorous test of the intracellular localization of the enzyme was not undertaken. Mazelis and Vennesland (64) found some PEP carboxylase activity in chloroplast fractions from spinach leaves, but they found higher activity in the small particulate fraction, which presumedly would correspond to a mitochondrial fraction. PEP Carboxykinase activity was also indicated to be present in this fraction.

Because no correlation could be drawn between the PEP carboxylase and glycolate oxidase activity, it was concluded that peroxisomes do not contain PEP carboxylase activity. Activity was found in fractions containing mitochondria and chloroplasts, but, because of the anomalous behavior of the PEP carboxylase activity with respect to the marker activites, conclusions as to chloroplast and/or mitochondrial localization can only be regarded as tentative at best.

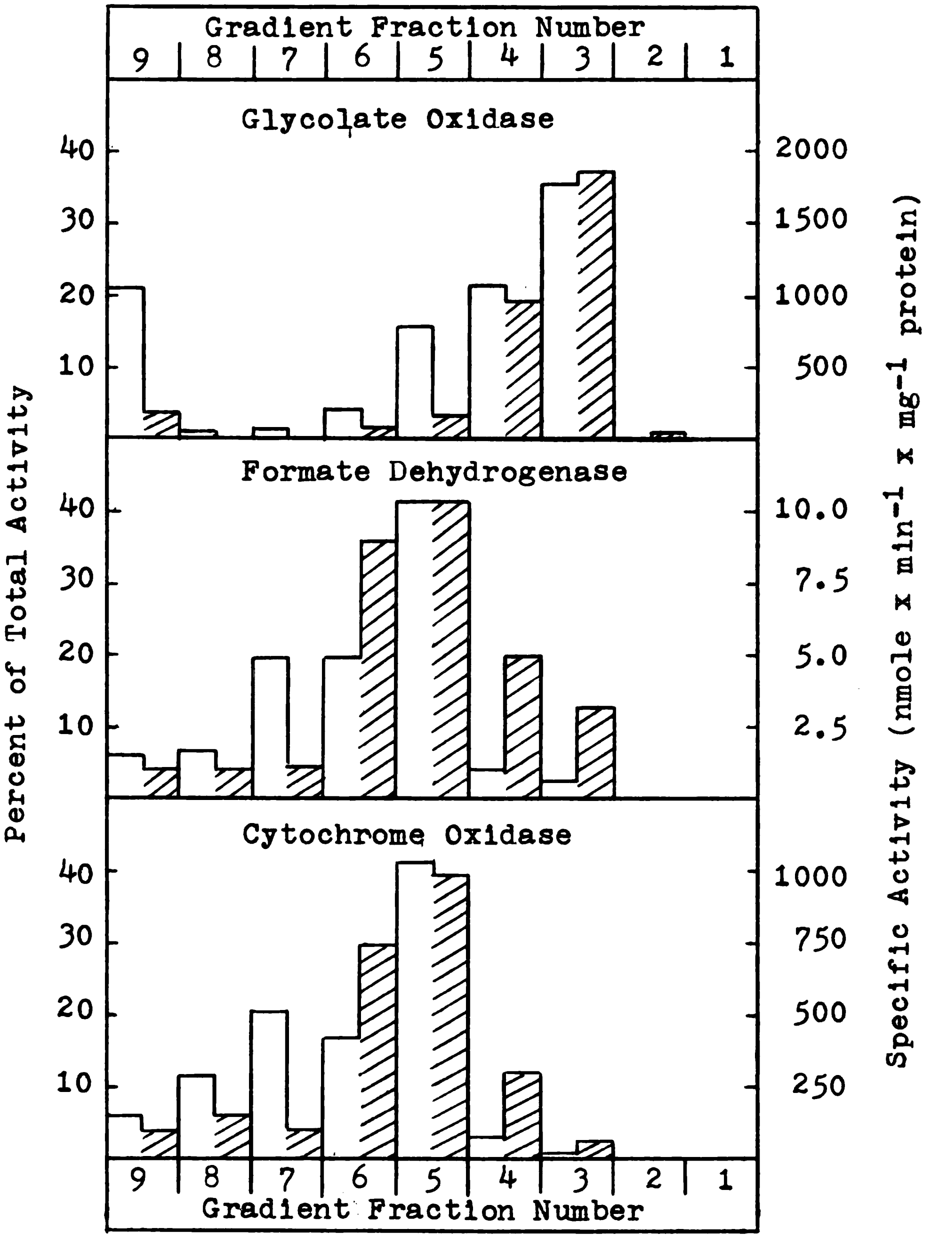
Formate Dehydrogenase

Because of the presence in the leaf peroxisomes of various organic acid dehydrogenases, and because of the implication by Zelitch (108) that formate is one of the products of the *in vivo* oxidation of glycolate, an investigation of the intracellular localization of formate dehydrogenase was made. The distribution of the activity as shown in Fig. 6 indicates that the particulate formate dehydrogenase activity is mitochondrial. Mazelis (63) had previously indicated a particulate localization for the enzyme from leaf tissue, but the exact nature of these particles was not determined. The specific activity figures for the formate dehydrogenase should be taken as minimal since optimum conditions were not determined for the assay. The assay pH used in this study was the same as that determined to be optimal for the cabbage leaf enzyme by Mazelis. Formate was determined to be saturating under the conditions used.

Figure 6

Distribution of Formate Dehydrogenase and Marker Activities After Sucrose Density Gradient Centrifugation

☐, Percent of total activity; ▨, specific activity expressed as nmole per min per mg protein.


Gradient Fraction Number
9
8
7
6
5
4
3
2
1
Glycolate Oxidase
40
30
20
10
2000
1500
1000
500
Formate Dehydrogenase
40
30
20
10
10.0
7.5
5.0
2.5
Cytochrome Oxidase
40
30
20
10
1000
750
500
250
Percent of Total Activity
Specific Activity (nmole x min^{-1} x mg^{-1} protein)

Preliminary experiments on the properties of the formate dehydrogenase indicated that the activity was membrane-bound since the addition of the non-ionic detergent Triton X-100 gave a 20% stimulation in the rate (Triton X-100 was routinely added in the assays). The addition of dithiothreitol gave a small (10%) stimulation of rate. Mazelis (63) observed similar stimulation by sulfhydryl-reducing agents with the preparation from cabbage leaves.

Considerations of the equilibrium indicate the reaction probably proceeds in the direction of formate oxidation. Certainly a role of the enzyme in the reductive-fixation of CO_2 during photosynthesis may be ruled out by energetic and locational factors. But the function of the formate dehydrogenase in the mitochondrial metabolism cannot be determined at this time.

Glyoxylate Oxidase

An enzyme activity catalyzing the oxidation of glyoxylate has been reported by Müller, Hogg and de Duve (67) to be present in *Tetrahymena* peroxisomes. The activity was shown to be a true oxidase producing hydrogen peroxide, but the question as to whether the enzyme involved was the same as the α-hydroxy-acid oxidase present in these peroxisomes was not resolved. No evidence has been presented for the existence of a separate enzyme for the oxidation of glyoxylate in leaves of higher plants. However, Richardson and Tolbert (80) have reported that plant glycolate oxidase will utilize glyoxylate as a substrate. The product of this oxidation was shown to be oxalic acid. When added to the assay, oxalate was shown to inhibit severely sugar beet glycolate oxidase activity with glyoxylate, but activity with glycolate was almost unimpaired.

In experiments on the oxidative activity of spinach leaf peroxisome fractions, certain anomalous results were obtained which may indicate the presence in the peroxisomes of a unique glyoxylate oxidase activity. Assays were performed on peroxisome fractions with both an anaerobic DCPIP spectrophotometric technique and a standard Warburg oxygen uptake method. In both cases the assay mixture contained 0.067 M TES (pH 7.4), 0.55 M sucrose, 0.01 M $MgCl_2$, and enzyme plus water to give a total volume of 3 ml after the addition of substrate. The spectrophotometric assay contained 0.005 M DCPIP. The final concentration of glyoxylate was 10^{-3}M in the spectrophotometric assay and 6.25 x 10^{-3}M in the Warburg assay. In both cases the reaction was initiated by the addition of glyoxylate.

In both the spectrophotometric and Warburg assays, the addition of 10^{-4}M NAD^+ was found to inhibit the rate of oxidation of glyoxylate about 30%. This inhibition cannot be due to diversion of reducing equivalents to NAD^+ from dye or oxygen, as evidenced by the fact that the addition of phenazine methosulfate did not alter the results. Phenazine methosulfate will mediate oxygen or dye reduction in the presence of NADH. The effect of NAD$^+$ on glycolate oxidation was not tested. In the Warbrug assay the addition of 10^{-3}M 2-pyridine-hydroxymethanesulfonate (PHMS), a known inhibitor of glycolate oxidase (107), was found to inhibit the rate of glyoxylate oxidation 27% whereas the rate of oxidation of glycolate (at a final concentration of 5×10^{-2}M) was inhibited 97%. The effects of PHMS could not be tested in the spectrophotometric assay since PHMS reacts with 2,6-dichlorophenolindophenol. None of the above experiments taken separately show the presence of glyoxylate oxidase, but taken together they may implicate the presence of a unique activity for the oxidation of glyoxylate in the peroxisome.

Malate Synthetase and Isocitrate Lyase

These two enzymes which are unique to the glyoxylate cycle were tested in leaf peroxisomes because of their occurrence in *Tetrahymena* peroxisomes (67) and glyoxysomes from castor bean endosperm (10, 11). Assay procedures are detailed in the Materials and Methods Section. Results of assays for both enzymes were negative with leaf peroxisomes although a malate synthetase activity was found to coincide with citrate synthetase activity in gradients. These data have already been discussed under the heading Citrate Synthetase. Yamamoto and Beevers (102) found isocitrate lyase activity only in homogenates of those tissues containing an active glyoxylate cycle. Traces of malate synthetase activity were found in leaf tissue by Carpenter and Beevers (12), but large amounts were found associated with an active glyoxylate cycle. The traces of activity found in leaf tissue may be a reflection of the "malate synthetase" activity of citrate synthetase as described in this thesis.

β-Oxidation

β-Oxidation is the process by which long-chain fatty acyl-CoA derivatives are oxidized to acetyl-CoA fragments with the concurrent reduction of pyridine nucleotide. This process has been shown by Cooper and Beevers (18) and Hutton and Stumpf (44) to occur in the glyoxysomes of germinating castor bean endosperm. The acetyl-CoA generated by this process is then utilized in the glyoxylate cycle to produce succinate which is eventually converted to carbohydrate. The presence of the β-oxidation system plus the glyoxylate cycle thus gives a clear gluconeogenic function to the glyoxysome.

β-Oxidation activity of leaf peroxisomes was checked using a modification of the method of Yamada and Stumpf (100). The reaction mixture contained 12 nmole of palmityl-CoA, 0.2 μmole of NAD^+ and $NADP^+$, 1 μmole ATP, 1 μmole $MnCl_2$, 100 μmole phosphate buffer (pH 7.3), 1 μmole $MgCl_2$, 5 μmole dithiothreitol, 0.02 μmole FAD, and 0.1 ml of a concentrated peroxisomal fraction in a total volume of 1.0 ml. This mixture was checked for oxygen uptake with a Clark oxygen electrode. A similar mixture without $MnCl_2$ was checked for pyridine nucleotide reduction in a spectrophotometer. In all cases no evidence for β-oxidation activity was found in the peroxisome fraction. Additions of Triton X-100, CoA-SH, or cyanide did not alter these results. It was thus concluded that β-oxidation activity was not present in leaf peroxisomes.

α-Oxidation

The process of α-oxidation has been reviewed by Stumpf (91). The first step in the process is thought to be a peroxidation of a long-chain free fatty acid to give a D-α-hydroxy-fatty acid. The hydroxy acid is then oxidatively decarboxylated to give the corresponding aldehyde and CO_2. Early experiments by Castelfranco, Stumpf, and Coutopoulou (13) indicated that glycolic acid activated α-oxidation in homogenates from peanut cotyledon. This activation was presumed to take place via the production of hydrogen peroxide by glycolate oxidase. Because of the presence of both α-hydroxy acid oxidase activity and hydrogen peroxide formation in the peroxisomes, α-oxidation activity of a peroxisome fraction was checked.

The assay was done in collaboration with A. Oeser. Activity was determined in two ways: stimulation of glycolate-dependent oxygen uptake on the Warburg respirometer, and trapping of $^{14}CO_2$ released from palmitic-1-^{14}C acid incubated with intact peroxisomes and glycolate. With both of these methods, no evidence was obtained for α-oxidation activity in leaf peroxisomes.

Other Enzyme Activities Not Found in Peroxisomes

The following enzyme activities could not be detected in preliminary experiments with spinach leaf peroxisomal fractions. Results are summarized in Table 5.

Glutamic and alanine dehydrogenase activities could not be detected as measured by following NADH oxidation by enzyme samples upon the addition of either α-ketoglutarate or pyruvate and ammonium ions. Significant activity with pyruvate was detected in mitochondrial fractions using the same procedure. Neither was activity found in peroxisomal fractions using NADPH and α-ketoglutarate. Aspartate dehydrogenase activity was checked through the addition of ammonium chloride in the standard NAD-malate dehydrogenase assay. No stimulation above a nonspecific salt effect was seen, indicating no detectable aspartate dehydrogenase activity in peroxisomal fractions.

Kornberg (48) has reported that *Micrococcus denitrificans* catabolizes glyoxylate by transamination to glycine and condensation of glycine and glyoxylate to give *erythro*-β-hydroxyaspartate. This compound then undergoes a dehydratase reaction to give ammonia and oxalacetate. A similar set of reactions might be postulated to take place in the peroxisomes because of the presence of glycine, glyoxylate and presumably oxalacetate. However, addition of DL-erythro-hydroxyaspartate to the standard NAD-malate dehydrogenase assay gave no rate of reaction. If a dehydratase activity were present, the oxalacetate formed would be reduced due to the presence of NAD-malate dehydrogenase in the peroxisomes.

Glyoxylate dehydrogenase activity of peroxisomes was assayed by the method of Quayle (76). The enzyme from *Pseudomonas* reversibly catalyzes the conversion of glyoxylate and free CoA-SH to oxalyl-CoA with the concomitant reduction of $NADP^+$. This activity could not be detected in spinach leaf peroxisome fractions.

Oxalate oxidase has been reported by Finkle and Arnon (28) to be a particulate enzyme in sugar beet leaves. As described by these authors, the enzyme produces hydrogen peroxide and CO_2. Using the Warburg respirometer, no oxalate-dependent oxygen uptake could be detected in any fractions from a sucrose density gradient preparation of spinach particulates.

Catechol oxidase (polyphenol oxidase) activity as determined by Warburg manometry on sucrose density gradient fractions coincided with the chlorophyll content of the fractions (data not shown). Little or no activity could be detected in peroxisome fractions. It was concluded that catechol oxidase activity was located in the chloroplast as found by Arnon (2) and Mayer (62).

PEP Carboxykinase activity of peroxisomal fractions was checked by looking for stimulation of fixation of radioactive bicarbonate into organic acids by the addition of ADP (64). The addition of 0.9 mM ADP to the PEP carboxylase assay (see Materials and Methods) was found to inhibit fixation 25% in peroxisomal fractions. The fixation initially present in the peroxisomal fractions has been attributed to other particles (see discussion of PEP carboxylase). Thus PEP carboxykinase activity was not detected in peroxisomal fractions.

Phenylalanine ammonia-lyase activity of a peroximal fraction was determined by the method of Zucker (116). The procedure follows the formation of <u>trans</u>-cinnamate, which absorbs at 290 mμ. No activity was detected in the peroxisomal fraction.

GENERAL DISCUSSION

In trying to determine the function of a new organelle such as the leaf peroxisome, two approaches may be used. The first involves supplying the organelle with starting material(s), allowing metabolism to take place, and isolating intermediates and end product(s). From the nature of the end product(s) found, one can then attempt to deduce the nature of the individual reactions and the function of the organelle as a whole. The second approach involves searching for the individual reactions of the organelle and then trying to deduce the function as a summation of the individual reactions.

Large limitations are inherent in both approaches. The first suffers from the fact that the *in vivo* starting material(s) and cofactor and other substrate requirements may not be known. In the case of the second approach, one can only guess as to which enzyme activities to try. Negative results may be obtained because of the use of suboptimal assay conditions. It would thus appear that a combination of both approaches might yield the most information.

Kisaki and Tolbert (47) supplied the isolated leaf peroxisomes with ^{14}C-labeled glycolate and glyoxylate and found only glycine to be labeled. Glycine was thus indicated to be an end product of the peroxisomal metabolism of glycolate. Investigation of other enzyme activities of the peroxisome, however, showed the presence of NAD-malate dehydrogenase as detailed in this thesis. Because an adequate explanation could not be given for the presence of this enzyme in the peroxisome, a study of other possible activities was initiated. In this study of the intracellular distribution of various enzyme activities, four were found in the peroxisome. The other activities could not be detected in the peroxisome. The divisions in this general discussion are based on this peroxisomal or extraperoxisomal localization. The results of these intracellular distribution studies are summarized in Table 5.

Activities Not Found in Leaf Peroxisomes

NAD-Isocitrate dehydrogenase, fumarase, citrate synthetase, and aconitase activities appear to be localized exclusively in the mitochondrial fraction after isopycnic centrifugation of spinach leaf particulate fractions. While the finding of TCA cycle enzyme activities in mitochondria does not constitute an increase in knowledge of the role of the mitochondrion, the absence of detectable activities of citrate synthetase and aconitase from the leaf peroxisomes infers a basic difference between glyoxysomes and leaf peroxisomes.

Citrate synthetase and aconitase have been shown by

TABLE 5

Summary of Intracellular Localization in Spinach Leaves of Enzymes Studied

Symbols: +, activity present; 0, activity not detected; -, activity not searched for; tr, trace activity.

Enzyme or activity	Peroxisome	Mitochondrion	Chloroplast
NAD-Malate dehydrogenase	+	+	0
NADP-Isocitrate dehydrogenase	+	+	0
Aspartate aminotransferase	+	+	+
Serine-pyruvate aminotransferase	+	0	0
Glyoxylate oxidase	?	-	-
NAD-Isocitrate dehydrogenase	0	+	0
Fumarase	0	+	0
Citrate synthetase	0	+	0
Aconitase	0	+	0
Malate synthetase	0	tr	-
Isocitrate lyase	0	-	-
Malic enzyme	0	+	0
Formate dehydrogenase	0	+	0
NAD-glutamate dehydrogenase	0	+	-
NAD-alanine dehydrogenase	0	-	-
NAD-aspartate dehydrogenase	0	-	-
PEP Carboxylase	0	?	+
PEP carboxykinase	0	-	-
Hydroxyaspartate dehydratase	0	-	-
α-Oxidation	0	-	-
β-Oxidation	0	-	-
Oxalate oxidase	0	0	0
Catechol oxidase	0	0	+
Phenylalanine ammonia-lyase	0	-	-

Breidenbach, Kahn, and Beevers (11) to be present in the castor bean endosperm glyoxysomes. Similarly, malate synthetase and isocitrate lyase, which are present in the glyoxysomes, could not be detected in leaf peroxisomes. From these results it is concluded that leaf peroxisomes do not contain a functional glyoxylate cycle. This conclusion can be inferred from the lack of isocitrate lyase and malate synthetase activities in crude homogenates of mature leaf tissue (7). Glyoxysomes, on the other hand, contain all the enzyme activities of the glyoxylate cycle (10, 11). Inability to detect β-oxidation activity in peroxisomes is taken as another indication of the difference between leaf peroxisomes and glyoxysomes which have been shown to contain β-oxidation activity (18).

The reaction of glyoxylate with acetyl-CoA as catalyzed by citrate synthetase appears not to have been reported previously in the case of the plant enzyme. The occurrence of such a reaction may explain formation of malate from glyoxylate as proposed by Asada _et_ _al_. (3) in spinach leaves.

The finding of NADP-isocitrate dehydrogenase activity in peroxisomes, mitochondria, and supernatant fractions suggests a transport or shuttle function for these locational isozymes. Since mitochondria do not readily oxidize exogenous citrate (78), one or both of the extra-mitochondrial forms may be active in the oxidation of externally-supplied citrate to α-ketoglutarate. This is perhaps more of a possibility in the case of the supernatant activity, since large amounts of aconitase activity have also been found in supernatant fractions from tobacco (74). In either case the extra-mitochondrial oxidation of isocitrate would supply the cytoplasm or peroxisome with NADPH which could then be utilized in biosynthetic sequences. Cleland (16) has also suggested that the extra-mitochondrial NADP-isocitrate dehydrogenase(s) may be carrying out the reductive carboxylation of α-ketoglutarate.

The presence of malic enzyme in spinach leaf mitochondria may also be an indication of a shuttle mechanism for reducing equivalents. Simpson and Estabrook (86) have suggested the operation of a malate-pyruvate shuttle in the transport of NADPH reducing equivalents into the mitochondria of bovine adrenal cortex. The possibility of similar shuttle mechanisms operating in plant tissue has not yet been fully explored. The mitochondrial localization of malic enzyme activity may argue against a significant contribution of this activity to the total photosynthetic CO_2 fixation of spinach. The localization in spinach mitochondria differs from that found in maize by Slack and Hatch (87). These authors found malic enzyme activity to be associated with the chloroplasts after nonaqueous isolation.

The presence of formate dehydrogenase and serine transhydroxymethylase (unpublished results of W. J. Bruin and N. E. Tolbert) in the mitochondria is suggestive of an active role of the mitochondrion in C_1 metabolism. An interrelation of C_1 metabolism and TCA cycle activity is suggested by the finding of Magar and Homi (60) that folate is an allosteric inhibitor of NADP-isocitrate dehydrogenase activity.

Inability to detect NAD-glutamate dehydrogenase, alanine dehydrogenase, and aspartate dehydrogenase activities in peroxisomal fractions is taken as an indication that peroxisomes are not able to carry out the reductive amination of α-keto acids. This implies that amino groups must be supplied from an external source for the aminotransferase activites found in the peroxisomes. A close relationship must therefore exist between the peroxisomes and mitochondria and/or chloroplasts with respect to amino acid metabolism. The close spatial association of peroxisomes, chloroplasts, and mitochondria as described by Frederick and Newcomb (29) is of special significance in this respect.

The absence of significant PEP carboxylase and PEP carboxykinase activities as well as malic enzyme in peroxisomal fractions may indicate inability of the peroxisome to convert C_4 compounds to C_3 compounds and vice versa. Gluconeogenesis from C_4 acids thus appears unlikely in the peroxisomes.

An unusual pathway for glyoxylate metabolism has been demonstrated in Micrococcus denitrificans by Kornberg (48). Glyoxylate is first transaminated to glycine. The glycine is then condensed with another glyoxylate to give erythro-hydroxyaspartate. Finally the hydroxyaspartate undergoes a dehydratase reaction to give oxalacetate. Because of the presence of glyoxylate and glycine in the peroxisome, hydroxyaspartate dehydratase activity of peroxisomal fractions was assayed. No activity was detected. This finding, plus the fact that Kisaki and Tolbert (47) did not find any other amino acids beside glycine in peroxisomal experiments with ^{14}C-labeled glycolate and glyoxylate, indicate that peroxisomes do not metabolize glyoxylate by the β-hydroxyaspartate pathway.

Glycolate and glycolate oxidase have been implicated in the generation of H_2O_2 needed for α-oxidation (13), but the failure to find α-oxidation activity in leaf peroxisomes may indicate that sources of H_2O_2 other than the peroxisomal glycolate oxidase must be utilized in this process. Such is probably also true of peroxidase activities since no peroxidase activity was detected in leaf peroxisomes (97).

The inability to demonstrate oxalate oxidase in spinach leaf particulate fractions is probably a reflection of the inertness of oxalic acid in this plant. Oxalic acid is known to make up about 80% of the total titratable acid content of spinach leaf (79). Finkle and Arnon (28) have indicated localization of oxalate oxidase in "cytoplasmic particles" from sugar beet leaves. Further investigation of this localization in sugar beet leaf seems merited, especially with respect to the possibility of a peroxisomal localization.

The enzyme phenylalanine ammonia-lyase catalyzes the deamination of phenylalanine to trans-cinnamic acid, the first step in the transformation of phenylalanine to chlorogenic acid (115). This activity was checked in peroxisomal fractions in an attempt to determine if the peroxisomes were involved in phenolic metabolism. The absence of detectable activity is taken to be an indication of the nonparticipation of the peroxisomes in phenolic metabolism.

Peroxisomal Activities

Comparison of the enzymic content of the peroxisomes from various sources and the glyoxysomes reveals that only the presence of catalase and a flavoprotein oxidase is common to all these microbodies (Table 6). The word "microbody" is used here as a generic term including all cellular organelles containing catalase and one or more H_2O_2-producing oxidases and bounded by a single membrane. This includes peroxisomes and glyoxysomes. If Acanthamoeba is excluded from this listing (the data from Acanthamoeba has been published in abstract form only), it is apparent that, more specifically, only catalase and an α-hydroxy acid oxidase are common to all these microbodies. This diversity with respect to other enzyme activities has been

TABLE 6

Enzyme Activities of Peroxisomes From Various Sources and Glyoxysomes

Symbols: +, activity present; 0, activity not detected; -, activity not searched for; tr, trace activity.

Enzyme Activity	Spinach leaves	Castor bean	*Tetrahymena*	Rat liver	Rat kidney	Acanthamoeba
Catalase	+	+	+	+	+	+
α-Hydroxy acid oxidase						-
Short-chain (glycolate oxidase)	+	+	0	+	0	
Long-chain	0	-	+	0	+	
Amino acid oxidase, D-	0	-	+	+	+	0
L-	0	-	0	tr	+	-
Urate oxidase	0	-	0	+	0	+
β-Oxidation	0	+	-	-	-	-
Malate synthetase	0	+	+	0	-	-

Isocitrate lyase	0	+	+	0	-	-
Citrate synthetase	0	+	0	0	-	-
Aconitase	0	+	0	-	-	-
NAD-Malate dehydrogenase	+	+	0	0	-	-
NADP-Isocitrate dehydrogenase	+	0[a]	+	+	-	-
NAD-Glycerate dehydrogenase (NAD-Glyoxylate reductase)	+	0[a]	0	+[b]	-	-
Aminotransferase			-	-	-	-
Glutamate-glyoxylate	+	0[a]				
Serine-pyruvate	+	-				
Aspartate-α-ketoglutarate	+	+[a]				
References outside this thesis	(47, 97, 98, 103)	(11, 18)	(24, 67)	(23, 24)	(23, 24)	(23)

[a] Personal communication, T. B. Cooper, Purdue University.

[b] Personal communication, S. L. Vandor and N. E. Tolbert, Michigan State University.

interpreted by de Duve (23) as being a reflection of selective enzyme loss during the course of evolution. Whether or not this is true is beyond the scope of this thesis. My interpretation of this enzymic diversity of microbodies is that the differences in enzyme content must be viewed as reflecting differences in particle metabolic function.

Since catalase and H_2O_2-producing oxidases are found in all the microbodies (by definition), it is apparent that H_2O_2 detoxification is a function common to all. Certainly the oxidative activities of the peroxisomes from various sources are expressed because of the ready availability of substrates (glycolate in the case of leaf tissue and lactate, D- and L-amino acids in the case of the other tissues). Likewise, Cooper and Beevers (18) have demonstrated that the acyl-CoA dehydrogenase activity in the β-oxidation sequence of the glyoxysomes produces H_2O_2.

With respect to carbon metabolism, however, gross differences in the microbodies can be inferred. In the case of the rat liver and kidney, the presence of the large number of oxidative activites is taken to indicate that the main function of the animal peroxisomes lies in the oxidation of α-hydroxy acids and amino acids to the corresponding α-keto acids. In the case of L-lactate and several of the amino acids, such an oxidation may represent the first step in a gluconeogenic sequence. A non-phosphorylating electron transport pathway to oxygen such as this might also be important in controlling the degree of reduction of the pyridine nucleotide pool, especially in the presence of excess reducing power and concentrations of molecular oxygen in excess of that which can be utilized by the mitochondria. This theory has been expressed by de Duve (23) in terms of oxygen detoxification by the peroxisomes. Regardless of the use or raison d'être of these oxidative activities, their relative abundance plus lack of evidence for other pathways of carbon metabolism are suggestive of a solely oxidative function for the animal peroxisomes.

The presence of malate synthetase and isocitrate lyase in the peroxisomes from Tetrahymena indicate a gluconeogenic function for these particles. As pointed out by Müller, Hogg, and de Duve (67), the apparent absence of the other glyoxylate cycle activities makes a close relationship with these peroxisomes and the mitochondria imperative for expression of the complete glyoxylate cycle.

In the case of the castor bean endosperm glyoxysomes, the presence of all the enzymes of the glyoxylate cycle plus the complete β-oxidation sequence strongly suggest that the function of the glyoxysomes lies in the conversion of lipid to succinate, a carbohydrate precursor. Mitochondria are required only for the conversion of succinate to oxalacetate in the gluconeogenic sequence. Further evidence for this gluconeogenic function of the glyoxysomes is found in the fact that the appearance and disappearance of the glyoxysomes during germination parallel the increase and decline of gluconeogenesis from lipid (31).

The presence of glycolate oxidase and glyoxylate reductase is certainly an indication of the central role of the leaf peroxisomes in glycolate metabolism. The peroxisomes may perhaps play a central role in the metabolism of the photosynthesizing leaf as a whole since Zelitch (107) has indicated that as much as 50% of the carbon being fixed in photosynthesis may pass through glycolate. Recent studies (33, 108, 109) of the process of photorespiration (the light-dependent oxygen uptake and CO_2 release seen in plants) have indicated that the oxygen uptake of photorespiration is due to the action of glycolate oxidase and that the CO_2 release is due to further metabolism of glycolate. Since the peroxisome is assumed to be the sole site of glycolate oxidase, it is evident that the peroxisomal function must be intimately associated with photorespiration.

The process of photorespiration is assumed to be detrimental to the plant in the sense that newly-fixed carbon is lost again to the atmosphere. Thus maize, which is reported to lack photorespiration (108, 109), is known to be a more efficient plant with respect to net photosynthesis. Such comparisons must, however, also take into account the apparent differences in pathways of CO_2 fixation in plants such as spinach and tobacco as compared with plants such as maize and sugarcane (87). A correlation has been shown by our laboratory between the amount of peroxisomal material isolated and the presence of photorespiration in various plants (98). Those plants exhibiting photorespiration were found to contain larger amounts of isolatable peroxisomes than those plants lacking photorespiration. This finding is indicative of a direct relationship between the leaf peroxisomes and the process of photorespiration.

As indicated above, the oxygen uptake component of photorespiration is probably due to the action of the peroxisomal glycolate oxidase. Thus the peroxisomes are thought to be the site of oxygen uptake. Zelitch (108) has implied that the CO_2 released by photorespiration arises from the non-enzymatic oxidation of glyoxylate by the hydrogen peroxide produced by glycolate oxidase. Kisaki and Tolbert (47), however, were not able to detect significant amounts of $^{14}CO_2$-release from peroxisomal preparations actively oxidizing glycolate-1-^{14}C. In addition, the large amount of catalase activity present in the peroxisomes would seem to preclude the use of H_2O_2 in other reactions. Glyoxylate thus appears to be a poor candidate as a substrate for the CO_2 release of photorespiration.

Kisaki and Tolbert (47) demonstrated that the end product of glycolate metabolism in isolated peroxisomes is glycine. No other amino acids were formed, and no CO_2 was released in the conversion of glycolate to glycine. These authors were further able to demonstrate that a specific aminotransferase activity in the peroxisome was responsible for the conversion of glyoxylate to glycine. Only glutamate and alanine were found to give appreciable rates of reaction when tested with glyoxylate. The leaf peroxisomes are thus able to convert glycolate to glycine.

The data of Kisaki and Tolbert (47) indicated that the conversion of glycine to serine did not occur in the peroxisomes. Data of W. J. Bruin and N. E. Tolbert (unpublished results) now indicate that this conversion may take place in the mitochondria. Serine hydroxymethylase activity has been located in the mitochondrial fraction from sucrose density gradient centrifugation.

It has been demonstrated in this thesis that the leaf peroxisomes contain a serine-pyruvate aminotransferase activity. This was accomplished by coupling hydroxypyruvate formation to the hydroxypyruvate reductase (D-glycerate dehydrogenase) activity of the peroxisomes. Localization of these activities in the peroxisome allows conversion of serine to D-glycerate in the particles.

It has been suggested in a diagram in Ref. 47 that the combined action of glycolate oxidase and the glyoxylate reductase activity of D-glycerate dehydrogenase would provide the peroxisome with a system for oxidizing NADH or malate through the action of NAD-malate dehydrogenase.

This possibility, however, appears unlikely in view of the high Km for glyoxylate (ca. 10^{-2}M) and the low rate of reaction at pH 7 for the glyoxylate reductase activity (43, 54, and unpublished results with A. Oeser and N. E. Tolbert). In view of the more favorable Km and pH behavior, it seems reasonable to assume that the glycerate dehydrogenase activity of the glyoxylate reductase-glycerate dehydrogenase protein is the one actually expressed in the peroxisome *in vivo*.

We have now established that peroxisomes, in concert with mitochondria, can carry out the conversion of glycolate to glycerate. It should be noted that the glycerate dehydrogenase action requires a source of NADH. This requirement for NADH might be met in two different ways: diffusion of NADH into the peroxisome, or generation of NADH within the peroxisome. Although the first possibility is simple and direct, the possibility of internal generation of NADH is attractive in several respects. First, control could be exerted within the peroxisome as to how the reducing power is used. Thus the NADH-generating system might be turned off within the peroxisome when glycerate dehydrogenase activity is not needed but glycine synthesis is required. Second, control of NADH-generation can be exerted outside the peroxisome. Thus, if organic acids are utilized in the shuttle for NADH-generation, control at the extra-peroxisomal reduction step is possible.

Although the permeability of the peroxisomal membrane has not been tested directly, it may be possible to infer certain properties from existing data. The fact that glycolate oxidase (when assayed by following dye reduction), NAD-glyoxylate reductase, and NAD-malate dehydrogenase all show 35% more activity in the presence of the non-ionic detergent TritonX-100 strongly suggests non-permeation of at least one of the substrates. Evidence that small molecules such as organic acids and oxygen can easily pass into the peroxisome is indicated in the observation that no stimulation by Triton X-100 is observed in the cases of catalase (assayed by following H_2O_2 disappearance spectrophotometrically) and glycolate oxidase when assayed on the Warburg respirometer (unpublished results of A. Oeser and N. E. Tolbert). In such cases there is no need for a "large" molecule to enter the peroxisome for assay purposes. A further indication of selective membrane permeation is found in the case of the glutamate-glyoxylate aminotransferase activity of the peroxisome. Kisaki and Tolbert (47) found isonicotinyl hydrazide to be much less effective as an inhibitor in the absence of Triton X-100. This suggests difficulty in permeation of isonicotinyl hydrazide which can be considered to be an analogue of NAD. The addition of Triton X-100 has no effect on the usual assay for this aminotransferase (unpublished results of W. J. Bruin and N. E. Tolbert). It this appears that a case can be made for the non-permeability of the peroxisomal membrane to specific or relatively large molecules such as pyridine nucleotides.

Evidence for the presence of a pyridine nucleotide pool within the peroxisome is also indirect. Experiments on malate oxidation by the peroxisomal NAD-malate dehydrogenase in the presence of DCPIP and diaphorase indicated that an absolute requirement for added NAD^+ could not be demonstrated. Addition of NAD^+ gave a 5-fold stimulation of the low rate in the absence of exogenous NAD^+. A zero rate would be expected in the absence of exogenous NAD^+. The presence of this low rate may indicate the presence of endogenous NAD in the peroxisomes.

It is suggested here that the NADH required for the reduction of hydroxypyruvate to D-glycerate is generated by the action of either NAD-malate dehydrogenase or an unknown dehydrogenase which is NAD-linked. A unified scheme utilizing the NAD-malate dehydrogenase for generation of NADH is presented in Figure 7. This scheme is based on demonstrated enzyme activities of the peroxisome. The heavy arrows of the figure indicate the path of carbon in the glycolate pathway. In this scheme it is assumed that the peroxisomal membrane is freely permeable to the organic and amino acids but is impermeable (or semipermeable at best) to pyridine nucleotide. Explanations for the individual steps are given in the text below. The circled numbers refer to the enzymatic activities of Figure 7.

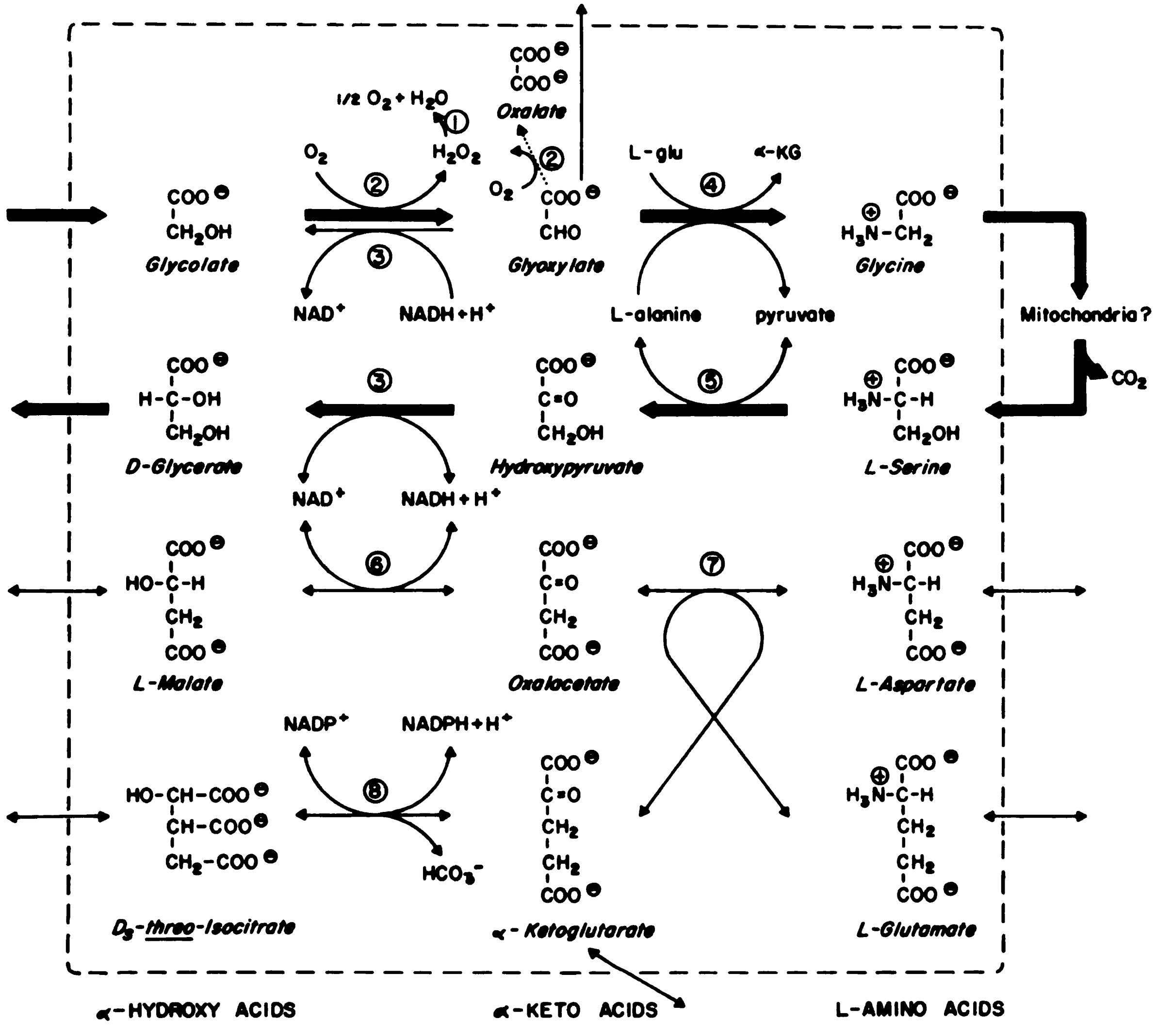
Figure 7

Enzyme Reactions of Spinach Leaf Peroxisomes

Heavy arrows indicate the flow of carbon in the glycolate pathway.

Number	Enzyme
1	Catalase (EC 1.11.1.6)
2	Glycolate oxidase (EC 1.1.3.1)
3	NAD-Glycerate dehydrogenase (EC 1.1.1.29) (NAD-Glyoxylate reductase)
4	Glutamate-glyoxylate aminotransferase (EC 2.6.1.-) (Alanine-glyoxylate aminotransferase)
5	Serine-pyruvate aminotransferase (EC 2.6.1.-)
6	NAD-Malate dehydrogenase (EC 1.1.1.37)
7	Aspartate aminotransferase (EC 2.6.1.1)
8	NADP-Isocitrate dehydrogenase (EC 1.1.1.42)

Enzyme Reactions of Spinach Leaf Peroxisomes



Glycolate from an external source, presumably the chloroplast, enters the peroxisome. By the action of glycolate oxidase ②, the glycolate is converted to glyoxylate with concomitant H_2O_2 production. The hydrogen peroxide is immediately broken down to molecular oxygen and water by the action of catalase ①. Under conditions where glutamate is limiting, glyoxylate may be further oxidized by glycolate oxidase to yield oxalate (dotted arrow of Figure 7). If the concentration of glyoxylate inside the peroxisome approaches 10^{-2}M, glyoxylate reductase activity ③ may be expressed in the presence of NADH. This reductase activity would yield glycolate which could again be oxidized to glyoxylate. The net effect of such a cycle would be the oxidation of NADH. It must be emphasized again that such an NADH-oxidase activity would be expressed only in the presence of very high concentrations of glyoxylate.

Under normal conditions glyoxylate would be transaminated to glycine. This glutamate-glyoxylate aminotransferase activity ④ requires glutamate to be supplied by an extra-peroxisomal source since reductive amination activity is apparently absent from the peroxisomes. Glycine then presumably passes out of the peroxisome and into the mitochondrion where it is converted to serine. This conversion of glycine to serine probably requires two glycine molecules for the formation of one serine molecule. One amino group is lost as ammonia, and one carbon is lost as CO_2. This CO_2 released is presumably that observed in measurements of photorespiration. The mitochondrion is thus implied as the site of CO_2 release during photorespiration.

Serine formed in the mitochondrion then passes back into the peroxisome. Here it loses its amino group by aminotransferase activity with pyruvate ⑤. Notice that the alanine formed in this reaction can now be utilized by the glyoxylate system for further formation of glycine. With the operation of such a cyclic system, the input of only one glutamate is required for the conversion of two glyoxylates to one hydroxypyruvate (one amino group is lost in the conversion of two glycines to one serine).

Hydroxypyruvate is next reduced to D-glycerate by the action of D-glycerate dehydrogenase ③. The NADH required for this step is generated by the action of NAD-malate dehydrogenase ⑥. L-Malate, which must be supplied from an extra-peroxisomal source, is the actual carrier of the NADH equivalents into the peroxisome. Oxalacetate produced by the action of the NAD-malate dehydrogenase is then converted to aspartate by aspartate aminotransferase activity ⑦. Aspartate then passes out of the peroxisome and undergoes transamination and subsequent reduction to L-malate. The α-ketoglutarate produced by the action of the glutamate-glyoxylate aminotransferase and aspartate aminotransferase can either pass out of the peroxisome or undergo reductive carboxylation by the action of NADP-isocitrate dehydrogenase ⑧. If α-ketoglutarate is assumed to pass out of the peroxisome, then the sum total of these reactions is as follows:

2 glycolate + L-malate + 2 L-glutamate ⟶
D-glycerate + L-aspartate + 2 α-ketoglutarate

If α-ketoglutarate undergoes reductive carboxylation by NADP-isocitrate dehydrogenase, a source of NADPH in the peroxisome would also be required. In this case "2 isocitrate" would replace "2 α-ketoglutarate" as products.

An alternate scheme utilizing an as yet unknown NAD-linked dehydrogenase to generate NADH within the peroxisome can also be envisioned. Hydroxypyruvate and oxalacetate might then compete for NADH. In this case the flow of carbon in the malate-aspartate and isocitrate-glutamate pathways as shown in Figure 7 might be reversed from that in the first scheme. The flow of carbon from glycolate to glycerate would remain unchanged in this alternate scheme, but more NADH would be required as a consequence of the conversion of aspartate to malate. If the substrate of this unknown dehydrogenase is designated as AH_2 and the product as A, then the sum of the reactions would be as follows:

$$2\ \text{glycolate} + \text{L-aspartate} + \text{Isocitrate} + \text{NADP}^{+} + \text{AH}_2 \longrightarrow$$
$$\text{D-glycerate} + \text{L-malate} + \text{L-glutamate} + \text{NADPH} + \text{A}$$

One feature of this alternate scheme is that the glycolate-glycerate and malate-aspartate pathways might conceivably operate independently.

The specific activities of the leaf peroxisomal enzyme activities are given in Table 7. Using a figure of 250 μmole CO_2 fixed per hr per mg chlorophyll in intact spinach leaves and assuming that 50% of the carbon fixed is going through glycolate (107), it is possible to calculate a rate of 30 nmole glycolate synthesized per min per mg protein in crude homogenates (assuming 30 mg protein per mg chlorophyll - Ref. 103). It has been estimated that the leaf peroxisomes may account for 1.5% of the total leaf protein (98). Using this figure, a rate of 2 μmole per min per mg peroxisomal protein can be calculated for glycolate synthesis in the intact leaf. Thus, in order to account for glycolate metabolism without accumulation of intermediates in the peroxisome, all enzyme specific activities based on mg peroxisomal protein must exceed the critical value of 2 μmole per min per mg protein. From Table 7 it can be seen that all the peroxisomal enzymes except the NADP-isocitrate dehydrogenase and the NADP-glyoxylate reductase activity have specific activities which exceed this critical limit. This implies that all the enzyme activities pictured in Figure 7 are active enough

TABLE 7

Calculated Specific Activities of Peroxisomal Enzyme Activities

Specific activity figures are based on Lowry mg protein, in the peroxisome. Data for catalase, NAD-malate dehydrogeanse, NAD-glyoxylate reductase, and glycolate oxidase taken from an experiment utilizing a continuous sucrose density gradient for peroxisome isolation. Little or no contamination from mitochondria or chloroplasts was detected. Data for other activities were taken from experiments using less pure peroxisome preparations and were extrapolated relative to the specific activity of glycolate oxidase given here.

Enzyme activity	Number in figure	Assay pH	Specific activity (μmole/min/mg protein, 25°)
Catalase	1	7.0	11,600.
NAD-Malate dehydrogenase	6	7.4	87.[b]
NAD-Glycerate dehydrogenase	3	6.2	47.[c]
NAD-Glyoxylate reductase[a]	3	6.2	11.
Glycolate oxidase	2	8.7	9.6[d]
Aspartate-α-ketoglutarate aminotransferase	7	7.4	7.3
NADP-Glycerate dehydrogenase[a]		5.1	4.2

Serine-pyruvate aminotransferase	5	7.4	4.1
Glutamate-glyoxylate aminotransferase	4	7.5	2.8[e]
NADP-Glyoxylate reductase[a]		5.1	0.27
NADP-Isocitrate dehydrogenase	8	7.4	0.12

---

[a] These activities may be a reflection of nonspecificity of NAD-glycerate dehydrogenase.

[b] Extrapolated value at saturating substrate concentration, assuming no substrate inhibition.

[c] Calculated from NAD-glyoxylate reductase data, assuming a ratio of the rates with hydroxypyruvate to glyoxylate of 4.3.

[d] Extrapolated value at saturating DCPIP concentrations.

[e] Unpublished data of W. J. Bruin and N. E. Tolbert.

to account for the conversion of glycolate (which may represent 50% of the total carbon fixed) to glycerate. This assumes that the rate of conversion of glycine to serine is not limiting and that L-malate and L-glutamate do not become limiting.

Regardless of the source of NADH in the leaf peroxisome, certain characteristics of the peroxisome are evident from this thesis. The main function of the peroxisome lies in the conversion of glycolate to glycerate, *i.e.*, the glycolate pathway. The specific activities of all the peroxisomal enzymes involved in the glycolate pathway are large enough to account for the complete conversion of glycolate to glycerate in the intact leaf, even if it is assumed that 50% of the carbon fixed during photosynthesis is passing through this pathway. Amino acid metabolism is intimately involved in this process. A close relationship must exist between the peroxisome, chloroplast, and mitochondrion with respect to generation of amino groups and reducing power (and the conversion of glycine to serine). It is also implied that the peroxisome is the site of oxygen uptake and that the mitochondrion is the site of CO_2 release during photorespiration.

If it is assumed that the peroxisomal membrane is impermeable to pyridine nucleotide, it follows that the reduction of hydroxypyruvate is linked in a compulsory manner to the NAD-specific oxidation of malate (or unknown compound). Such a theory is attractive as an explanation of the inhibition of dark respiration which apparently takes place during photosynthesis (33, 35). If we rephrase the compulsory linking described above, it can be stated that oxalacetate formation in the peroxisome depends immediately upon glycerate formation and ultimately upon photosynthetic glycolate formation. Hence, during photosynthesis and accompanying glycolate formation, the peroxisomes can produce glyoxylate and oxalacetate. This combination of keto acids has been found to produce a concerted inhibition of NADP-isocitrate dehydrogenase from Bacillus subtilis, Escherichia coli, and pig heart (85). Ruffo and coworkers (83) have demonstrated that non-enzymatic condensation of glyoxylate and oxalacetate at neutral pH produces a six carbon acid, oxalomalate. This compound has been demonstrated to be a highly-effective inhibitor of NADP-isocitrate dehydrogenase, aconitase, and α-ketoglutarate dehydrogenase from pig heart. Laties (54) has demonstrated the inhibition of citrate isocitrate, and α-ketoglutarate oxidation in potato slices by the addition of γ-hydroxy-α-ketoglutarate, a product of decarboxylation of oxalomalate. All these reports are suggestive of the fact that a combination of glyoxylate and oxalacetate may be utilized to inhibit TCA cycle activity. It is possible that such a control mechanism for mitochondrial oxidation may operate in the plant leaf. Certainly mitochondrial oxidation would seem to be unnecessary during photosynthesis.

The observation of Asada *et al*. (3) that glycolate is not metabolized beyond serine in the dark suggests that the source of reducing power for the peroxisome is related to photosynthetic production of reducing power. If malate is utilized as the shuttle for reducing power, it may be that the ultimate source of the peroxisomal NADH equivalents is the chloroplast. Hatch and Slack (38) have reported the presence of an NADP-malate dehydrogenase activity in spinach and other plants. In the case of maize, this activity was found to be localized in the chloroplast. A shuttle mechanism utilizing the chloroplastic NADP-malate dehydrogenase and the peroxisomal NAD-malate dehydrogenase might provide the peroxisome with NADH equivalents. This shuttle would not operate in the dark since NADPH formation would cease in the chloroplast. Thus the peroxisome would not be supplied with malate, and glycolate metabolism would stop at serine.

Little is known concerning the development of the leaf peroxisomes. The observation (95, 96) that glycolate oxidase activity of wheat increases greatly upon greening is suggestive of an increase in either the number of peroxisomes or the activity of pre-existing peroxisomes. Coordinate control of all the activities of the peroxisome is suggested by the observation of Zucker (114) that glycolate oxidase, glyoxylate reductase, and malate dehydrogenase activities of tobacco plants grown in full sunlight were higher than of those tobacco plants grown in shade. The fact that the specific activities of these enzymes also were somewhat higher in sun-grown plants may indicate an increase of peroxisomal activity over the general increase in leaf protein.

SUMMARY AND CONCLUSIONS

The enzymic content of leaf peroxisomes has been surveyed with respect to a number of activities. The leaf peroxisomes have been found to differ from other peroxisomes and glyoxysomes in enzyme content. The differences among these microbodies with respect to enzyme content are suggested to reflect differences in function of these variour particles.

The presence of all the enzyme activities necessary for the conversion of glycolate to glycerate (except for the conversion of glycine to serine) strongly suggests that the function of the leaf peroxisomes lies in the glycolate pathway. Evidence has been presented which indicates that the peroxisomal enzyme activities are sufficient to account for this gluconeogenic conversion of glycolate to glycerate. Amino acid metabolism has been found to be intimately associated with this process. Close cooperation with the chloroplasts and mitochondria is necessitated.

A scheme, assuming impermeability of the peroxisomal membrane to pyridine nucleotide, has been formulated from demonstrated enzyme activities. A shuttle mechanism involving malate transport has been invoked to explain internal generation of NADH. Consequences of such a shuttle mechanism have been discussed.

Enzyme activities found to be external to the peroxisome have been discussed in terms of known organelle function.

BIBLIOGRAPHY

1. Anderson, D. E., P-Glycolate phosphatase and aconitase from tobacco leaves, Ph.D. thesis, Michigan State University, 1969.

2. Arnon, D. I., Cu enzymes in isolated chloroplasts - Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. *24*, 1-15 (1949).

3. Asada, K., K. Saito, S. Kitoh, and Z. Kasai, Photosynthesis of glycine and serine in green plants. Plant Cell Physiol. *6*, 47-59 (1965).

4. Atkinson, D. E., The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry *7*, 4030-34 (1968).

5. Baker, A. L., and N. E. Tolbert, Purification and some properties of an alternate form of glycolate oxidase. Biochim. Biophys. Acta *131*, 179-87 (1967).

6. Bandurski, R. S., and Clare M. Greiner, The enzymatic synthesis of oxaloacetate from phosphoryl-enolpyruvate and carbon dioxide. J. Biol. Chem. *204*, 781-86 (1953).

7. Beevers, H., Metabolic production of sucrose from fat. Nature *191*, 433-36 (1961).

8. Bergmeyer, H. U., and E. Bernt, Glutamate-oxalacetate transaminase, in Methods of Enzymatic Analysis, H. U. Bergmeyer, Ed., Academic Press, New York, 1965, pp. 837-46.

9. Björkman, O., Carboxydismutase activity in shade-adapted and sun-adapted species of higher plants. Physiol. Plant. *21*, 1-10 (1968).

10. Breidenback, R. W., and H. Beevers, Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem. Biophys. Res. Commun. *27*, 462-69 (1967).

11. Breidenbach, R. W., A. Kahn, and H. Beevers, Characterization of glyoxysomes from castor bean endosperm. Plant Physiol. *43*, 705-13 (1968).

12. Carpenter, W. D., and H. Beevers, Distribution and properties of isocitritase in plants. Plant Physiol. 34, 403-09 (1959).

13. Castelfranco, P., P. K. Stumpf, R. Coutopoulou, Fat metabolism in higher plants. V. A soluble palmitate oxidase requiring glycolic acid as activator. J. Biol. Chem. 214, 567-77 (1955).

14. Cheung, G. P., I. Y. Rosenblum, and H. J. Sallach, Comparative studies of enzymes related to serine metabolism in higher plants. Plant Physiol. 43, 1813-20 (1968).

15. Clagett, C. O., N. E. Tolbert, and R. H. Burris, Oxidation of α-hydroxy acids by enzymes from plants. J. Biol. Chem. 178, 977-87 (1949).

16. Cleland, W. W., Enzyme kinetics. Ann. Review Biochem. 36, 77-112 (1967).

17. Conn, E. C., B. Vennesland, and L. M. Kraemer, Distribution of a triphosphopyridine nucleotide-specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid in higher plants. Arch. Biochem. Biophys. 23, 179-97 (1949).

18. Cooper, T. G., and H. Beevers, β-Oxidation in glyoxysomes from castor bean endosperm. Fed. Proc. 28, 539 (1969).

19. Danner, J., and I. P. Ting, CO_2 metabolism in corn roots. II. Intracellular distribution of enzymes. Plant Physiol. 42, 719-24 (1967).

20. Davies, D. D., The specificity of malic dehydrogenase from higher plants. Biochem. J. 80, 93-99 (1961).

21. Davies, D. D. and R. J. Ellis, Enzymes of the Krebs cycle, the glyoxalate cycle and related enzymes. in Modern Methods of Plant Analysis, Vol. VII, H. F. Linskens, B. D. Sanwal, and M. V. Tracey, Springer Verlag, Berlin, 1964, pp. 616-46.

22. Davison, Daphne C., Distribution of formic and alcoholic dehydrogenases in the higher plant - Their variation in the pea plant during its life cycle. Proc. Linnean Soc. N. S. Wales 74, 26-36 (1949).

23. de Duve, C., The peroxisome. A new cytoplasmic organelle. Nature, in press (1969).

24. de Duve, and P. Baudhuin, Peroxisomes (Microbodies and related particles). Physiol. Rev. 46, 323-57 (1966).

25. Dennis, D. T., and T. P. Coultate, Regulatory properties of the NAD-specific isocitrate dehydrogeanse from a higher plant. Life Sci (Oxford) 6, 2353-8 (1967); from C. A. 68, 27106e (1968).

26. Dixon, G. H., and H. L. Kornberg, Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. 72, 3P (1959).

27. Fine, I. H., and L. A. Costello, The use of starch electrophoresis in dehydrogenase studies. in Methods of Enzymology, S. P. Colowick and N. O. Kaplan, eds., Vol. VI, Academic Press, New York, 1963, pp. 958-72.

28. Finkle, B. J., and D. I. Arnon, Metabolism of isolated cellular particles from photosynthetic tissues. II. Oxidative decarboxylation of oxalic acid. Physiol. Plant. 7, 614-24 (1954).

29. Frederick, Sue Ellen, and E. H. Newcomb, Microbody-like organelles in leaf cells. Science 163, 1353-55 (1969).

30. Frigerio, N. A., and H. A. Harbury, Preparation and some properties of crystalline glycolic acid oxidase of spinach. J. Biol. Chem. 231, 135-57 (1958).

31. Gerhardt, B. P., and H. Beevers, Developmental studies on glyoxysomes in castor bean endosperm. Plant Physiol. 43 (Supplement), S-41 (1969).

32. Glentka-Rychter, Anna, and J. H. Cherry, De novo synthesis of isocitritase in peanut (Arachis hypogaea L.) Cotyledons. Plant Physiol. 43, 653-59 (1968).

33. Goldsworthy, A., Experiments on the origin of CO_2 released by tobacco leaf segments in the light. Phytochemistry 5, 1013-19 (1966).

34. Graham, D., and Judith E. Cooper, Changes in levels of nicotinamide adenine nucleotides and Krebs cycle intermediates in mung bean leaves after illumination. Aust. J. Biol. Sci. 20, 319-27 (1967).

35. Graham, D., and D. A. Walker, Some effects of light on the interconversion of metabolites in green leaves. Biochem. J. 82, 554-60 (1962).

36. Harrop, Linda C., and H. L. Kornberg, The role of isocitrate lyase in the metabolism of algae. Proc. Royal Soc., B, 166, 11-29 (1966).

37. Harvey, M. J., and A. P. Brown, Nicotinamide cofactors of intact chloroplasts isolated on a sucrose density gradient. Biochim. Biophys. Acta 172, 116-25 (1969).

38. Hatch, M. D., and C. R. Slack, NADP-specific malate dehydrogenase and glycerate kinase in leaves and evidence for their location in chloroplasts. Biochem. Biophys. Res. Commun. 34, 589-93 (1969).

39. Hathaway, J. A., and D. E. Atkinson, Kinetics of regulatory enzymes: effect of adenosine triphosphate on yeast citrate synthetase. Biochem. Biophys. Res. Commun. 20, 661-65 (1965).

40. Heber, U., Vergleichende Untersuchen an Chloroplasten, die durch Isolierungs0Operationen in nicht-wässrigem und in wässrigem Milieu erhalten wurden. II. Kritik der Reinheit und Ferment-Lokalisationen in Chloroplasten. Z. Naturforsch. 15b, 100-09 (1960).

41. Hock, B., and H. Beevers, Development and decline of the glyoxylate-cycle enzymes in watermelon seedlings (*Citrullus vulgaris* Schrad.). Effects of Dactinomycin and cycloheximide. Z. Pflanzenphysiol. Bd. 55, 405-14 (1966).

42. Hogg, J. F., and H. L. Kornberg, The metabolism of C_2-compounds in micro-organisms. 9. Role of the glycoxylate cycle in protozoal glyconeogenesis. Biochem. J. 86, 462-68 (1963).

43. Holzer, H., and A. Holldorf, Isolierung von D-Glyceratdehydrogenase, einige Eigenschaften des Enzymes und seine Verwendung zur enzymatisch-optischen Bestimmung von Hydroxypyruvat neben Pyruvat. Biochem. Z. 329, 292-312 (1957).

44. Hutton, D., and P. K. Stumpf, Characterization of the β-oxidation systems from maturing and germinating castro bean seeds. Fed. Proc. 28, 863 (1969).

45. John, P. C. L., and P. J. Syrett, The inhibition, by intermediary metabolites, of isocitrate lyase from *Chlorella pyrenoidosa*. Biochem. J. 110, 481-84 (1968).

46. Kenten, R. H., and P. J. G. Mann, Hydrogen peroxide formation in oxidations catalyzed by plant α-hydroxyacid oxidase. Biochem. J. 52, 130-34 (1952).

47. Kisaki, T., and N. E. Tolbert, Glycolate and glyxoylate metabolism by isolated peroxisomes or chloroplasts. Plant Physiol. 44, 242-50 (1969).

48. Kornberg, H. L., Anaplerotic sequences and their role in metabolism. in Essays in Biochemistry, Vol. 2, P. N. Campbell and G. D. Greville, Eds., Academic Press, New York, 1966, pp. 1-31.

49. Kornberg, H. L., and H. Beevers, The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. Biochim. Biophys. Acta 26, 531-37 (1957).

50. Kornberg, H. L., and H. A. Krebs, Synthesis of cell constituents from C-2 units by a modified tricarboxylic acid cycle. Nature 179, 988-91 (1957).

51. Kuczmak, M., and N. E. Tolbert, Glycolic acid oxidase formation in greening leaves. Plant Physiol. 37, 729-34 (1962).

52. Lado, P., M. Schwendimann, and E. Marre, Repression of isocitrate lyase synthesis in seeds germinated in the presence of glucose. Biochim. Biophys. Acta 157, 140-48 (1968).

53. Lardy, H. A., V. Paetkau, and P. Walter, Paths of carbon in gluconeogenesis and lipogenesis: the role of mitochondria in supplying precursors of phosphoenolpyruvate. P. Nat. Acad. Sci. U.S. 53, 1410-15 (1965).

54. Laties, G. G., The inhibition of citrate, isocitrate and α-ketoglutarate oxidation in aged potato slices by γ-hydroxy-α-ketoglutarate. Phytochemistry 6, 181-85 (1967).

55. Laudahn, G., Vergleichende Untersuchungen zum Umsatz von Pyruvat, Hydroxypyruvat und Glyoxylat durch die NAD-Oxydo-reduktasen Lactat-dehydrogenase, Glyoxylsaure-Reduktase und D-Glycerat-dehydrogenase. Biochem. Z. 337, 449-61 (1963).

56. Leech, Rachel M., Comparative biochemistry and comparative morphology of chloroplasts isolated by different methods. in Biochemistry of Chloroplasts, T. W. Goodwin, Ed., Academic Press, New York, 1966, pp. 65-74.

57. Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve, The large scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR1339. J. Cell Biol. *37*, 482-513 (1968).

58. Longo, C. P., Evidence for *de novo* synthesis of isocitritase and malate synthetase in germinating peanut cotyledons. Plant Physiol. *43*, 660-64 (1968).

59. Longo, C. P., Development of peroxysomes in cotyledons of germinating peanut. Plant Physiol. *43* (Supplement), S-41 (1969).

60. Magar, M. E., and M. L. Homi, Allosteric inhibition of TPN-linked isocitrate dehydrogenase by folate. Biochem. Biophys. Res. Commun. *31*, 665-70 (1968).

61. Marcus, A., and J. Velasco, Enzymes of the glyoxylate cycle in germinating peanut and castor beans. J. Biol. Chem. *235*, 563-67 (1960).

62. Mayer, A. M., Catechol oxidase: enzymic liberation from sugar beet chloroplasts. Phytochemistry *5*, 1297-1301 (1966).

63. Mazelis, M., Formate oxidation by particulate preparations from higher plants. Plant Physiol. *35*, 386-91 (1960).

64. Mazelis, M., and B. Vennesland, Carbon dioxide fixation into oxalacetate in higher plants. Plant Physiol. *32*, 591-600 (1957).

65. Mukerji, S. K., and I. P. Ting, Intracellular localization of CO_2 metabolism enzymes in cactus phylloclades. Phytochemistry *7*, 903-11 (1968).

66. Mukerji, S. K., and I. P. Ting, Malate dehydrogenase (decarboxylating (NADP) isoenzymes of *Opuntia* stem tissue. Biochim. Biophys. Acta *167*, 239-49 (1968).

67. Müller, M., J. F. Hogg, and C. de Duve, Distribution of tricarboxylic acid cycle enzymes and glyoxylate cycle enzymes between nitochondria and peroxisomes in *Tetrahymena pyriformis*. J. Biol. Chem. *243*, 5385-95 (1968).

68. Munkres, K. D., Physiocochemical identity of *Neurospora* malate dehydrogenase and aspartate aminotransferase. Arch. Biochem. Biophys. *112*, 347-54 (1965).

69. Nagamachi, K., M. Fujii, and K. Honda, Studies on isocitrate lyase of the germinating castor bean endosperm. A possible metabolic control of NADP-specific isocitrate dehydrogenase and isocitrate lyase. J. Agr. Chem. Soc. Japan 41, 381-85 (1967).

70. Ochoa, S., "Malic" enzyme. in Methods in Enzymology, Vol. I, S. P. Colowick, and N. O. Kaplan, Eds., Academic Press, New York, 1955, pp. 739-53.

71. Ogren, W. L., and D. W. Krogmann, Studies of pyridine nucleotides in photosynthetic tissue. Concentrations, interconversions, and distribution. J. Biol. Chem. 240, 4603-08 (1965).

72. Penner, D., and F. M. Ashton, Hormonal control of isocitrate lyase synthesis. Biochim. Biophys. Acta 148, 481-85 (1967).

73. Pinfield, N. J., The promotion of isocitrate lyase activity in hazel cotyledons by exogenous gibberellin. Planta 82, 337-41 (1968).

74. Pierpoint, W. S., Mitochondrial preparation from the leaves of tobacco (Nicotiana tabacum). 3. Glycollic oxidase and fumarase activity. Biochem. J. 75, 511-15 (1960).

75. Pierpoint, W. S., The distribution of succinate dehydrogenase and malate dehydrogenase among components of tobacco leaf extracts. Biochem. J. 88, 120-25 (1963).

76. Quayle, J. R., Glyoxylate dehydrogenase. in Methods in Enzymology, Vol. IX, W. A. Wood, Ed., Academic Press, New York, 1966, pp. 342-46.

77. Quayle, J. R., Formate dehydrogenase. in Methods in Enzymology, Vol. IX, W. A. Wood, Ed., Academic Press, New York, 1966, pp. 360-64.

78. Racker, E., Mechanisms in Bioenergetics, Academic Press, New York, 1965.

79. Ranson, S. L., The plant acids. in Plant Biochemistry, J. Bonner, and J. E. Varner, Eds., Academic Press, New York, 1965, pp. 493-525.

80. Richardson, K. E., and N. E. Tolbert, Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase. J. Biol. Chem. 236, 1280-84 (1961).

81. Richardson, K. E., and N. E. Tolbert, Phosphoglycolic acid phosphatase. J. Biol. Chem. 236, 1285-90 (1961).

8

8

8

8

90

91

92

82. Rosenberg, L. L., J. B. Capindale, and F. R. Whatley, Formation of oxalacetate and aspartate from phosphoenol-pyruvate in spinach leaf chloroplast extract. Nature 181, 632-33 (1958).

83. Ruffo, A., E. Testa, A. Adinolfi, G. Pelizza, and R. Moratti, Control of the citric acid cycle by glyoxylate. Mechanism of the inhibition by oxalomalate and γ-hydroxy-α-oxoglutarate. Biochem. J. 103, 19-23 (1967).

84. Shannon, L. M., Plant isoenzymes. in Ann. Rev. Plant Physiol. 19, 187-210 (1968).

85. Shiio, I., and H. Ozaki, Concerted inhibition of isocitrate dehydrogenase by glyoxylate plus oxalacetate. J. Biochem. (Tokyo) 64, 45-53 (1968).

86. Simpson, E. R., and R. W. Estabrook, A possible mechanism for the transfer of cytosol-generated NADPH to the mitochondrial mixed-function oxidases in bovine adrenal cortex: a malate shuttle. Arch. Biochem. Biophys. 128, 977-78 (1968).

87. Slack, C. R., and M. D. Hatch, Comparative studies on the activity of carboxylases and other enzymes in relation to the new pathway of photosynthetic carbon dioxide fixation in tropical grasses. Biochem. J. 103, 660-65 (1967).

88. Srere, P. A., H. Brazil, and L. Gonen, The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. Acta Chem. Scand. 17, S129-S134 (1963).

89. Stafford, Helen A., and Anna Magaldi, A developmental study of D-glycerate dehydrogenase. Plant Physiol. 29, 504-08 (1954).

90. Stafford, Helen A., Anna Magaldi, and Birgit Vennesland, The enzymatic reduction of hydroxypyruvic acid to D-glyceric acid in higher plants. J. Biol. Chem. 207, 621-29 (1954).

91. Stumpf, P. K., Lipid metabolism in higher plants. Nature 194, 1158-60 (1962).

92. Tanner, W. H., and H. Beevers, Competition between the glyoxylate cycle and the oxidative breakdown of acetate in Ricinus endosperm. I. The metabolism of isocitrate in endosperm extracts. Z. Pflanzenphysiol. 53, 72-85 (1965).

93. Thompson, C. M., and C. P. Whittingham, Intracellular localisation of phosphoglycollate phosphatase and glyoxalate reductase. Biochim. Biophys. Acta 143, 642-44 (1967).

94. Tolbert, N. E., Formic acid metabolism in barley leaves. J. Biol. Chem. 215, 27-34 (1955).

95. Tolbert, N. E., Glycolate pathway. in Photosynthetic Mechanisms in Green Plants, Publication 1145, Nat. Acad. Sci. Nat. Res. Council, 1963, pp. 648-62.

96. Tolbert, N. E., and Marjorie S. Cohan, Activation of glycolic acid oxidase in plants. J. Biol. Chem. 204, 639-48 (1953).

97. Tolbert, N. E., A. Oeser, T. Kisaki, R. H. Hageman, and R. K. Yamazaki, Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism, J. Biol. Chem. 243, 5179-84 (1968).

98. Tolbert, N. E., A. Oeser, R. K. Yamazaki, R. H. Hageman, and T. Kisaki, A survey of plants for leaf peroxisomes. Plant Physiol. 44, 135-47 (1969).

99. Willis, J. E., and H. J. Sallach, Serine biosynthesis from hydroxypyruvate in plants. Phytochemistry 2, 23-28 (1963).

100. Yamada, M., and P. K. Stumpf, Fat metabolism in higher plants. XXIV. A soluble β-oxidative system from germinating seeds of Ricinus communis. Plant Physiol. 40, 653-58 (1965).

101. Yamamoto, Y., Formic dehydrogenase of bean seed embryo in the germinating stage. J. Biochem. (Tokyo) 41, 551-65 (1954).

102. Yamamoto, Y., and H. Beevers, Malate synthetase in higher plants. Plant Physiol. 35, 102-08 (1960).

103. Yamazaki, R. K., and N. E. Tolbert, Malate dehydrogenase in leaf peroxisomes. Biochim. Biophys. Acta 178, 11-20 (1969).

104. Yue, S. B., Isozymes of malate dehydrogenase from barley seedlings. Phytochemistry 5, 1147-52 (1966).

105. Zelitch, I., Oxidation and reduction of glycolic and glyoxylic acids in plants. II. Glyoxylic acid reductase. J. Biol. Chem. 201, 719-26 (1953).

106. Zelitch, I., The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. J. Biol. Chem. 216, 553-75 (1955).

107. Zelitch, I., The relationship of glycolic acid to respiration and photosynthesis in tobacco leaves. J. Biol. Chem. 234, 3077-81 (1959).

108. Zelitch. I., Increased rate of net photosynthetic carbon dioxide uptake caused by the inhibition of glycolate oxidase. Plant Physiol. 41, 1623-31 (1966).

109. Zelitch, I., Investigations on photorespiration with a sensitive ^{14}C-assay. Plant Physiol. 43, 1829-37 (1968).

110. Zelitch, I., and G. A. Barber, Oxidative phosphorylation and glycolate oxidation by particles from spinach leaves. Plant Physiol. 35, 205-09 (1960).

111. Zelitch. I., and G. A. Barber, Oxidative enzymes of spinach chloroplasts. Plant Physiol. 35, 626-31 (1960).

112. Zelitch, I., and A. M. Gotto, Properties of a new glyoxylate reductase from leaves. Biochem. J. 84, 541-46 (1962).

113. Zelitch, I., and S. Ochoa, Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. J. Biol. Chem. 201, 707-18 (1953).

114. Zucker, M., Alteration of the protein components and other constituents in the tobacco leaf by shading. Plant Physiol. 34, 653-59 (1959).

115. Zucker, M., Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol. 40, 779-84 (1965).

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

BBA 65860

MALATE DEHYDROGENASE IN LEAF PEROXISOMES

R. K. YAMAZAKI AND N. E. TOLBERT
Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823 (U.S.A.)

(Received October 25th, 1968)

SUMMARY

The technique of isopycnic centrifugation of leaf homogenates has allowed a separation of chloroplasts, mitochondria, and peroxisomes according to their respective densities. Chlorophyll, cytochrome *c* oxidase, and glycolate oxidase, respectively, were used as markers for these organelles. Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) showed peaks of activity in the mitochondrial and peroxisomal fractions. The peroxisomal enzyme as assayed by following reduced pyridine nucleotide oxidation had a broad pH optimum from 6.4 to 7.4 and was specific for NADH. The peroxisomal and mitochondrial forms of malate dehydrogenase were differentiated by their kinetic and electrophoretic behavior. The mitochondrial form had a K_m (oxalacetate) of $5.7 \cdot 10^{-6}$ M and was inhibited by oxalacetate concentrations above $7 \cdot 10^{-5}$ M. The peroxisomal form had a K_m (oxalacetate) of $1.4 \cdot 10^{-5}$ M and was inhibited by oxalacetate concentrations in excess of $2 \cdot 10^{-4}$ M. The supernatant malate dehydrogenase showed kinetic characteristics intermediate to those of the peroxisomal and mitochondrial forms. Starch-gel electrophoresis of the supernatant fraction showed the presence of both the peroxisomal and mitochondrial forms together with another isozyme.

INTRODUCTION

Although the presence of multiple forms of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) in leaf tissue is a well-established fact, there are conflicting reports concerning the location(s) of the nonmitochondrial form(s). Early reports[1,2] excluded the possibility of malate dehydrogenase activity in the chloroplast, whereas later reports[3-9] have localized activity in the chloroplasts or plastid fraction. In addition several workers have characterized 'soluble' malate dehydrogenases from leaf tissue and have shown these forms to be kinetically and electrophoretically distinct from the mitochondrial form[10-12].

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Our laboratory has recently reported the isolation and partial characterization of peroxisomes from various leaf tissues[13–15]. The peroxisomes were shown to contain the enzymes glycolate oxidase, NAD glyoxylate reductase, and catalase. This report describes the presence and some of the properties of the particulate malate dehydrogenase in leaf peroxisomes.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L.) was purchased locally. The leaf tissue was washed, deribbed, and cut into small strips. Subsequent operations were carried out at 0–4° either in a cold room or in ice buckets. The tissue (100 g) was homogenized in a Waring blender for 10 sec with 1 vol. by weight of grinding medium (0.5 M sucrose, 0.02 M potassium glycyl-glycine, pH 7.5). The resulting slurry was squeezed through twelve layers of cheesecloth, and the pH (about 7) was readjusted to 7.5 with KOH. The sap was then centrifuged at 100 × *g* for 20 min, and the resulting pellet resuspended in the grinding medium and designated the 'Chloroplast' fraction. The sap was further centrifuged at 6000 × *g* for 20 min, and this pellet, after resuspension in the grinding medium, was designated the 'Peroxisome and broken chloroplast' fraction, for it contained besides the peroxisomes large amounts of chloroplast fragments and some mitochondria. A 'Mitochondrial' fraction was prepared by centrifugation at 37 000 × *g* for 20 min and resuspension of the pellet. The supernatant fluid after the last centrifugation was designated the 'Supernatant' fraction. Preparations were made with other plants using substantially the same procedure, although in some cases the volume of the grinding medium was increased to facilitate homogenization.

A discontinuous sucrose density gradient was prepared in the cold by pipetting successively 4 ml of 2.5 M, 8 ml of 2.3 M, 10 ml of 1.8 M, 15 ml of 1.5 M, and 13.5 ml of 1.3 M sucrose in a cellulose nitrate tube designed for the Spinco SW 25.2 swinging-bucket rotor (all sucrose solutions were 0.02 M in potassium glycyl-glycine, pH 7.5). A portion of the 'Peroxisomal and broken chloroplast' fraction (4 ml) was then layered on top of the gradient, and the sample was centrifuged in a Spinco Model L centrifuge at 25 000 rev./min for 3 h at 4°. Fractions were collected from the bottom of the tube after puncturing and are numbered in the order of collection.

Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) was assayed spectrophotometrically by following the oxidation of NADH at 340 mμ using a Gilford automatic recording spectrophotometer. The assay mixture contained 0.67 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.03 ml of 0.5% Triton X-100, 0.04 ml of 0.01 M oxalacetic acid, neutralized to pH 7.4 with KOH, 0.02 ml of $2.81 \cdot 10^{-3}$ M NADH, and enzyme *plus* water to give a total volume of 1 ml in the cuvette. The reaction was initiated by the addition of oxalacetate. A unit of activity has been defined as the oxidation of 1 μmole NADH per min at 25°.

Glycolate oxidase (EC 1.1.3.1) was used as a marker for peroxisomes and was assayed by following the anaerobic reduction of 2,6-dichlorophenolindophenol as described previously[14]. Cytochrome *c* oxidase (EC 1.9.3.1) was used as a marker for mitochondria and was assayed by following the oxidation of reduced cytochrome *c* (ref. 14). Chlorophyll was estimated by the method of ARNON[16] and protein by the method of LOWRY[17].

Starch-gel electrophoresis was carried out following the method of FINE AND COSTELLO[18] using Triton X-100 extracts of the fractions. Clear separation of the isozymes was noted after electrophoresis at 27 mA for 18 h at 4°. Enzymatic activity on the gel was detected by dark incubation in a buffer containing malate, NAD$^+$, phenazine methosulfate, and Nitro Blue Tetrazolium for 30 min at 37° as described by FINE AND COSTELLO[18].

Crystalline glyoxylate reductase (glycolate:NAD oxidoreductase, EC 1.1.1.26) was purchased from C. F. Boehringer and Söhne, Mannheim, Germany and was used without further purification. Cytochrome *c* and pyridine nucleotides were purchased from Sigma, (St. Louis, Mo., U.S.A.). Other reagents were purchased from Calbiochem (Los Angeles, Calif., U.S.A.).

RESULTS

Distribution of malate dehydrogenase among the particles

In Table I are shown data from a typical experiment, giving the volumes of the fractions collected as well as the approximate sucrose concentrations and appearance of the fractions collected by differential and isopycnic gradient centrifugation. It should be noted here that Fractions 3, 5, and 7 included the interface between layers of different sucrose concentration. At these interfaces were concentrated particles whose density was less than that of the next sucrose layer.

The distribution of malate dehydrogenase among the 4 fractions of the original

TABLE I

FRACTIONS FROM DIFFERENTIAL AND SUCROSE DENSITY GRADIENT CENTRIFUGATION

Volumes for the whole chloroplast, broken chloroplast, and mitochondrial fractions are those after resuspension of the pellets in the grinding medium.

Fraction	*Sucrose (M)*	*Vol. (ml)*	*Comments*
Differential centrifugation			
Original		125	118 ml used for centrifugation
Chloroplast		8.1	
Peroxisome and broken chloroplast		10.0	4.0 ml used for gradient
Mitochondrial		7.8	
Supernatant		104	
Isopycnic gradient centrifugation			
1	2.5	3.4	Clear
2	2.5–2.3	6.7	Clear
3	2.3–1.8	3.1	Peroxisomes at interface, yellow-brown band
4	1.8	5.3	Clear
5	1.8–1.5	4.4	Whole chloroplasts and mitochondria at interface, green band
6	1.5	9.6	Clear
7	1.5–1.3	7.3	Chloroplast fragments at interface, green band
8	1.3	9.9	Chloroplast fragments, green band
9	1.3–0.5	4.2	Supernatant, clear yellow

TABLE II

MALATE DEHYDROGENASE AND OTHER ASSAYS FOR CHARACTERIZING FRACTIONS FROM DIFFERENTIAL CENTRIFUGATION

Specific activity as nmoles · min^{-1} · mg^{-1} protein.

	Original	*Whole chloroplast*	*Peroxisome and broken chloroplast*	*Mito-chondria*	*Supernatant*
Malate dehydrogenase					
Percent		6.9	41.3	3.7	48.1
Specific activity	560	326	1216	521	540
Glycolate oxidase					
Percent		4.7	31.2	2.5	61.3
Specific activity	21	10.7	44.4	16.9	33.2
Cytochrome *c* oxidase					
Percent		8.0	46.2	16.5	28.4
Specific activity	25	15.3	54.9	93.9	13.3
Chlorophyll					
Percent		25.6	63.6	5.5	5.3
mg · ml^{-1}	0.58	2.3	4.6	0.5	0.0
Protein					
Percent		14.0	22.4	4.7	58.5
mg · ml^{-1}	19.5	33.5	43.6	11.7	11.0

homogenate after differential centrifugation is compared in Table II with other characteristics used to distinguish the various particles. Three points should be noted. (1) Although malate dehydrogenase is a known constituent of the tricarboxylic acid cycle of the mitochondrion, among the particulate fractions, malate dehydrogenase showed the highest specific activity in the peroxisome fraction. In contrast, cytochrome *c* oxidase, a mitochondrial marker, showed the highest specific activity in the mitochondrial fraction. This indicates that there must be another source of malate dehydrogenase activity beside the mitochondria in the particulate fractions. (2) Although 25% of the chlorophyll was found in the whole chloroplast fraction, less than 7% of the malate dehydrogenase activity was found in this fraction. This low activity can be attributed to the presence of peroxisomes and mitochondria in this fraction, as indicated by the percentages of glycolate oxidase and cytochrome *c* oxidase. Further it should be noted that the specific activity of the malate dehydrogenase was greater in the original sap than in the chloroplast fraction. (3) The percentage of malate dehydrogenase in each fraction ranged between those of the marker enzymes glycolate oxidase and cytochrome *c* oxidase. If an enzyme is located in two different organelles, the percentage of this enzyme in each fraction should be close to an average of the percentages for the other organelle markers, assuming similar specific activities for the isozymes of the particular enzyme.

Fig. 1 shows the distribution of the enzyme activities, chlorophyll and protein in the isopycnic sucrose density gradient. Glycolate oxidase served as the marker enzyme for peroxisomes which were mainly in Fraction 3. Cytochrome *c* oxidase, the marker enzyme for mitochondria, was mainly in Fraction 5 and partly in Fraction 7.

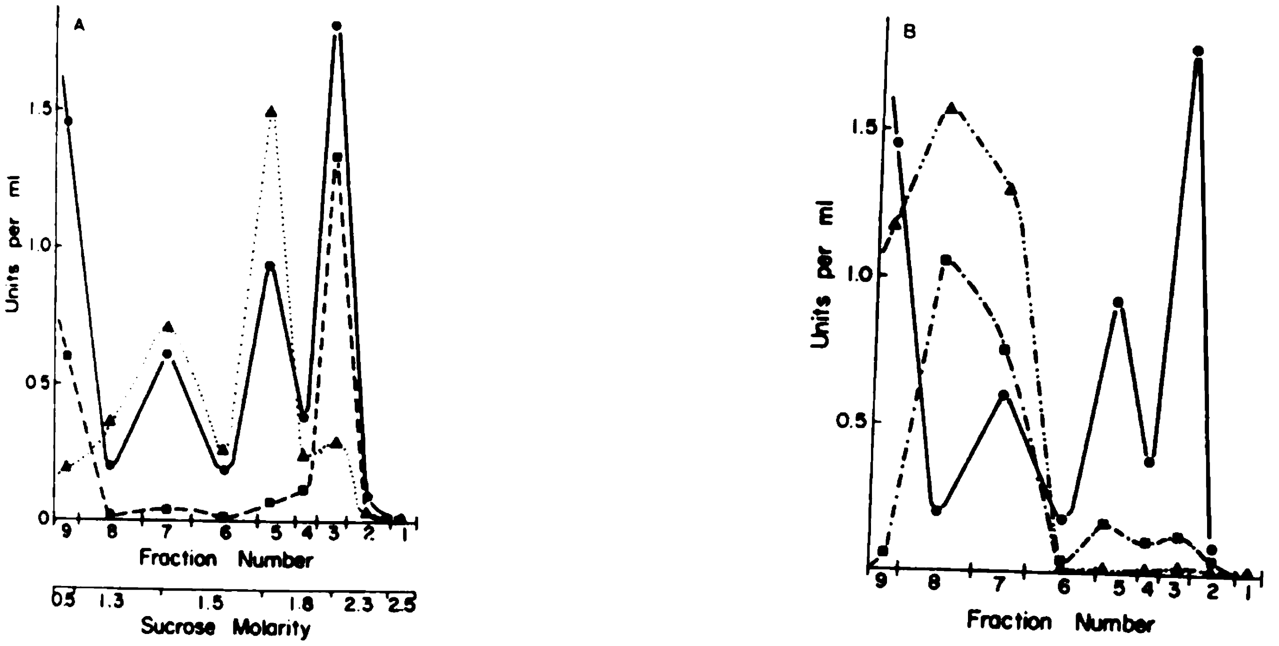


Fig. 1. Distribution of malate dehydrogenase and other marker enzymes among fractions from isopycnic sucrose gradient centrifugation. Malate dehydrogenase, glycolate oxidase and cytochrome *c* oxidase units expressed as μmoles substrate transformed per min per ml at 25°. Protein and chlorophyll expressed as mg/ml. ●—●, malate dehydrogenase; ■— — —■, glycolate oxidase; ▲·····▲, cytochrome *c* oxidase; ▲—··—▲, protein ($^1/_5$ scale); ■—·—■, chlorophyll.

Most of the chloroplasts, as indicated by chlorophyll, were in Fractions 7 and 8. Similar results were obtained with sunflower, morning glory, wheat, tobacco and perilla. Some of these data have been presented elsewhere[15]. The trimodal nature of the malate dehydrogenase distribution is evident. The two particulate malate dehydrogenases were in the peroxisomes and mitochondria and not in the chloroplasts. Two mitochondrial peaks, as measured by cytochrome *c* oxidase activity, occur because of the presence of interfaces in the gradient. A significant portion of the total malate dehydrogenase activity on the gradient was found in Fraction 9, the gradient supernatant fraction. Whether this activity arose from leakage of the peroxisomes and/or mitochondria or from actual soluble enzyme could not be ascertained. In earlier work with glycolate oxidase and glyoxylate reductase, it was noted that the ratio of the percent activity found in particulate fractions to that found in the supernatant fraction was similar for all three enzymes[14]. This suggested that the enzymes were present initially in the same particle and were released to the same extent upon isopycnic centrifugation. Thus, at least part of the malate dehydrogenase activity in the gradient supernatant fraction may be accounted for by leakage from the peroxisomes.

Peroxisomal malate dehydrogenase properties

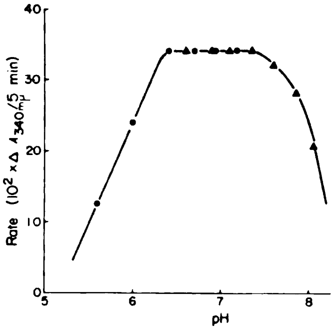
Since spinach leaves are readily available and give high yields of peroxisomes, this tissue was used to study the characteristics of the peroxisomal malate dehydrogenase. Peroxisomes isolated by isopycnic centrifugation were used for these experiments without further purification.

Latency. As in the cases of glycolate oxidase and glyoxylate reductase[14], the peroxisomal malate dehydrogenase activity was somewhat latent when the particle membrane was intact. When the nonionic detergent Triton X-100 was added to the assay mixture, an increase in activity of about 30% was noted. To insure that all malate dehydrogenase activity would be measured, 0.01% Triton X-100 was routinely added in all assays.

pH dependence. Two zwitterionic buffers were used to minimize changes in ionic strength with variation of the pH. The pH curve (Fig. 2) is very similar to that shown for a malate dehydrogenase preparation made from acetone powders of spinach leaves by HIATT AND EVANS[19]. These authors found a rather broad pH optimum, with activity slightly increasing from pH 6.4 to 7.4. The use of sodium phosphate buffer in their study may account for this increase in activity with increasing pH, since the ionic strength of the medium would increase as the pH was raised and malate dehydrogenase activity is affected by ionic concentration.

Pyridine nucleotide specificity. The peroxisomal malate dehydrogenase is specific for NADH. At a reduced pyridine nucleotide concentration of 6 mM, the rate of NADPH oxidation was only 0.6% that of NADH.

Malate dehydrogenase activity in purified glyoxylate reductase. Since the enzyme NAD-glyoxylate reductase (EC 1.1.1.26) has been shown to be present in spinach peroxisomes, it was of importance to determine whether or not any of the malate dehydrogenase activity of the peroxisomes could be attributed to this enzyme. Consequently a crystalline preparation of glyoxylate reductase was tested in the normal assay for malate dehydrogenase activity. The rate of oxalacetate reduction in the preparation was 2.6% of that of glyoxylate. This low level might be attributed to the presence of contaminating amounts of malate dehydrogenase which has a very high turn-over number.



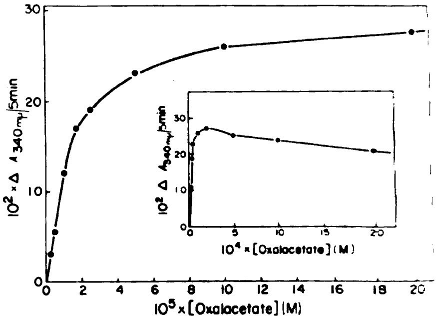


Fig. 2. pH dependency of peroxisomal malate dehydrogenase. Buffer pH adjusted by addition of KOH. Buffers: ●—●, 0.2 M *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES ▲—▲, 0.6 M HEPES. Other additions as described in the MATERIALS AND METHODS section.

Fig. 3. Effect of oxalacetate concentration on peroxisomal malate dehydrogenase activity. The standard assay procedure was used except that the oxalacetate concentration was varied as indicated. Peroxisomes purified by isopycnic centrifugation were used.

Kinetic behavior. In order to differentiate the various forms of malate dehydrogenase in the cell and to ascertain possible functions, the kinetic properties of the major malate dehydrogenase fractions were compared. The dependence of the reaction rate of the peroxisomal fraction (sucrose density gradient Fraction 3) on the oxalacetate concentration is given in Fig. 3. At oxalacetate concentrations greater than

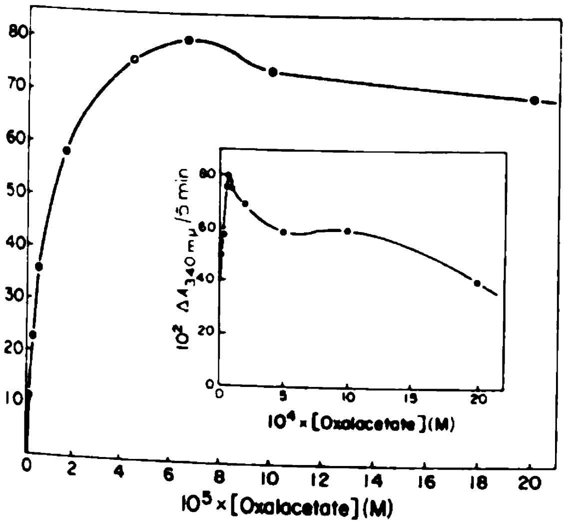
$3 \cdot 10^{-4}$ M, some substrate inhibition was observed. From a Lineweaver–Burk plot the K_m (oxalacetate) was $1.4 \cdot 10^{-5}$ M, which is similar to that of $1.8 \cdot 10^{-5}$ M obtained by Hiatt and Evans[19]. This suggests that the form isolated from acetone powders of spinach leaves was in fact the peroxisomal form.

A plot of the rate as a function of the substrate concentration for the mitochondrial fraction (sucrose density gradient Fraction 5) is shown in Fig. 4. Substrate inhibition began at an oxalacetate concentration of $7 \cdot 10^{-5}$ M and reached 50% at $2 \cdot 10^{-3}$ M. A Lineweaver–Burk plot gave a K_m (oxalacetate) of $5.7 \cdot 10^{-6}$ M for the mitochondrial malate dehydrogenase. The rate dependence of the supernatant fraction on the substrate concentration is shown in Fig. 5. A comparison of Figs. 3 and 4 with Fig. 5 gives the impression that the kinetic properties of the supernatant enzyme may be composite of the properties of the peroxisomal and mitochondrial forms, with the peroxisomes making the larger contribution.

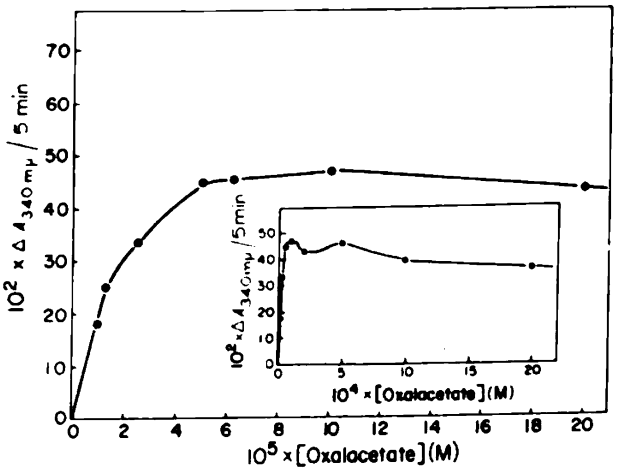
Malate oxidation. Oxidation of L-malate by peroxisomal fractions could not be observed by following NAD$^+$ reduction at pH 7.4, probably because of the unfavorable equilibrium of the reaction. It was possible, however, to observe malate oxidation at pH 7.4, using 2,6-dichlorophenolindophenol and NAD$^+$ if diaphorase was added to the assay.

Salt effects. The effects of added salts in the assay were not studied extensively. Because of the similarities in properties of the peroxisomal malate dehydrogenase to the preparation described by Hiatt and Evans[19], care was taken to exclude the addition of extraneous cations and anions in the assay medium. It was observed that the addition of 0.05 M NH_4Cl to the medium increased the rate of the reaction approx. 33%. It is interesting to note that another peroxisomal enzyme, glyoxylate reductase, also shows pronounced salt effects when hydroxypyruvate is utilized as the substrate[20].

Starch-gel electrophoresis. In Fig. 6 is shown a schematic representation of the patterns obtained upon starch-gel electrophoresis of the major malate dehydrogenase-containing fractions. The peroxisomal and mitochondrial forms were clearly differentiated by their mobility during electrophoresis, the mitochondrial form migrating



ig. 4. Effect of oxalacetate concentration on mitochondrial malate dehydrogenase activity. Con- tions as in Fig. 3 except that mitochondria purified by isopycnic centrifugation were used.



ig. 5. Effect of oxalacetate concentration on supernatant malate dehydrogenase. Conditions as Fig. 3 except that the supernatant from 37 000 × *g* centrifugation was used.

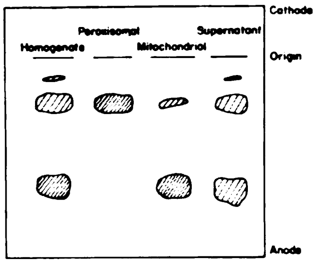


Fig. 6. Schematic representation of starch-gel electrophoresis patterns of the various malate dehydrogenases. Separation and staining techniques are described in the MATERIALS AND METHODS section.

the faster of the two. The supernatant fraction contained the peroxisomal and mitochondrial forms, with the peroxisomal form being the major component. In addition the supernatant contained a minor component which was not observed in particles. This component was not investigated further.

Comparison of specific activity of peroxisomal enzymes. Malate dehydrogenase, when measured in the direction of oxalacetate reduction, had a specific activity approx. 30 times greater than that of glycolate oxidase or glyoxylate reductase (Table III). Catalase had a still higher level of activity, which was about 25-fold greater than malate dehydrogenase.

TABLE III

SPECIFIC ACTIVITY OF PEROXISOMAL ENZYMES

Spinach peroxisomes isolated by isopycnic centrifugation were used without further purification.

Enzyme	*Specific activity (μmoles · min^{-1} · mg^{-1} protein)*
Glycolate oxidase	0.3–1.0
Glyoxylate reductase	0.3–1.0
Malate dehydrogenase	25 –39
Catalase	500 –600

DISCUSSION

In reports which locate malate dehydrogenase activity in chloroplasts, a common feature is the use of differential centrifugation for the isolation of chloroplast-enriched fractions. This use of only the sedimentation velocity properties of the cellular organelles for their purification has been shown by us to be unsatisfactory in the separation of chloroplasts and peroxisomes[14]. Thus with differential centrifugation of leaf homogenates at 1000 × *g*, one obtains a fraction enriched in both chloroplasts and peroxisomes. When this fraction is subjected to sucrose density gradient centrifugation at 1000 × *g* for short times, resolution of chloroplasts from peroxisomes is still not possible, because the parameter used for separation is again sedimentation velocity. Apparently the sedimentation coefficients of the peroxisomes and chloroplasts are very similar. It is only through the use of another, distinct, physical property, such as buoyant density, that satisfactory separation of these particles can be achieved.

After differential centrifugation we have used isopycnic or equilibrium density centrifugation to obtain a separation of the chloroplasts, mitochondria and peroxisomes. A clear separation upon sucrose density gradient centrifugation resulted from the differences in the densities of the organelles. After equilibrium was attained (centrifugation at 100 000 × *g* for 3 h), the three types of particles were found in distinct bands centering about their own respective buoyant densities. Of the three organelles, the peroxisomes were the most dense, banding in the 1.95 M sucrose layer ($\varrho \cong 1.25$). The mitochondria banded in the 1.5 M sucrose ($\varrho \cong 1.20$) and the chloroplasts or chloroplast fragments banded in the 1.3 M layer ($\varrho \cong 1.17$). Collection of fractions of the appropriate volumes thus separated the organelles.

By these techniques malate dehydrogenase activity was found in the peroxisomal and mitochondrial fractions, whereas no malate dehydrogenase activity could be attributed to the chloroplasts. Chloroplasts which had been washed three times by differential centrifugation lacked malate dehydrogenase activity, although phosphoglycolate phosphatase, a chloroplast enzyme[13,21], remains with the chloroplasts (unpublished results of R. K. Yamazaki, S. L. Vandor and N. E. Tolbert). We conclude that chloroplasts do not contain any malate dehydrogenase activity.

Similarities in pH behavior, pyridine nucleotide specificity, kinetic behavior and the effect of salts all suggest that the malate dehydrogenase activity isolated from acetone powders of spinach leaves by Hiatt and Evans[19] was the peroxisomal form. This fact, together with the intracellular malate dehydrogenase distribution (Table II) and the comparison of the activities of the isozymes of the leaf homogenate after electrophoresis, suggests that the peroxisomal form contributes a major part of the total cellular malate dehydrogenase activity in the leaf.

The enzymes which have been found to date in leaf peroxisomes are glycolate oxidase, NAD-glyoxylate reductase, catalase, glutamate-glyoxylate transaminase (unpublished results of T. Kisaki and N. E. Tolbert) and malate dehydrogenase. All of these enzymes except malate dehydrogenase are associated with the direct metabolism of glycolate to glycine, *i.e.*, the glycolate pathway. Malate dehydrogenase is the only enzyme so far detected in the leaf peroxisomes which is not directly associated with glycolate metabolism.

As shown in Fig. 4, the spinach leaf mitochondrial malate dehydrogenase was noticeably inhibited by oxalacetate concentrations higher than $7 \cdot 10^{-5}$ M. This result is in accord with those found by other workers, using the mammalian mitochondrial enzyme, and has been interpreted as indicating that the physiological role of the mitochondrial enzyme is to provide for the oxidation of L-malate. On the other hand, he peroxisomal malate dehydrogenase was found to be much less sensitive to oxalacetate concentration. Similar differences in kinetic behavior between mitochondrial and 'soluble' malate dehydrogenases have been found in beef heart[22] and corn root ip[23]. In both cases, the nonmitochondrial form was thought to be catalyzing the eduction of oxalacetate to L-malate. Because of the similarities between these 'soluble' orms and the leaf peroxisomal malate dehydrogenase it is inviting to speculate that the peroxisomal enzyme may likewise be utilized in the reduction of oxalacetate. Definite conclusions as to the function of the peroxisomal enzyme, however, must await further studies.

ACKNOWLEDGMENTS

This research was supported in part by Grant GB 4154 from the National Science Foundation. This is journal article 4536 of the Michigan Agricultural Experiment Station. Parts of this work were abstracted in *Plant Physiology*, 43 (1968) S-9.

REFERENCES

1 C. A. Price and K. V. Thimann, *Plant Physiol.*, 29 (1954) 113.
2 W. O. James and V. S. R. Das, *New Phytologist*, (1957) 325.
3 R. M. Leech and R. S. Ellis, *Nature*, 190 (1961) 790.
4 R. M. Leech, in T. W. Goodwin, *Biochemistry of Chloroplasts*, Vol. I, Academic Press, New York, 1966, pp. 65–74.
5 M. V. Laycock, D. Boulter and D. A. Thurman, in T. W. Goodwin, *Biochemistry of Chloroplasts*, Vol. I, Academic Press, New York, 1966, pp. 293–298.
6 I. Zelitch and G. A. Barber, *Plant Physiol.*, 35 (1960) 626.
7 W. S. Pierpoint, *Biochem. J.*, 88 (1963) 120.
8 S. K. Mukerji and I. P. Ting, *Phytochemistry*, 7 (1968) 903.
9 V. Rocha, S. K. Mukerji and I. P. Ting, *Biochem. Biophys. Res. Commun.*, 31 (1968) 890.
10 D. D. Davies, *Biochem. J.*, 80 (1961) 93.
11 G. R. Honold, V. Macko and M. A. Stahmann, *Naturwissenschaften*, 54 (1967) 169.
12 R. Weimberg, *Plant Physiol.*, 43 (1968) 622.
13 N. E. Tolbert, A. Oeser, T. Kisaki, R. H. Hageman and R. K. Yamazaki, *Federation Proc.*, 27 (1968) 344.
14 N. E. Tolbert, A. Oeser, T. Kisaki, R. H. Hageman and R. K. Yamazaki, *J. Biol. Chem.*, 243 (1968) 5179.
15 N. E. Tolbert, A. Oeser, R. K. Yamazaki, R. H. Hageman and T. Kisaki, *Plant Physiol.*, 44 (1969), in the press.
16 D. I. Arnon, *Plant Physiol.*, 24 (1949) 1.
17 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
18 I. H. Fine and L. A. Costello, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, pp. 958–972.
19 A. J. Hiatt and M. J. Evans, *Plant Physiol.*, 35 (1960) 662.
20 I. Zelitch, *J. Biol. Chem.*, 216 (1955) 553.
21 C. M. Thompson and C. P. Whittingham, *Biochim. Biophys. Acta*, 143 (1967) 642.
22 F. C. Grimm and D. G. Doherty, *J. Biol. Chem.*, 236 (1961) 1980.
23 I. P. Ting, *Arch. Biochem. Biophys.*, 126 (1968) 1.

Reprinted from Plant Physiology, Vol. 44, No. 1, January, 1969, pages 135-147.
(PRINTED IN U.S.A.)

A Survey of Plants for Leaf Peroxisomes[1]

**N. E. Tolbert, A. Oeser, R. K. Yamazaki,
R. H. Hageman, and T. Kisaki**
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

Received September 30, 1968.

Abstract. **Leaves of 10 plant species, 7 with photorespiration (spinach, sunflower, tobacco, pea, wheat, bean, and Swiss chard) and 3 without photorespiration (corn, sugarcane, and pigweed), were surveyed for peroxisomes. The distribution pattern for glycolate oxidase, glyoxylate reductase, catalase, and part of the malate dehydrogenase indicated that these enzymes exist together in this organelle. The peroxisomes were isolated at the interface between layers of 1.8 to 2.3 M sucrose by isopycnic nonlinear sucrose density gradient centrifugation or in 1.95 M sucrose on a linear gradient. Chloroplasts, located by chlorophyll, and mitochondria by cytochrome *c* oxidase, were in 1.3 to 1.8 M sucrose.**

In leaf homogenates from the first 7 species with photorespiration, glycolate oxidase activity ranged from 0.5 to 1.5 μmoles × min^{-1} × g^{-1} wet weight or a specific activity of 0.02 to 0.05 μmole × min^{-1} × mg^{-1} protein. Glyoxylate reductase activity was comparable with glycolate oxidase. Catalase activity in the homogenates ranged from 4000 to 12,000 μmoles × min^{-1} × g^{-1} wet weight or 90 to 300 μmoles × min^{-1} × mg^{-1} protein. Specific activities of malate dehydrogenase and cytochrome oxidase are also reported. In contrast, homogenates of corn and sugarcane leaves, without photorespiration, had 2 to 5 % as much glycolate oxidase, glyoxylate reductase, and catalase activity. These amounts of activity, though lower than in plants with photorespiration, are, nevertheless, substantial.

Peroxisomes were detected in leaf homogenates of all plants tested; however, significant yields were obtained only from the first 5 species mentioned above. From spinach and sunflower leaves, a maximum of about 50 % of the marker enzyme activities was found to be in these microbodies after homogenization. The specific activity for peroxisomal glycolate oxidase and glyoxylate reductase was about 1 μmole × min^{-1} × mg^{-1} protein; for catalase, 8000 μmoles × min^{-1} × mg^{-1} protein, and for malate dehydrogenase, 40 μmoles × min^{-1} × mg^{-1} protein. Only small to trace amounts of marker enzymes for leaf peroxisomes were recovered on the sucrose gradients from the last 5 species of plants. Bean leaves, with photorespiration, had large amounts of these enzymes (0.57 μmole of glycolate oxidase × min^{-1} × g^{-1} tissue) in the soluble fraction, but only traces of activity in the peroxisomal fraction. Low peroxisome recovery from certain plants was attributed to particle fragility or loss of protein as well as to small numbers of particles in such plants as corn and sugarcane.

Homogenates of pigweed leaves (no photorespiration) contained from one-third to one-half the activity of the glycolate pathway enzymes as found in comparable preparations from spinach leaves which exhibit photorespiration. However, only traces of peroxisomal enzymes were separated by sucrose gradient centrifugation of particles from pigweed. Data from pigweed on the absence of photorespiration yet abundance of enzymes associated with glycolate metabolism is inconsistent with current hypotheses about the mechanism of photorespiration.

Most of the catalase and part of the malate dehydrogenase activity was located in the peroxisomes. Contrary to previous reports, the chloroplast fractions from plants with photorespiration did not contain a concentration of these 2 enzymes, after removal of peroxisomes by isopycnic sucrose gradient centrifugation.

Many plants exhibit photorespiration, which is ibuted to the oxidation of glycolate as it is formed dly during photosynthesis (6, 13, 26). Such its apparently produce glycolate from sugar phos- tes of reductive photosynthetic CO_2 fixation and abolize it by the glycolate pathway (15, 18). A n CO_2 compensation point in these plants reflects the balance achieved by these 2 competing processes of CO_2 fixation by photosynthesis and CO_2 production by photorespiration (4, 23).

Another group of plants characterized by corn, sugarcane, and pigweed, do not exhibit photorespiration. These plants fix CO_2 in the light into C_4-dicarboxylic acids, and this pathway seems to be superimposed upon the photosynthetic carbon cycle involving the production of 3-P-glycerate (7, 8, 17). Plants without photorespiration have a very low compensation point (5), and do not convert added glycolate at a significant rate to CO_2 (12, 22, 27),

---

Supported in part by NSF Grant GB 4154 and pub- d as journal article No. 4521 of the Michigan Agri- ıral Experiment Station.

Consequently, it might be predicted that such plants would contain little or no glycolate oxidase. Although 1 investigation failed to observe significant glycolate oxidase activity in corn (22), the enzyme has been reported in this plant by other workers (10, 19, 20). The metabolism of added glycolate-^{14}C by corn leaves to glycine and serine also suggests the existence of an active glycolate pathway in this species (15, 27). Further evidence that glycolate metabolism occurs in corn leaves is the accumulation of glycolate after the application of a specific glycolate oxidase inhibitor to the tissue (26) and the stimulation of respiration by addition of glycolate to the leaves (2).

The discovery in spinach leaves of the microbodies, peroxisomes, which contain the enzymes of the glycolate pathway and catalase (21) suggested there should be a correlation between the presence of peroxisomes in leaves and the phenomenon of photorespiration. We have now surveyed 10 plants, representative of those with and without photorespiration, for quantitative amounts of certain enzymes of the glycolate pathway and for the presence of peroxisomes.

Materials and Methods

Plant Species. The plants used for experimental material, their source, and their classification with respect to photorespiration are given in table I. Most material was grown in greenhouse or growth chamber environments. The growth medium was a soil-peat mixture supplemented twice weekly with Hoagland nutrient solution and watered as needed. Only mature leaves were used. Similar results were obtained with sugarcane leaves from young plants and from older leaves of more mature plants. Sugarcane leaves were processed within 36 hr of harvest after being shipped to us in ice by air freight. Similar results were obtained with spinach leaves freshly harvested from the growth chamber, leaves from the grocery store, or after storage for 10 days at 4°. With spinach leaves that had been stored, a slightly lower percentage of the enzymes of the glycolate pathway were found in the particulate fraction. Leaves from all the other plants were used immediately after harvesting.

Preparation of Fractions by Differential Centrifugation. Fresh leaves were washed with distilled water, excess water removed by blotting, the midribs cut out, weighed, and chilled on ice. From 75 to 100 ml of grinding medium and 50 g of leaf tissue, cut into small pieces, were ground for 10 sec in a Waring Blendor at maximum speed. For corn and sugarcane leaves, cut into smaller pieces, it was necessary to use the grinder at low speed for 2 min and then for 10 sec at high speed. The grinding medium contained 0.5 M sucrose in 0.02 M glycylglycine buffer at pH 7.5. Homogenates were squeezed through 9 layers of cheesecloth and the pH adjusted to 7.5. All operations were conducted in the cold room at 2 to 4° or in ice baths.

A known portion of the original extract, generally 75 ml, was subjected to differential centrifugation. Whole chloroplasts were removed at 100*g* for 30 min, broken chloroplasts and peroxisomes at 6000*g* for 20 min, and mitochondria at 39,000*g* for 20 min, leaving the supernate. The nomenclature for the pelleted fraction is only for convenience and is based upon the presence of a major component in each fraction. All fractions were mixtures of the various particles as was shown by subsequent sucrose density gradient centrifugation. Each pellet was resuspended in the grinding medium and the final volume (3–5 ml) of each recorded. Enzymatic assays were run with the original extract after filtration through cheesecloth, and with subsequent fractions obtained by differential centrifugation. The yield of the activities in the fractions was equal within 10 % or better of activities in the original extract. In table II, the percent of total activity in the particulate fractions was calculated on the basis of the sum of the activities in the 4 fractions obtained by differential centrifugation.

Sucrose Density Gradient Centrifugation. A known volume (2.0–2.5 ml) of the peroxisomal and broken chloroplast fraction (6000*g* pellet) was layered on a non-continuous sucrose gradient in glycylglycine buffer, pH 7.5. This fraction was selected because previous work (21) had shown that it con-

Table I. *Plants Surveyed*

	Source	Photorespiration
Spinach, *Spinacia oleracea* L.	Growth chamber or store bought	Present (24)
Sunflower, *Helianthus annuus* L.	Greenhouse	Present (11)
Tobacco, *Nicotiana tabacum* L.	Greenhouse	Present (8,11,23)
Wheat, *Triticum vulgare* L., var. thatcher	Greenhouse or growth chamber	Present (5,8,9)
Pea, *Pisum sativum* L., var. little marvel	Growth chamber	Unknown
Bean, *Phaseolus vulgaris*, var. sanilac	Greenhouse	Present (4,8)
Swiss chard, *Beta vulgaris*, var. cicla	Greenhouse	Unknown[1]
Corn, *Zea mays* L., W64A X OL43	Greenhouse and growth chamber	Absent (2,3,5,8,11,17)
Sugarcane, *Saccharum*, CL 41-223	Field grown[2]	Absent (17)
Pigweed, *Amaranthus hybridus* L.	Greenhouse	Absent (3,24)

[1] Swiss chard probably has photorespiration since it is a species related to beets which exhibit photorespiration (24).

[2] Courtesy of L. P. Hebert, United States Sugarcane Field Station, Canal Point, Florida.

tained the bulk of the peroxisomes. The gradient of 5 layers was prepared at 4° by pipetting 4 ml of 2.5 M sucrose (85 % w/v), 8 ml 2.3 M sucrose (68 % w/v), 10 ml of 1.8 M sucrose (61 % w/v), 15 ml of 1.5 M sucrose (51 % w/v) and 15 ml of 1.3 M sucrose (44 % w/v). These percent values for w/v are based upon g sucrose per 100 ml final volume at room temperature. The 2.5 M sucrose had been prepared in 0.02 M glycylglycine and it was diluted with 0.02 M glycylglycine for the other sucrose concentrations. The gradient was centrifuged for 3 hr at 4° at 25,000 rpm (44,600*g* to 106,900*g*) in a swinging bucket rotor SW 25.2 of the Spinco centrifuge, Model L. In this time, the particles had sedimented to a sucrose molarity which had high enough specific gravity to stop the particles, and longer periods of centrifugation could not alter their distribution. In recent literature citations concerning the location of glycolate oxidase (14) and malate dehydrogenase (16) in chloroplast preparations, investigators have used non-isopycnic sucrose gradient centrifugation procedures which, when checked by us, did not separate the peroxisomes from the chloroplast fractions.

The gradient was divided into aliquots of 3 to 12 ml by draining from the bottom of the centrifuge tube through a needle, and fractions 1 (at the bottom) to 9 (at the top) were collected as indicated in figure 2. This procedure was selected on the basis of an investigation with spinach leaves (21). The 0.5 M sucrose fraction which had been layered on the gradient (fraction 9) retained the soluble enzymes. In the upper portion of the 1.3 M sucrose layer and at the interface region between 1.3 M and 1.5 M sucrose were bands of chloroplasts as observed microscopically and by chlorophyll analysis (fractions 7 and 8). The interface region between the 1.5 and 1.8 M sucrose layers (fraction 5) contained the mitochondria and some whole chloroplasts. At the interface between 1.8 and 2.3 M sucrose (fraction 3) were located the peroxisomes. Although a continuous gradient has produced a cleaner separation of peroxisomes completely free of chlorophyll, a discontinuous gradient was more convenient and faster for this survey because of the viscosity of 1.8 to 2.5 M sucrose. A 2.3 M sucrose layer was used rather than one of 2.0 M in order to stop the peroxisomes in a sharper band. No significant amount of enzymatic activity other than peroxisomal activity has been found below the 1.8 M sucrose layer.

The percent activity among the sucrose gradient fractions in figure 2 was calculated on the basis of the total activity recovered from the gradient. In general, nearly 100 % recovery on the gradient of the activity from the 6000*g* pellet fraction was achieved, except for cytochrome *c* oxidase and malate dehydrogenase, for which an increase in total activity was observed. This increase suggests that the values obtained with the initial homogenates for these 2 enzymes are too low.

Glycolate Oxidase (*EC 1.1.3.1*). Glycolate:O_2 oxidoreductase was assayed anaerobically by following 2,6-dichlorophenolindophenol reduction (21, 28). Additions were made to a Thunberg cuvette (d = 10 mm) in the following order: 2 ml 0.1 M pyrophosphate, pH 8.5, containing 1.5×10^{-4} M dye; 0.05 ml 5×10^{-3} M FMN (final concentration, 1.0×10^{-4} M); 0.05 ml 0.5 % Triton X-100 (0.01 % final); 0.01 to 0.2 ml of enzyme, and in the side arm, 0.1 ml of 0.125 M sodium glycolate (final concentration, 5×10^{-3} M). The final volume was 2.5 ml. The cuvette was evacuated and flushed 10 times with N_2 which had passed through Fieser's solution to remove traces of O_2. Dye reduction at 25° was measured at 600 mμ by an automatic recording Gilford spectrophotometer. A unit of activity was expressed as 1 OD change per min, which was equivalent to 4.78 nmoles of dye reduced per min.

The use of Triton X-100, which probably solubilizes the particulate membranes, increased glycolate oxidase, glyoxylate reductase, and malate dehydrogenase activity in the peroxisomal fraction by 30 to 40 %. A final concentration of 0.01 % Triton X-100 was found to be sufficient, even with the high sucrose concentration from the gradient. Catalase activity was not increased by Triton X-100, although catalase is the only enzyme whose activity is increased in liver peroxisomes by use of Triton X-100 (1).

All attempts to run the glycolate oxidase assay aerobically with a high concentration of cyanide to inhibit peroxidase reoxidation of the reduced dye were unsuccessful, as discussed previously (21). In the aerobic assay, the peroxidase in the soluble fractions resulted in lower total enzyme units. The peroxisome fraction (No. 3) from the sucrose gradient contained so little peroxidase that the aerobic assay could be used with this fraction. However, aerobic assays gave incorrect overall recoveries because of low values in fractions with peroxidase activity such as the supernate. Only by the anaerobic assay could a quantitative recovery of the enzyme from the various fractions be obtained.

Glyoxylate Reductase (*EC 1.1.1.26*). The assay for this enzyme involved following the oxidation of NADH at 340 mμ (25). Additions were made to a 1-ml Beckman cuvette (d = 10 mm) in the following order; 0.4 ml of 0.02 M glycylglycine buffer, pH 7.5; 0.05 ml NADH containing 0.2 mmole; Triton X-100 (final 0.01 %); enzyme, water, and substrate to make a final volume of 1.0 ml. After determining the endogenous rate for 1 to 3 min, 0.04 ml 0.125 M sodium glyoxylate (final concentration, 5×10^{-3} M) was added. A unit of activity was expressed as 1 OD change per min, which was equivalent to 161 nmoles of NADH oxidized per min.

After completing this survey, it was found that glyoxylate reductase, as obtained from the peroxisome fraction of spinach leaves by Triton X-100 treatment, was not assayed at optimal conditions. The pH optimum was found to be 5.8 as compared to 7.5 with the soluble enzyme or 6.3 to 6.6 for the

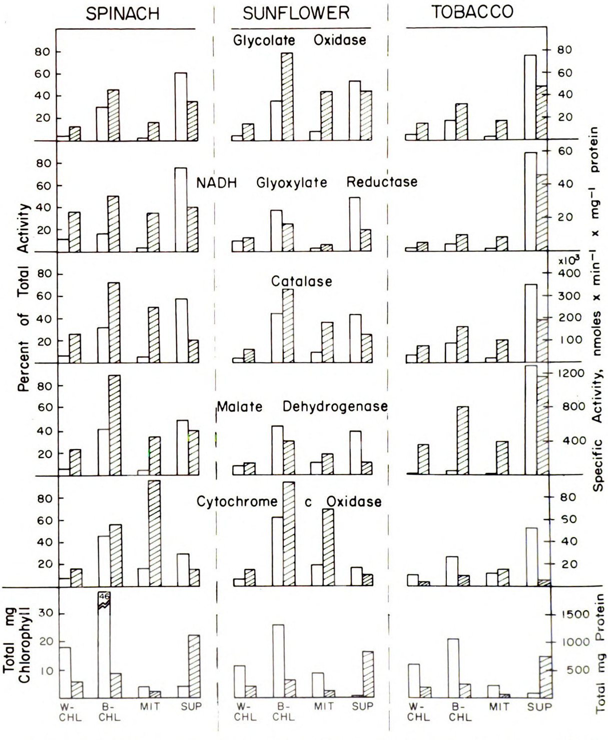
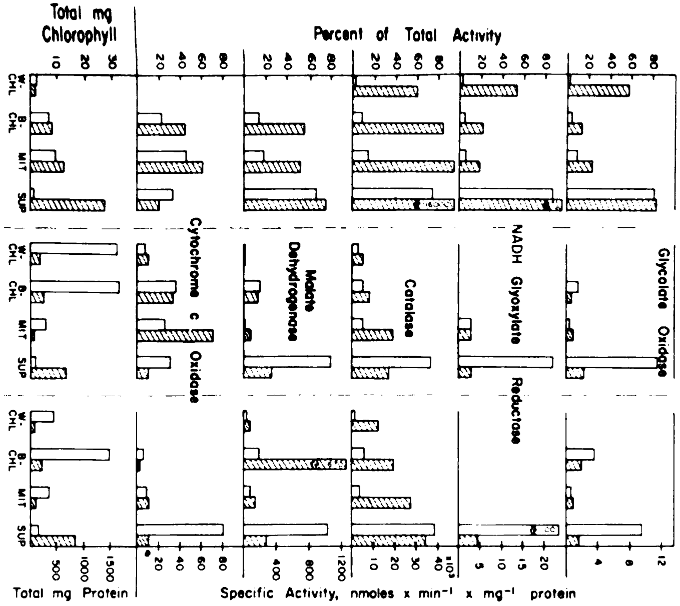


FIG. 1. Enzymic assays of fractions from differential centrifugation. The leaf homogenates were separated into a 100*g* pellet containing whole chloroplasts (W-Chl), a 6000*g* pellet containing peroxisomes and broken chloroplasts (B-Chl), a 39,000*g* pellet containing mitochondria (Mit), and supernate (Sup). For the enzyme assays of each fraction, the open bar represents the percent of the total activity of the homogenate and the hatched bar the specific gravity. At the bottom of the figure the open bar represents chlorophyll and the hatched bar protein.

Figures continued next page



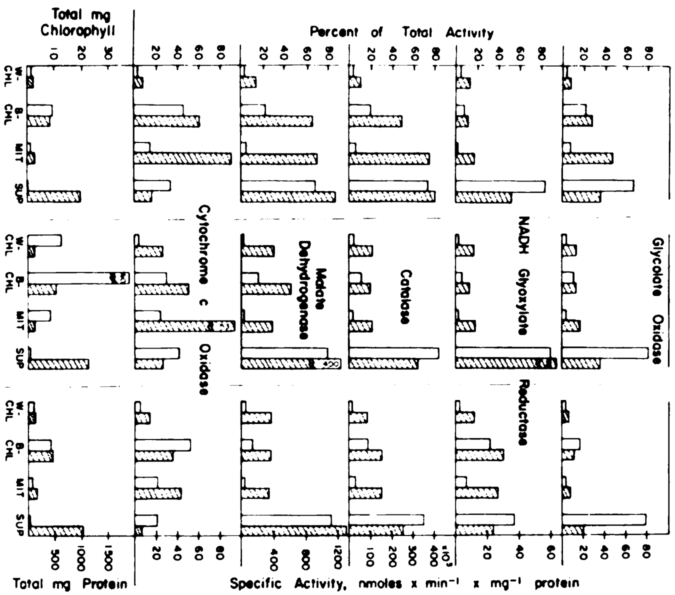
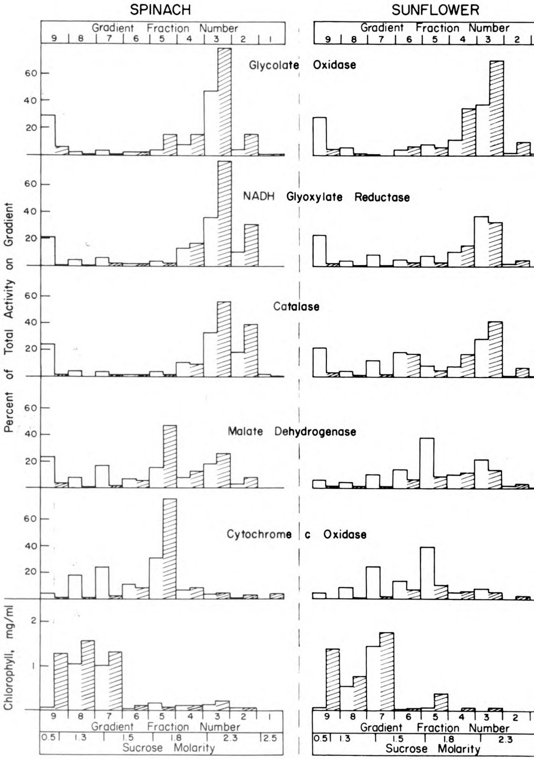


Table II. *Total Enzymatic Activities in Homogenate*

	Glycolate oxidase			Glyoxylate reductase			Catalase			Malate dehydrogenase			Cytochrome *c* oxidase		
	nmoles × min⁻¹ ×		% In	nmoles × min⁻¹ ×		% In	nmoles × min⁻¹ ×		% In	nmoles × min⁻¹ ×		% In	nmoles × min⁻¹ ×		% In
	g⁻¹ wet weight	mg⁻¹ protein	particulate fractions	g⁻¹ wet weight	mg⁻¹ protein	particulate fractions	g⁻¹ wet weight	mg⁻¹ protein	particulate fractions	g⁻¹ wet weight	mg⁻¹ protein	particulate fractions	g⁻¹ wet weight	mg⁻¹ protein	particulate fractions
Spinach	514	21	39	902	21	25	4.2×10^6	94,900	43	13.7×10^3	560	51	610	25	72
Sunflower	1450	49	47	382	13	51	5.3×10^6	181,500	57	6.6×10^3	223	62	1340	45	84
Tobacco	1060	39	25	578	21	32	6.0×10^6	222,700	30	29.8×10^3	1108	4	1413[1] (314)	54[1] (12)	 (48)
Pea	1220	31	33	758	19	16	11.8×10^6	302,800	27	32.4×10^3	832	30	1788	46	64
Wheat	815	29	18	1300	47	8	8.7×10^6	313,500	16	34.9×10^3	1261	20	1637	59	58
Swiss chard	600	21	17	1012	36	9	7.0×10^6	244,900	24	20.7×10^3	728	46	1092	38	84
Bean	574	17	22	754	23	45	8.3×10^6	248,900	29	22.1×10^3	664	17	741	22	77
Pigweed	266	7	18	454	12	14	4.1×10^6	105,100	24	23.2×10^3	600	33	1410	36	67
Corn	30	1	15	53	2	12	0.5×10^6	18,800	26	7.6×10^3	271	18	653	23	68
Sugarcane	48	3	30	39	2	trace	0.6×10^6	35,300	22	3.5×10^3	205	21	680[1] (81)	42[1] (5)	 (18)

[1] Recovery of cytochrome *c* oxidase activity from the broken chloroplast fraction of the homogenate during sucrose density gradient centrifugation (fig 2) was 840 % for sugar cane and 450 % for tobacco. Apparently the cytochrome *c* oxidase assay with the crude homogenate from these plants was not accurate, giving values much too low. The values are corrected for these factors; values in parentheses are uncorrected.


SPINACH
SUNFLOWER
Gradient Fraction Number
9 8 7 6 5 4 3 2 1
Glycolate Oxidase
NADH Glyoxylate Reductase
Catalase
Malate Dehydrogenase
Cytochrome c Oxidase
Percent of Total Activity on Gradient
60
40
20
Chlorophyll, mg/ml
2
1
Sucrose Molarity
0.5 1.3 1.5 1.8 2.3 2.5

continu

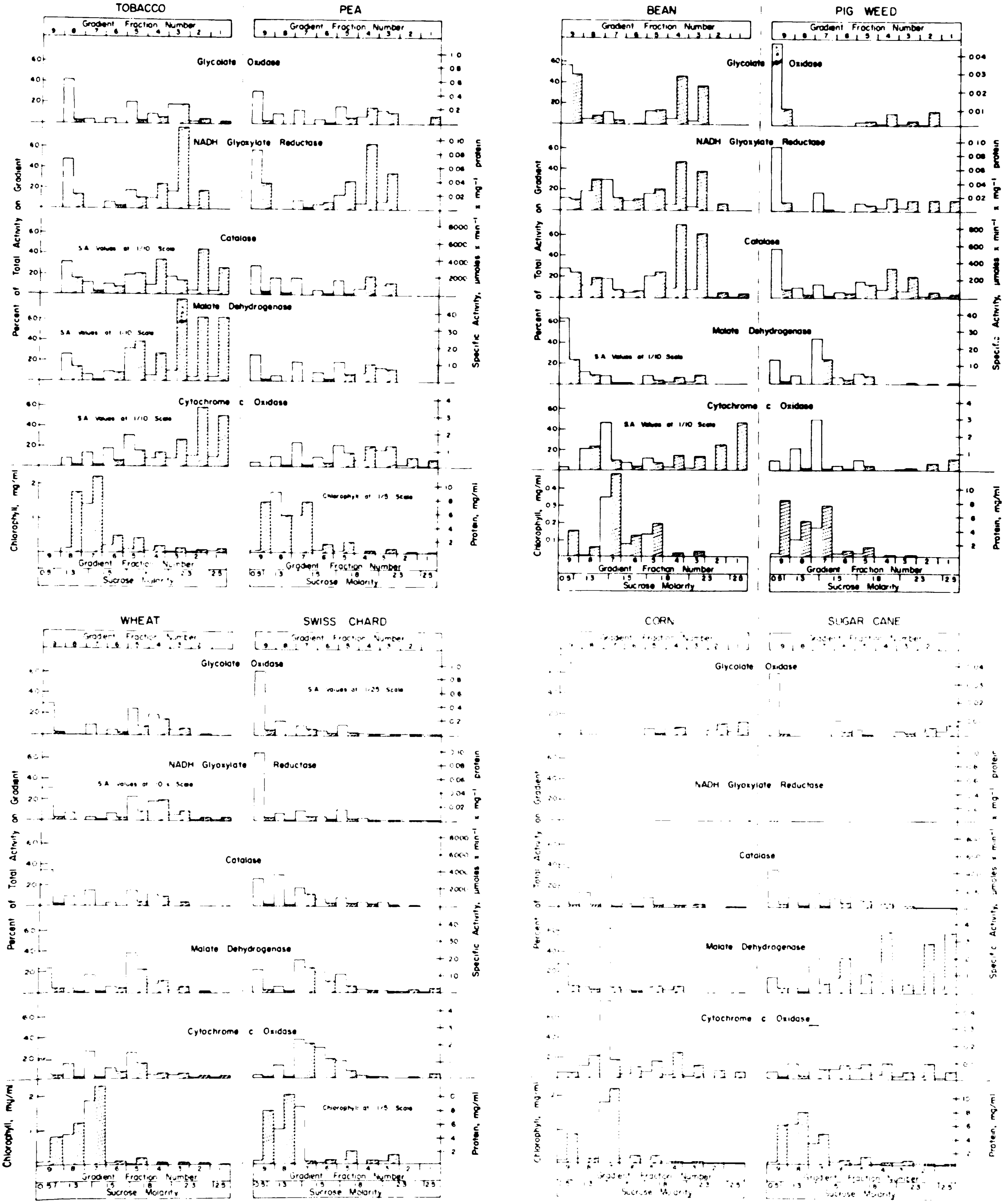


FIG. 2. Enzymic assays of sucrose gradient fractions from broken chloroplasts. The fractions are numbered on the horizontal axis as removed from the bottom of the centrifuge tube. The designated sucrose molarity is that used to prepare the gradient, and the scale is proportional to the volume of each layer of sucrose solution. The total volume of each gradient was about 54 ml and the actual volume of each fraction from the gradient is depicted by its band width. Consequently, the total chlorophyll (open bar) and protein (hatched bar) in each fraction is represented by the width plus height of the bar. With respect to the enzyme assays, only the height of the bars are to be considered. The percent of the total activity on the gradient is indicated by the open bars, while the hatched bars show the specific activity of the enzyme in each fraction.

crystalline enzyme (25). For optimal conditions, a final substrate concentration of 5×10^{-2} M was indicated, which is comparable to previous values of 9.1×10^{-3} M (25). Thus, the specific activities for glyoxylate reductase reported here (except for wheat which was assayed at pH 5.8 with 5×10^{-2} M substrate) are estimated to be about 40 % of maximum values. Sufficient reevaluation of the data were completed to verify that the reported results did not alter the conclusions concerning the distribution of this enzyme among the various fractions.

Other Assays. These procedures have been detailed in a paper on the characterization of peroxisomes from spinach leaves (21). Catalase (EC 1.11.1.6) was assayed by the disappearance of H_2O_2 as measured spectrophotometrically at 240 mμ. A unit of activity was a change of 1 OD in 1 min at 25°, and was equal to 2.76 μmoles of H_2O_2. In the cytochrome *c* oxidase (EC 1.9.3.1) assay, the rate of oxidation of reduced cytochrome was measured at 550 mμ. Malate dehydrogenase (EC 1.1.1.37) was measured by the spectrophotometric rate of NADH oxidation with 4×10^{-4} M oxalacetate as substrate. A more detailed characterization of malate dehydrogenase in leaf peroxisomes will be published elsewhere. Protein was determined by the Lowry procedure and chlorophyll by its absorption at 652 mμ according to Arnon's procedure.

Results

Activity in Homogenates and Fractions From Differential Centrifugation. In table II, the specific activity of each enzyme in nmoles × min^{-1} × g^{-1} wet weight or mg^{-1} protein in the different leaf homogenates and the percentage of this activity in the combined particulate fractions have been recorded. The distribution of these enzymic activities in the whole homogenate among the 4 cellular fractions obtained by differential centrifugation of the leaf homogenates is plotted in figure 1. The fractions have been designated by the centrifugational procedure (Methods) after their major component. Thus the 100*g* pellet contained whole chloroplasts (W-Chl). The bulk of the protein in the 6000*g* pellet was associated with broken chloroplasts (B-Chl), yet this fraction contained in addition most of the peroxisomal activity and a substantial part of the mitochondrial cytochrome *c* oxidase activity. The percent distribution of the different enzymatic activities (open bars), and the specific activity (hatched bars) of each enzyme showed a significant difference among the plants.

The amount and specific activity of glycolate oxidase and glyoxylate reductase were of the same order of magnitude. Glycolate oxidase values ranged from 1.450 μmoles × min^{-1} × g^{-1} wet weight for sunflower to 0.030 for corn leaves, and glyoxylate reductase from 1.3 for wheat to 0.039 for sugarcane. Due to its high turnover number, the specific activity of catalase was immense, and catalase activity, like glycolate oxidase activity, varied among the plants
from 11,800 μmoles × min^{-1} × g^{-1} we
pea to 500 for corn leaves. Total mal
genase also varied among the plants.
of cytochrome *c* oxidase was more ne
among the plants and reflects the ubiquit
of mitochondria.

Sucrose Density Gradient Centrif
Peroxisomes. Figure 2 shows the di
the various enzymes among the 9 fracti
from the isopycnic non-linear sucrose den
separation of the 6000*g* or broken chlo
tion. The whole chloroplast and mitoc
tions from the differential centrifugati
subjected to the sucrose gradient separa
they contained less of the enzymes ass
peroxisomes. However, from a previo
tion (21), the small amount of glycolat
peroxisomal activity in the whole chlo
mitochondrial fractions had banded
sucrose gradients to that in the broke
fraction.

As indicated by the chlorophyll
analysis (fig 2), most of the chlorop
sucrose gradient were found in fractio
which consisted of 1.3 M to 1.5 M suc
fractions were visible as 3 distinct gree
the upper part of the 1.3 M sucrose la
at the interface with the 1.5 M sucrose
small one at the interface between 1.
sucrose (fraction 5). The chloroph
particles in fraction 5 appeared to
observed with the light microscope, w
fractions 7 and 8 appeared broken. Wh
chloroplast fraction from the differentia
tion was used instead of the broken chl
tion, more chlorophyll was present i
(data not shown).

Mitochondria were predominantly lo
tion 5, as illustrated by the location of
oxidase and 1 of the malate dehydro
(fig 2).

Peroxisomes were found in fracti
sucrose gradient which represented
between 1.8 and 2.3 M sucrose (fig
particles in fraction 3 from spinach
examined by electron microscopy, th
spheroid, 0.5 to 1.0 μ in diameter, and
dense granular matrix or stroma surr
single membrane which was ruptured i
(21). In this survey, the peroxisomes
terized by their location on the sucr
and by their enzymic content of glyco
glyoxylate reductase, catalase, and part
dehydrogenase activity. The presence
each of these enzymes at the top of th
fraction 9, which corresponds to the
particulate region of the gradient, may
of the fragility of these particles. Since
were not washed, some activity from
enzymes of the original homogenate w
pear in fraction 9 of the gradient.

To estimate the relative distribution of each zyme between the peroxisomes and the cytoplasm, e sum of the particulate activities obtained by dif- rential centrifugation can be compared to the tivity in the supernate (fig 1). This estimation es not account for breakage of the particles during inding which is undoubtedly severe. A maximum about 50 % of the glycolate oxidase in homogenate spinach and sunflower leaves was found in the mbined particulate fractions, whole chloroplasts, roxisomes plus broken chloroplasts, and mitochon- ia. Examination of these 3 fractions indicated it the same particle, peroxisomes, moving to the me place on the sucrose gradient, contained this colate oxidase activity. In subsequent handling the particles during gradient development, more the enzymatic activity was solubilized and ap- ared in the soluble fraction, No. 9, of the sucrose adient. Movement across boundaries of different crose molarities has a shearing effect upon par- les. How much of the activity in the top soluble action 9 of the sucrose gradient is from disruption the particles or from removal of supernatant zymes occluded with the particle cannot be re- lved. This degree of apparent breakage varied tween 20 to 30 % for sunflower and spinach and to 90 % for pigweed, corn, and sugarcane. Ex- mination of the spinach peroxisomes by electron croscopy showed that nearly all of them had a oken membrane or were more severely ruptured 1).

The small amount of peroxisomal activity which s found in fraction 2 of 2.3 M sucrose is an arti- t of the procedure. Removal of the fractions m the gradient was based upon visual observations. attempt was made to stop the collection of ction 2, after this very light green solution had ined from the delivery tubing. This slight color med to be due to traces of chlorophyll-containing terial. Under the best of conditions, fraction 3 s faintly yellow in color. It was never possible completely separate fractions 2 and 3 after 1 dient run which started with a concentrated mix- e of particles. Some contamination of fraction 3 o fraction 2 resulted in enzyme activity in fraction f lower specific activity, probably due to the green terial seen in it. Contamination of fraction 3 o occurred, and the enzymatic specific activities orted for it are not maximum.

Isopycnic Linear Gradient. Fractions 2 and 3 m a spinach preparation were combined and rerun a second isopycnic linear gradient between 2.3 1.7 M sucrose. The peroxisomal activity banded ween an estimated sucrose molarity of 1.95 to 0, and the peroxisomes were completely devoid chlorophyll and cytochrome oxidase activity which nained at the top of the gradient. No activity s found below 2.1 M sucrose. In this experiment, peroxisomes in about 2.0 M sucrose of fraction 3 re diluted only to 1.5 M sucrose before rerunning the linear gradient. During this recentrifugation, about 30 % of the glycolate oxidase activity was solubilized and was found at the top of the second gradient. This result gives some measure of the loss of enzymatic activity from the spinach particles. In the original grinding medium, more severe rupture of the particles could be expected.

Fraction of Total Protein as Peroxisomes. From the data only a limited approximation can be made concerning the total amount of cellular protein which may be in the peroxisomes from spinach and sunflower leaves. About 1.5 to 2.0 % of the protein of the 6000*g* pellet was found in the peroxisomal fraction, No. 3, after sucrose density gradient centrifugation. According to the marker enzymes this fraction was reasonably pure, but its actual purity on the basis of protein from other particulate material was not known. This value should be corrected to 3 to 4 % of the total protein of the 6000*g* pellet on the basis of the amount of marker enzymic activity which was found in other fractions on the gradient, particularly the solubilized top fraction. About 20 % of the total protein of the homogenate was generally in the 6000*g* or broken chloroplast pellet. Thus 0.6 to 0.8 % of the total protein of the homogenate appeared to be in the peroxisomes. This value may be twice as large on the basis of an estimated 50 % rupture of the peroxisomes during grinding. Thus about 1 to 1.5 % of the protein of the spinach homogenate may be peroxisomal.

Grouping of Plants by Photorespiration, Enzyme Activity, and Peroxisomes. In the plants surveyed, the activity of the enzymes associated with the peroxisomes varied over a wide range. In an attempt to categorize the data, the plants have been grouped with respect to photorespiration (table I), total glycolate oxidase activity (table II), and the relative amount of this enzyme which remained in peroxisomes during the isolation procedure (figs 1 and 2).

Sunflower and spinach leaves, which exhibit photorespiration, contained a high level of glycolate oxidase (1450 and 514 nmoles × min^{-1} × g^{-1} wet tissue or 49 and 21 nmoles × min^{1-} × mg^{-1} protein) in the crude extract. Likewise, values for the specific activity of glyoxylate reductase, catalase, and malate dehydrogenase activity were high in these plants. In the original extract, 40 to 50 % of the activity of these enzymes was found in particulate fractions and 50 to 60 % in the supernate. On the sucrose gradient of the broken chloroplast fraction, a single peroxisome fraction (No. 3) contained most of these enzymatic activities. These plants, then, are characterized by the presence of the most stable peroxisomal fraction, active enzymes for glycolate metabolism, and photorespiration. As such, these plants provide a basis for comparison with the other plants.

Corn and sugarcane leaves contained only 2 to 5 % as much total glycolate oxidase, glyoxylate reductase, or catalase as spinach or sunflower leaves, and only traces of these enzymic activity were found in the area on the sucrose gradient where peroxisomes should be located. These 2 plants represent the best example of plants without photorespiration and the least amount of peroxisomal enzymes. It is noteworthy that the total activity of each of the peroxisomal enzymes was similarly low in these plants compared to sunflower and spinach. In figure 1 for the fractions from the crude extract, the specific activity scale for glycolate oxidase is one-seventh that used for plants with photorespiration. The specific activity of glycolate oxidase in the supernate from corn and sugarcane leaf extracts was about 2 nmoles × min^{1-} × mg^{-1} protein as compared to values between 40 and 80 for plants with photorespiration. On a wet weight basis, corn oxidized 29 nmoles glycolate × min^{-1} × g^{-1} and sugarcane 48, which is to be compared with values of 500 to 1500 for other plants. These low values are not due to poor homogenation of these fibrous tissues since amounts of protein and chlorophyll in the extracts were comparable with amounts found in other leaves (fig 1). However, it must be emphasized that the activity of glycolate oxidase and catalase from corn and sugarcane leaves was still sufficiently great to be measured easily and reliably. Significant amounts of these enzymes are present in corn and sugarcane, whereas very large amounts of these enzymes are present in spinach and sunflower.

For corn, sugarcane, and pigweed, there were only trace amounts of marker enzymes on the sucrose gradient at the location for peroxisomes. Again, note the scale change for specific activity in figure 2 when comparing these plants with sunflower or spinach. The smaller amount of enzyme activity which was first separated with the 6000*g* pellet or broken chloroplast fraction by differential centrifugation was not stable to repeated centrifugation and after sucrose gradient centrifugation was located at the top of the sucrose gradient. The presence of trace amounts of activity in fraction 3 from all the plants indicate that there are some peroxisomes in these plants. The results are qualified because of the difficulty in grinding corn and sugarcane leaves which might have resulted in greater rupture of the particles. For this reason, preparations were made with old leaves as well as very young leaves, but similar results were obtained. Although the necessary longer grinding period could have ruptured most of the peroxisomes, it should not have destroyed such enzymes as glycolate oxidase and catalase and the values for total activity should not be in serious error.

Although pigweed, like corn and sugarcane, does not exhibit photorespiration, its complement of glycolate pathway enzymes was greater. The crude pigweed homogenate oxidized glycolate at the rate of 266 nmoles × min^{-1} × g^{-1} wet weight, or 7 nmoles × min^{-1} × mg^{-1} protein. This amount of activity was half that found in spinach leaves. Similar results were obtained with leaves from green house-grown plants that were flowering or from vegetative or florally induced plants grown in growth chambers. It was hoped that pigweed leaves, without photorespiration, would be easy material from which to extract enzymes. However, the leaf homogenate from greenhouse leaves was so gelatinous that only part of it could be squeezed through cheesecloth, and from plants in the growth chamber, the homogenates were rich with saponifiers and extremely foamy. The total amount of enzyme activity on a leaf weight basis is thus minimal and true values are probably similar to those from spinach leaves.

From the pigweed broken chloroplast fraction, only traces of peroxisome activity were observed, and of this small amount, most of it was found in the soluble protein fraction of the sucrose gradient. In this respect, the pigweed preparations gave gradient distribution patterns similar to that from corn and sugarcane and differed from those obtained with spinach and sunflower preparations.

Tobacco, pea, and wheat leaves, plants with photorespiration, contained relatively high levels of glycolate oxidase, glyoxylate reductase, catalase, and malate dehydrogenase activity. However, from these species, more of the enzyme activity was found in the supernate (65–80 %) rather than in the particulate fractions of the original extracts. Thus, either there were fewer peroxisomes in these leaves or else the yield of peroxisomes by the isolation procedure employed was poor. The activity in the broken chloroplasts was located on the sucrose density gradient in 2 bands, 1 in fraction 3 as for spinach and sunflower peroxisomes and 1 in fraction 5 at the interface of the 1.5 to 1.8 M sucrose layer. Peroxisomes in fraction 5 could be accounted for by more broken particles, by less dense whole particles, or by 2 species of peroxisomes. Further study is needed to delineate between these possibilities or other explanations of the results. Currently, it is assumed that fraction 5 contained broken peroxisomes to account for its glycolate oxidase activity.

Homogenates of bean leaves, a plant which has photorespiration, and Swiss chard leaves contained high levels of glycolate oxidase and glyoxylate reductase. However, 80 % or more of these activities were found in the supernate after differential centrifugation. Of the amount of activity in the broken chloroplast fraction, only traces were found in the peroxisome fraction 3 as well as in fraction 5 after sucrose gradient centrifugation, and most of the activity was recovered in the top layer which is assumed to be soluble protein. Note that the specific activity scale for these for the plots in figure 2 were magnified 25-fold. Again, these low yields of peroxisomes could be due to rupture of the particles during grinding.

These results seem to be inconsistent with the concept that peroxisomes are characteristic of plants with photorespiration. In fact, bean leaves are particularly active for glycine synthesis. In previous studies, the leaves of the same bean variety incorporated 50 % of the total $^{14}CO_2$ fixed in the first

10 to 30 sec of photosynthesis into glycine by the glycolate pathway (unpublished). Thus, bean and Swiss chard may be characterized as having an active glycolate metabolizing system and active enzymes of the glycolate pathway which mainly appear in the soluble fraction after grinding the leaves.

The results with the last 2 groups of plants could be reconciled by the assumption that the differences reflect the degree of rupture of the peroxisomes or the inefficiency of isolating the peroxisomes by the single procedure developed for spinach leaves and used for all the plants. All 3 groups of plants with photorespiration contained active enzymes of the glycolate pathway. The major difference was in the distribution of the enzymes between peroxisomes and the supernate.

Similar Distribution Pattern for Glycolate Oxidase and Glyoxylate Reductase. Glycolate oxidase and glyoxylate reductase in the isolated peroxisomes had about equal total and specific activities and the same distribution on the sucrose gradient. A somewhat similar percentage of both enzymes was found in the broken chloroplast fraction from each plant. Each of these activities in the broken chloroplast fraction, when put on a sucrose gradient, was found to distribute similarly between a peroxisome fraction (No. 3 or No. 5) and the soluble or non-particulate fraction (No. 9). In all cases, a substantial part of the activity was in the soluble fraction even though this part of the activity had originally sedimented with the broken chloroplasts. Electron microscopic examination of spinach peroxisomes had indicated that they were nearly all sheared or ruptured, and, thus, partial solubilization of activity is to be expected. The data suggest that the 2 enzymes are associated in the cell in the same organelle; *i.e.*, the peroxisomes. From the data, it is not possible to ascertain whether there are 2 pools of each of these enzymes, 1 soluble and 1 particulate.

Distribution of Catalase and Malate Dehydrogenase. Distribution of catalase activity was similar to that for glycolate oxidase and glyoxylate reductase. We wish to emphasize that catalase did not follow the chlorophyll-containing bands on the sucrose gradient. With sunflower, spinach, and tobacco, it was present mainly in peroxisomes and it was nearly absent in chloroplasts. The data suggest that for sunflower and spinach leaves, at least, peroxisomes may be the only site of catalase activity. A similar distribution of glycolate oxidase and catalase is consistent with the concept that, during the oxidation of glycolate by peroxisomes, the H_2O_2 produced is immediately decomposed by the catalase. For sugarcane, corn, Swiss chard, and bean, from which poor yields of peroxisomes were obtained, a higher percent of the catalase activity was found with the soluble fractions, as was true for glycolate oxidase. From corn and sugarcane leaves, only one-tenth as much total catalase activity was found as in other plants, and this fact was also true for glycolate oxidase.

Characterization of malate dehydrogenase of the peroxisomes is being published elsewhere. There are different malate dehydrogenase isozymes, in leaves (16), so that the percentage of the total malate dehydrogenase activity found in the peroxisome fraction is affected by the recovery of the peroxisomes as well as that fraction of the total cell activity attributed to the peroxisomes. The present data clearly indicate that in all the plants, malate dehydrogenase activity is in both peroxisomes and mitochondria. Like catalase, the specific activity of malate dehydrogenase in the peroxisomes was high.

Discussion

Peroxisomes containing glycolate oxidase, glyoxylate reductase, catalase, and malate dehydrogenase were isolated in good yields from 5 of the 10 plant species analyzed. Spinach and sunflower leaves were the best plants so far examined from which to isolate peroxisomes, and about half of these enzymatic activities remained with the peroxisomal particles after grinding in 0.5 M sucrose. Only small to trace amounts of peroxisomes, as indicated by total enzymatic activity for glycolate oxidation, were isolated from the other 5 species. As discussed later, the low yield of peroxisomes from some of these species may be due to the fragility or loss of protein from these particles under the isolation techniques used. The results establish that peroxisomes are widely distributed in plant leaves.

Our data support the concept that plant species with relatively high levels of glycolate pathway enzymes are obligate photorespiring plants, with the exception of pigweed. Of the plant species which have been stated to exhibit photorespiration (references in table I), all contained a relatively high but variable level (500–1500 nmoles × min^{-1} × g^{-1} wet weight) of glycolate oxidase. Swiss chard might be included in this group, but no published literature was found with respect to its photorespiration. The data from corn and sugarcane also supports this view as they have but 2 to 5 % of the glycolate oxidase and glyoxylate reductase activity of plants that exhibit photorespiration. Both of these species do not photorespire. Pigweed is an exception as it does not exhibit photorespiration (24), yet its leaves had 25 to 50 % of the level (266 nmoles min^{-1} × g^{-1} wet weight) of glycolate oxidase as had photorespiring plants such as spinach, bean, and wheat. Even this specific activity value for pigweed is too low because of difficulty in filtering the leaf homogenate. Obviously, data from pigweed on the absence of photorespiration yet abundance of enzymes associated with glycolate metabolism necessitates continuing the search for a better understanding of photorespiration.

As a corollary, it can be proposed that *in vivo* all the glycolate pathway enzymes are functionally organized and exclusively contained in the peroxisomes. This proposition is based on the supposition that the stability of the peroxisomes is a function of the plant species and the isolation techniques. The isolation procedure used was developed for spinach and used without change for all other species. Electron micrograph observations have indicated that the membranes of the isolated peroxisomes or the peroxisomes themselves were nearly all broken. Thus, the peroxisome yields, as measured by enzymatic activity, are at best very minimal. It is easy to visualize that the observed relative distribution for glycolate oxidase between the supernate and the peroxisomes may not represent the actual *in vivo* distribution. Thus, the results may be considered quantitative for the amount of total enzyme present in each plant, but only indicative of the presence of peroxisomes.

The grouping of plants by peroxisome stability used in the results should not be considered as any type of botanical classification. The results emphasize the great range of total activity for glycolate oxidation and variability in the amount and stability of the peroxisomes. If more plants were surveyed, other groups may become evident. Before any classification can be done on the basis of peroxisomes, it would be necessary to work out isolation procedures for maximum yield of active peroxisomes from each plant. The magnitude of this task is suggested by the fact that procedures to isolate active whole chloroplasts from many different plants is still not available. It is interesting to note that spinach provides a good yield of both chloroplasts and peroxisomes. At best, our present results indicated the diversity that exists among plants for glycolate oxidation. In no case were peroxisomes entirely absent, and it is possible that the enzymes of the glycolate pathway are present in peroxisomes in all plants. Plants such as bean and Swiss chard, which had much total glycolate oxidase, yielded no more peroxisomes than corn or sugarcane. For bean and Swiss chard, particular effort should be made to improve the isolation procedure for the peroxisomes.

The function of the peroxisomes and its relationship to photosynthetic efficiency is of interest. These data prove that photosynthesis and photorespiration, as measured by glycolate oxidation, occur in distinct and separate entities in the cell. The data obtained with those plant species that have photorespiration indicates a great diversity in the level of glycolate pathway enzymes (peroxisomes). This enzymatic data indicates that there should be a wide variation in the magnitude of photorespiration among plants. Such has been the case for compensation point analyses. However, any attempt to correlate the compensation point as CO_2 production with the amount of glycolate oxidase activity in different leaves would also have to consider the efficiency of CO_2 fixation.

Although corn and sugarcane leaves do not exhibit photorespiration, the presence of 2 to 5 % as much glycolate oxidase, as in sunflower or spinach, is still a substantial amount of activity which could catalyze a rapid flow of carbon through the glycolate pathway. Homogenates of sugarcane leaves oxidized glycolate at the rate of 48 nmoles $\times$ min^{-1} $\times$ g^{-1} wet weight of tissue or 3 nmoles $\times$ min^{-1} $\times$ mg^{-1} protein. Previous results cited in the introduction for glycolate-^{14}C metabolism by corn leaves can be accommodated by this level of enzyme activity. The complete absence of photorespiration in corn and sugarcane and the failure to release $^{14}CO_2$ during glycolate-^{14}C metabolism in the light cannot be due to the absence of these enzymes. The absence of photorespiration in these leaves may be accounted for by a combination of a lower rate of glycolate metabolism and the highly efficient CO_2 fixation process catalyzed by P-enolpyruvate carboxylase (7, 8, 17).

We would interpret our data to indicate that peroxisomes and chloroplasts are separate entities. It seems unlikely that the peroxisomes are in whole chloroplasts and break out of the chloroplasts during centrifugation as do starch grains. In all the plants tested, the ratio of amount of chlorophyll or protein to amount of glycolate oxidase or glyoxylate reductase in the "whole" chloroplast fraction was small compared to the ratio in the "broken" chloroplasts. If the peroxisomes were inside the chloroplasts, this ratio ought to be largest for the whole chloroplasts. However, a large part of the starch grains from inside the chloroplasts are pelleted by themselves, even by centrifugation at a low gravitation field to remove whole chloroplasts. Thus, the more dense bodies readily escape from chloroplasts during isolation, and the peroxisomes become much denser than the chloroplasts in a sucrose solution.

In previous discussions about the enzymes of the glycolate pathway (15, 18), catalase usually was not included because of the assumption that the universal presence of catalase would easily dispense with H_2O_2 produced by glycolate oxidase. In this survey, a similar distribution pattern between peroxisomes and supernate for all the plants was found for glycolate oxidase, glyoxylate reductase, and catalase. This data emphasizes that these 3 enzymes appear to be closely associated in the same organelle. The possibility of several catalases in the cell is not excluded, but little catalase activity was found with the mitochondria or chloroplasts from sunflower or spinach leaves. Soluble cytoplasmic catalase may also not exist because, in plants with the most stable peroxisomes, catalase activity in the supernate of the original leaf homogenate was no greater on a percent basis than glycolate oxidase.

Literature Cited

1. DE DUVE, C. AND P. BAUDHUIN. 1966. Peroxisomes (microbodies and related particles). Physiol. Rev. 46: 323–57.
2. DOWNTON, W. J. S. AND E. B. TREGUNNA. 1968. Photorespiration and glyoxylate metabolism: A re-examination and correlation of some previous studies. Plant Physiol. 43: 923–29.

El-Sharkawy, M. A., R. S. Loomis, and W. A. Williams. 1967. Apparent reassimilation of respiratory carbon dioxide by different species. Physiol. Plantarum 20: 171–86.

Forrester, M. L., G. Krotkov, and C. D. Nelson. 1966. Effect of oxygen in photosynthesis, photorespiration, and respiration in detached leaves. I. Soybean. Plant Physiol. 41: 422–27.

Forrester, M. L., G. Krotkov, and C. D. Nelson. 1966. Effect of oxygen on photosynthesis, photorespiration and respiration in detached leaves. II. Corn and other monocotyledons. Plant Physiol. 41: 428–31.

Goldsworthy, A. 1966. Experiments on the origin of CO$_2$ released by tobacco leaf segments in the light. Phytochemistry 5: 1013–19.

Hatch, M. D. and C. R. Slack. 1966. Photosynthesis by sugar cane leaves. A new carboxylation reaction and the pathway of sugar formation. Biochem. J. 101: 93–111.

Hatch, M. D., C. R. Slack, and H. S. Johnson. 1967. Further studies on a new pathway of photosynthetic carbon dioxide fixation in sugar cane and its occurrence in other plant species. Biochem. J. 102: 417–22.

Jolliffe, P. A. and E. B. Tregunna. 1968. Effect of temperature, CO$_2$ concentration, and light intensity on oxygen inhibition of photosynthesis in wheat leaves. Plant Physiol. 43: 902–06.

Noll, C. R., Jr. and R. H. Burris. 1953. Nature and distribution of glycolic acid oxidase in plants. Plant Physiol. 29: 261–65.

Moss, D. N. 1966. Respiration of leaves in light and darkness. Crop Science 6: 351–54.

Moss, D. N. 1967. High activity of the glycolic acid oxidase system in tobacco leaves. Plant Physiol. 42: 1463–64.

Moss, D. N. 1968. Photorespiration and glycolate metabolism in tobacco leaves. Crop Science 8: 71–76.

Pierpont, W. S. 1962. Mitochondrial preparations from the leaves of tobacco. IV. Separation of some components by density gradient centrifugation. Biochem. J. 82: 143–48.

Rabson, R., N. E. Tolbert, and P. C. Kearney. 1962. Formation of serine and glyceric acid by the glycolate pathway. Arch. Biochem. Biophys. 98: 154–63.

16. Rocha, V., S. K. Mukerji, and I. P. Ting. 1968. Chloroplast-malic dehydrogenase: A new malic dehydrogenase isozyme from spinach. Biochem. Biophys. Res. Commun. 6: 890–94.

17. Slack, C. R. and M. D. Hatch. 1967. Comparative studies in the activity of carboxylases and other enzymes in relation to the new pathway of photosynthetic carbon dioxide fixation in tropical grasses. Biochem. J. 103: 660–65.

18. Tolbert, N. E. 1963. Glycolate pathway. In photosynthetic mechanisms in green plants. NSF-NRC publication 1145. p 648–62.

19. Tolbert, N. E. and R. H. Burris. 1950. Light activation of the plant enzyme which oxidizes glycolic acid. J. Biol. Chem. 186: 791–804.

20. Tolbert, N. E. and M. S. Cohen. 1953. Activation of glycolic acid oxidase in plants. J. Biol. Chem. 204: 639–48.

21. Tolbert, N. E., A. Oeser, T. Kisaki, R. H. Hageman, and R. K. Yamazaki. 1968. Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. J. Biol. Chem. 243: 5179–84.

22. Tregunna, E. B. 1966. Flavin mononucleotide control of glycolic acid oxidase and photorespiration in corn leaves. Science 151: 1239–41.

23. Tregunna, E. B., G. Krotkov, and C. D. Nelson. 1966. Effect of oxygen on the rate of photorespiration in detached tobacco leaves. Physiol. Plantarum 19: 723–33.

24. Tregunna, E. B. and J. Downton. 1967. Carbon dioxide compensation in members of the *Amaranthaceae* and some related families. Can. J. Botany 45: 2385–87.

25. Zelitch, I. 1955. The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. J. Biol. Chem. 216: 553–75.

26. Zelitch, I. 1958. The role of glycolic acid oxidase in the respiration of leaves. J. Biol. Chem. 233: 1299–1303.

27. Zelitch, I. 1966. Increased rate of net photosynthetic carbon dioxide uptake caused by the inhibition of glycolate oxidase. Plant Physiol. 41: 1623–31.

28. Zelitch, I. and S. Ochoa. 1953. Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. J. Biol. Chem. 201: 707–18.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 243, No. 19, Issue of October 10, pp. 5179-5184, 1968
Printed in U.S.A.

Peroxisomes from Spinach Leaves Containing Enzymes Related to Glycolate Metabolism*

(Received for publication, February 26, 1968)

N. E. TOLBERT,‡ A. OESER, T. KISAKI, R. H. HAGEMAN, AND R. K. YAMAZAKI
From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

SUMMARY

Microbodies, designated as peroxisomes because of their enzyme complement, have been isolated from spinach leaves. After grinding leaves in 0.5 M sucrose, the peroxisomes were removed with the broken chloroplast fraction by differential centrifugation. During sucrose density gradient centrifugation, the peroxisomes banded in about 1.9 M sucrose and were separated from mitochondria and chloroplasts. The particles, 0.5 to 1.0 μ in diameter, contained a dense granular stroma surrounded by a single membrane.

The leaf peroxisomes contained glycolate oxidase, DPNH-glyoxylate reductase, and catalase. Up to 55% of the activity for these enzymes in spinach leaves have been found in the particulate fractions after the initial centrifugation. The leaf peroxisomes are probably the site of oxygen uptake during photorespiration. No catalase activity was present in chloroplasts after removal of the peroxisomes by density gradient centrifugation. P-Glycolate phosphatase, TPNH-glyoxylate reductase, D-amino acid oxidase, urate oxidase, and peroxidase were not present in leaf peroxisomes.

Glycolate oxidase was first isolated from clarified extracts of tobacco leaves (1, 2). Subsequent investigations (3, 4) supported the concept that the oxidase, as well as DPNH-glyoxylate reductase (5) were soluble cytoplasmic enzymes. Chloroplast (6, 7) and mitochondrial fractions (8, 9) have been reported to contain only a small portion of the total glycolate oxidase activity. Upon examination by sucrose gradient centrifugation, Pierpoint (10) and, most recently, Thompson and Whittingham (11) have concluded that there was no glycolate oxidase in the particulate fractions. A reinvestigation of the intracellular localization of glycolate oxidase in leaves was prompted by our own observations of large amounts of glycolate oxidase in broken chloroplast preparations.

De Duve and Baudhuin (12) have designated the microbodies from liver and kidney, which contain α-hydroxy acid oxidase and catalase, as peroxisomes. The metabolic function of peroxisomes is unknown. Particles of unknown composition have been reported in plants and designated as cytosomes (13), but there are no reports of particles from leaves with enzymic activity characteristic of peroxisomes. Particles containing enzymes of the glyoxylate cycle have been isolated from germinating caster bean cotyledons (14) and from *Tetrahymena* (15). A similarity between these microbodies and peroxisomes is suggested by the fact that microbodies from *Tetrahymena* contain enzymes which are also characteristic of liver and kidney peroxisomes (16).

EXPERIMENTAL PROCEDURE

Preparation of Fractions—Spinach (*Spinacia oleracea* L.) leaves were purchased locally. Leaves have been stored at 4° for up to 1 month before use. The chilled leaves were washed and deribbed, and then 40 g of tissue were chopped into small segments before grinding at maximum speed for 10 sec in a Waring Blendor with 80 ml of grinding medium. All work was done at about 4°. The standard grinding medium was 0.5 M sucrose in 0.02 M glycylglycine, pH 7.5. The homogenate was hand squeezed through six layers of cheesecloth, and the pH of the sap immediately readjusted to 7.5. Particles from 70 ml of this sap were then precipitated by differential centrifugation at 0° for 20 min at each step. The first pellet (120 × *g*) contained mostly whole chloroplasts, starch grains, and large cell fragments. The second pellet (3,000 × *g*) was designated as "broken chloroplasts," although it also contained some mitochondria and a large part of the peroxisomes. The third pellet (35,000 × *g*), designated as "mitochondria" contained the remaining microbodies and broken chloroplasts. The remaining solution was designated "supernatant." Each pellet was resuspended in grinding medium by stirring, and the final volume of about 4 ml for the first and second pellet and about 2 ml for the third pellet was recorded.

Sucrose Density Gradient Centrifugation—A noncontinuous sucrose density gradient of five layers was prepared at 4° by pipetting 4 ml of 2.5 M sucrose (85.5%), 10 ml of 2.0 M sucrose (68.4%), 10 ml of 1.8 M sucrose (61.6%), 10 ml of 1.5 M sucrose (51.3%), and 20 ml of 1.3 M sucrose (44.5%). All sucrose fractions contained 0.02 M glycylglycine at pH 7.5. After

---

* This research was supported in part by Grant GB 4151 from the National Science Foundation. This is journal article 4307 of the Michigan Agricultural Experiment Station. Parts of this work were abstracted in the *Fed. Proc.*, 27, 34 (1968).

‡ To whom all inquiries should be addressed.

TABLE I

Sucrose density gradient centrifugation

Experimentally, fractions were numbered as removed from the bottom of the gradient.

Fraction	Sucrose	Volume	Type of particles in band
	M	*ml*	
9	0.5	4.3	Supernatant
8	1.3	8.6	Broken chloroplasts
7	1.3–1.5	11.9	Broken chloroplasts
6	1.5	5.4	None
5	1.5–1.8	3.8	Whole chloroplasts; mitochondria
4	1.8	6.4	None
3	1.8–2.0	4.0	Peroxisomes
2	2.0	7.6	None
1	2.5	3.6	None

layering 2.0 to 2.5 ml of the appropriate pellet fraction on top of the gradient, the samples were centrifuged for 3 hours at 4° at 25,000 rpm (44,700 $\times g$ to 106,900 $\times g$) in a swinging bucket rotor SW 25.2 of the Spinco centrifuge, model L. Samples of 3 to 12 ml were removed from the bottom of the centrifuge tube through a needle. The approximate location of the various particles after development of the gradient are illustrated in Table I.

Electron Microscopy—The peak fraction containing the peroxisomes was withdrawn from the gradient and fixed for 1 hour in 3.0% glutaraldehyde. The sucrose concentration was then lowered to 1 M and the particles were pelleted. The sediment was rinsed in 0.5 M sucrose buffered at pH 7.5 with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and then postfixed for 2 hours with 1% OsO_4. All fixation steps were carried out in the cold. The pellet was then dehydrated in a graded series of ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate.

Glycolate Oxidase—Glycolate-O_2 oxidoreductase (EC 1.1.3.1) was assayed anaerobically by 2,6-dichloroindophenol reduction (4). Additions were made to a 3-ml Thunberg Beckman cuvette (10 mm in diameter) in the following order: 2 ml of 0.3 M pyrophosphate, pH 8.3, containing 1.5×10^{-4} M dichloroindophenol; 0.05 ml of 0.1 M KCN in 0.01 M NH_4OH (final concentration of KCN, 2×10^{-3} M), at times 0.1 ml of 2×10^{-3} M FMN (final concentration, 0.8×10^{-4} M); water so that the final volume with enzyme would be 2.5 ml; in the side arm, 0.1 ml of 0.125 M sodium glycolate (final concentration, 5×10^{-3} M). Between 0.05 to 0.2 ml of enzyme was also placed in the cuvette before it was evacuated and flushed three times with N_2 which had passed through Fieser's solution to remove traces of O_2. Dye reduction at 25° was measured at 600 mμ by an automatic recording Gilford spectrophotometer. A unit of activity was expressed as 1 O.D. change per min, which was equivalent to 4.78 nmoles of reduced dye. Pyrophosphate buffer with dichloroindophenol should be prepared fresh weekly to avoid an initial lag in the assay.

Since the soluble glycolate oxidase is a FMN protein, FMN was used in the assays. Aerobic assays were unreliable since the H_2O_2 generated by the glycolate oxidase could be used by contaminating peroxidases to oxidize any reduced dichloroindophenol which was generated. Competing reactions then occurred: H_2O_2 production, dichloroindophenol reduction, and peroxidase oxidation of the reduced dye. Inhibition of peroxidase by high concentrations of KCN, as originally recommended (4) for an aerobic assay, did not give maximum rates. Peroxidases were so active in sap and supernatant that no aerobic reduction of dichloroindophenol could be detected if KCN were omitted. Consequently, anaerobic assays were the only reliable method, and KCN was left in the assay to ensure against peroxidase activity in case of incomplete anaerobiosis.

Cytochrome c Oxidase—From 5 to 25 μl of enzyme was pipetted into a bottom corner of a 0.3-ml spectrophotometer cuvette (diameter, 10 mm) and 5 μl of 4.0% digitonin were added, mixed, and allowed to stand for 1 min. Then 200 μl of 0.1 M phosphate buffer (pH 7.0) and 50 μl of 1.5 mM cytochrome *c* reduced with dithionite were added successively and mixed (17). Readings of optical density at 550 mμ were obtained with the Gilford recording spectrophotometer. The first order rate constant for the disappearance of reduced cytochrome *c* was calculated according to the method of Smith (18).

Other Enzymes—Glyoxylate reductases were assayed by the rate of oxidation of either DPNH or TPNH in the presence of 0.005 M substrate (19, 20). Catalase was assayed by the disappearance of H_2O_2 as measured spectrophotometrically at 240 mμ (21). A unit of activity was a change of 1 O.D. in 1 min, and based on the extinction coefficient was equal to 2.76 μmoles of H_2O_2. Peroxidase was assayed by the method of Gregory which was based upon the length of time to oxidize a standard amount of ascorbic acid (22). The assay for P-glycolate phosphatase has been described (23).

Protein and Chlorophyll—Protein was determined by the Lowry procedure. Although the green color in aliquots from the chloroplast fractions interfered, the recovery of protein in the various fractions indicated that the method gave a valid estimate of protein content. Chlorophyll was determined by its absorption at 652 mμ (24). Aliquots from 1 μl to 1 ml were diluted to 5 ml with water and acetone to make a final concentration of 80% acetone. They were allowed to stand in the dark at 0° with occasional stirring for several hours to completely solubilize the chlorophyll. In samples with high sucrose concentration, the acetone extracted so much of the water that the sucrose and chlorophyll formed a second phase. Since sucrose is more soluble in cold acetone, it was necessary to let these samples stand overnight at −18° in order to obtain effective chlorophyll extraction. Samples were centrifuged or filtered before reading the extinction at room temperatures.

RESULTS

Distribution of Glycolate Oxidase among Particles—Spinach leaves were ground in the sucrose grinding medium and separated by centrifugation into whole chloroplasts, broken chloroplasts, mitochondria, and supernatant fractions as described under "Experimental Procedure." The distribution of certain enzymes among these fractions is shown in Table II. In this type of experiment, the broken chloroplast fraction contained 29 to 34% of the total oxidase activity and had the highest specific activity. About 40 to 55% of all the oxidase activity was in the total particle fractions. Most of our investigations have been done with the broken chloroplast fraction, because glycolate oxidase from spinach leaves was greatest in this fraction on the basis of amount and specific activity. Removal of the whole chloroplast fraction was beneficial for reducing the load put on the subsequent sucrose gradient.

The broken chloroplast and mitochondrial fractions were subjected separately to sucrose density gradient centrifugation

TABLE II

Distribution of enzymes among fractions obtained by differential centrifugation

Data, from a typical experiment, are designated as Experiment A. The percentage of distribution from a second experiment, B, is shown to illustrate the maximum activity found in the pellet fractions. Specific activity is expressed as micromoles per min per mg of protein.

Fraction	Total protein	Glycolate oxidase				Catalase				DPNH-glyoxylate reductase		
			Specific activity	Distribution			Specific activity	Distribution			Specific activity	Distribution
				Experiment A	Experiment B			Experiment A	Experiment B			
	mg	*μmoles/min*		%	%	*μmoles/min*		%	%	*μmoles/min*		%
Whole chloroplasts	360	5.5	0.015	5.5	5.6	34,500	96	5.9	6.6	7.7	0.023	6.8
Broken chloroplasts	618	28.9	0.047	28.9	34.5	184,800	299	31.3	30.7	17.6	0.033	15.3
Mitochondria	208	5.2	0.025	5.2	15.0	29,700	143	5.0	15.5	2.6	0.022	2.2
Supernatant	2,730	60.5	0.022	60.4	44.9	341,000	125	57.8	47.3	86.4	0.026	75.7

TABLE III

Distribution of enzymes upon sucrose density gradient centrifugation of broken chloroplast fraction

Fractions were numbered in the order in which they were drained from the bottom of the centrifuge tube. Fraction 1 was in the bottom and 9 is the top supernatant. For approximate sucrose molarity see Fig. 1. The broken chloroplasts were from Experiment A of Table II. Specific activity is expressed as micromoles per min per mg of protein.

Fraction	Total protein	Glycolate oxidase			Catalase			DPNH-glyoxylate reductase		
			Specific activity			Specific activity			Specific activity	
	mg	*μmoles/min*		%	*μmoles/min*		%	*μmoles/min*		%
9	247.2	3.39	0.014	23.9	27,200	110	24.4	3.74	0.015	21.0
8	109.0	0.77	0.007	5.4	5,310	49	4.8	0.83	0.008	4.6
7	35.8	0.47	0.013	3.3	3,680	102	3.3	1.05	0.029	5.9
6	14.5	0.31	0.021	2.2	1,570	107	1.4	0.43	0.029	2.3
5	22.5	0.78	0.035	5.5	43	184	3.7	0.81	0.036	4.7
4	9.6	1.80	0.190	12.7	11,450	1,190	10.3	2.48	0.259	13.9
3	5.5	5.07	0.920	35.8	37,400	6,783	33.6	6.57	1.191	36.8
2	4.1	1.53	0.370	10.8	19,300	4,689	17.3	1.96	0.478	11.0
1	2.0	0.07	0.030	0.5	130	68	1.2	0	0	0

(Table III and Fig. 1). The glycolate oxidase activity was found in a band (No. 3) sedimenting in about 1.9 M sucrose. Activity, also present at the top of the gradient (No. 9), was attributed to soluble protein from the supernatant and from broken particles. The specific activity of the oxidase in the particles was about 92-fold greater than the specific activity in the top fraction. The oxidase activity in the 1.9 M sucrose band was distinctly separated from both the chloroplast bands, as indicated by chlorophyll analyses, and from the mitochondrial fraction, as indicated by cytochrome *c* oxidase activity (Fig. 1).

The particulate fraction containing the plant glycolate oxidase has been designated as peroxisomes after the terminology of De Duve and Baudhuin (12). These plant peroxisomes sedimented similarly in the sucrose gradient and contained similar types of enzymes as those found in peroxisomes from liver or kidney.

The α-hydroxy acid oxidase activity of the particles has been designated glycolate oxidase because it was more active with glycolate than lactate or α-hydroxybutyrate. With sufficient substrate for maximum activity, the relative rates of glycolate, lactate, and α-hydroxybutyrate oxidation at pH 8.3 by the peroxisomes were about 100:40:30. Since this activity ratio with the three substrates was the same in each fraction from the sucrose gradient, it is assumed that their oxidation was catalyzed by the same enzyme. This ratio of activity is similar to that reported earlier for the soluble spinach glycolate oxidase (25), but the oxidase from tobacco leaves is much more specific for glycolate than for α-hydroxybutyrate (1). Peroxisomes from rat liver have a similar specificity for glycolate, the shortest carbon chain of the α-hydroxy acid series, while peroxisomes from rat kidney contained an α-hydroxy acid oxidase which oxidizes the longer chain substrates (α-hydroxybutyrate) faster than glycolate (12). The reason for these substrate differences is not apparent.

The prosthetic group of glycolate oxidase, as prepared from soluble extracts of spinach leaves, is FMN (3, 4). Addition of excess (10^{-4} M) FMN generally increases the activity of glycolate oxidase in crude extracts or a fraction precipitated by ammonium sulfate. Similarly, the oxidation of glycolate by peroxisomes generally was stimulated as much as 50% by added FMN.

Catalase—Catalase activity had nearly the identical distribution among the particulate fractions as did glycolate oxidase (Table II and Fig. 1). About 40 to 50% of the catalase activity was in the particulate fractions. Upon subsequent sucrose density gradient centrifugation of the broken chloroplast or mitochondria fraction, about 70% of the activity in these fractions was present in the peroxisome particles. The presence of glycolate oxidase and catalase together in leaf peroxisomes is consistent with data on liver peroxisomes and with the concept

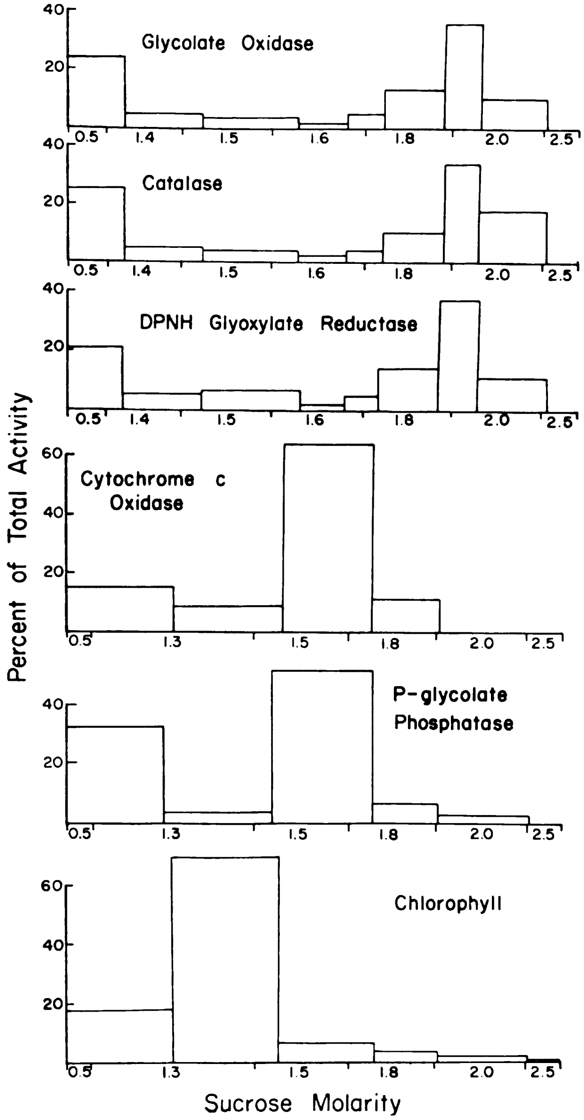


FIG. 1. Distribution of enzymic activities from broken chloroplast fraction of spinach leaves after sucrose gradient centrifugation. The range of sucrose molarity is proportional to the volume of each molarity used to prepare the gradient. The width of the columns is proportional to the volume of the fraction removed from the gradient for assay.

that the catalase is present to destroy the H_2O_2 formed by the FMN oxidase. Total catalase activity was at least 10^3 times greater than the activity of glycolate oxidase or glyoxylate reductase (Table III).

Catalase has often been recorded as present in chloroplasts and in cytoplasm. Little catalase activity was present in the chloroplast fractions from the sucrose gradient, but rather catalase was associated with the peroxisome fraction (Fig. 1). Since the bulk of the peroxisomes was removed initially from the sap in the broken chloroplast fraction, previous reports on chloroplast catalase could be due to peroxisome particles in the chloroplast preparations. If so, the unknown role for catalase in the photosynthetic apparatus need not be of concern. While isolating plant mitochondria by sucrose density gradient centrifugation, Plesnicar, Bonner, and Storey observed, but did not comment on, catalase activity at a location on the gradient, which would be characteristic of peroxisomes (see Table III of Reference 26).

DPNH-Glyoxylate Reductase—This enzyme was located in the peroxisomes similarly to glycolate oxidase and catalase (Tables II, III, and Fig. 1). On the basis of total or specific activity in the peroxisomes (Table III), glyoxylate reductase activity was the same order of magnitude as glycolate oxidase. Glyoxylate reductase has not been reported to be a constituent of liver peroxisomes (12), and Zelitch, when isolating this enzyme from plants, believed that it was a cytoplasmic component (5).

Enzymes Not Detected in Peroxisomes—The leaf peroxisomes were tested for other enzymes associated with the glycolate pathway (27), or with liver peroxisomes (12). D-Amino acid oxidase and urate oxidase, which have been found in liver peroxisomes, were absent in leaf peroxisomes. P-Glycolate phosphatase and TPNH-glyoxylate reductase were absent in the peroxisomes, but some activity for each was associated with the whole chloroplasts. The activity of P-glycolate phosphatase was found on the sucrose gradient in the same area as cytochrome *c* oxidase. This area contained the mitochondria as well as a small band of whole chloroplasts. The broken chloroplast bands containing the bulk of the chlorophyll did not contain P-glycolate phosphatase.

Peroxidases are extremely active and abundant in leaves. Both chloroplast and mitochondria fractions contained some peroxidase activity; however, the bulk of this activity was found in the supernatant. When the particulate fractions were further separated on sucrose gradient, no peroxidase activity by the ascorbate assay was detected in the peroxisome fraction. The catalase activity of the peroxisomes did not show peroxidase activity by the assay employed. Recently, Plesnicar *et al.* (26) with the use of sucrose gradient centrifugation, also concluded that peroxidase from mung bean hypocotyls was mostly soluble, although some activity was apparently located in microbodies.

Peroxisome Morphology—An electron micrograph of spinach leaf peroxisomes is shown in Fig. 2. The particles are characterized as containing a dense granular stroma surrounded by a single membrane. In some cases, a denser area was visible within the particles. The shape of the peroxisomes varied, but most often they appeared spherical and about 0.5 to 1.0 μ in diameter. The fact that the membranes of the particles were often broken is attributed to shearing forces as they passed through the discontinuous sucrose gradient. Thus, it was not surprising that a portion of the enzymic activity attributed to the peroxisomes was found at the top of the gradients. The preparation also contained chromatin material, broken chloroplasts, and other particles, presumably mitochondria. The amount of chlorophyll and cytochrome *c* oxidase (Fig. 1) parallels these visual observations.

Grinding Medium and Particle Stability—The grinding medium of 0.5 M sucrose in 0.02 M glycylglycine at pH 7.5 has been changed, but so far no variation has altered the primary conclusions or improved significantly the yield of peroxisomes. Without sucrose to prevent osmotic shock, much more of the glycolate oxidase activity was in the supernatant fraction, suggesting that the particles were labile. This also provides an explanation for previous literature citations that glycolate oxidase was a soluble enzyme. Substitution of NaCl or mannitol in the grinding medium for sucrose resulted in poorer recovery of peroxisomes, and shifted their location on the sucrose gradient to 1.8 M sucrose as if they were somewhat lighter in weight. Substitution of Carbowax 4000 for sucrose or addition of polyvinylpyrrolidone with sucrose was not beneficial.

The final concentration of sucrose in the leaf homogenate after grinding was lowered by the dilution from the leaf sap, and an unknown amount of peroxisome destruction may have occurred. Best results were obtained when the particles were removed as rapidly as possible by centrifugation and further separated on the sucrose gradient. If the leaf homogenate or the resuspended broken chloroplasts stood at 0° for several hours before the sucrose gradient centrifugation, a large part of the enzyme activity was found in the supernatant or top fraction. Stability of the particles was estimated by resuspending them in media with different concentrations of sucrose, and then removing them again at different time periods (Fig. 3). If resuspended in buffer without sucrose, 60% of the activity was immediately found in the supernatant, and it is assumed that the osmotic shock ruptured the particles. When resuspended in 0.5 M sucrose, the peroxisomes appear stable for 2 hours and in 1.0 M sucrose, most of the glycolate oxidase activity remained with the microbody pellet for about 8 hours. Enzyme activity lost from the pellet fraction was found in the supernatant or suspending medium. We do not understand why a constant level of about 40% of the glycolate oxidase activity remained in the microbody fraction even after 21 hours in buffer without sucrose.

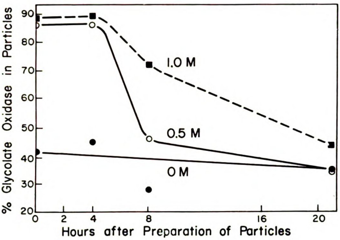


Fig. 3. Stability of peroxisomes in grinding media of different sucrose concentration. Equal aliquots of the chloroplast fraction were sedimented by centrifugation from sap prepared in the standard grinding medium of 0.5 M sucrose and buffer. The broken chloroplast fractions were resuspended and stored in different grinding media which contained 0, 0.5, and 1.0 M sucrose and buffer. At designated times, aliquots of these suspensions were recentrifuged at 39,000 × g for 20 min before measuring glycolate oxidase activity in the pellet or peroxisomes and in the soluble supernatant grinding media. For assay purposes, the particles were resuspended in the corresponding grinding medium in which they had been stored. Both the supernatant and the resuspended microbodies were assayed for glycolate oxidase with added FMN and KCN. There was little decrease in total activity during the experiment; decrease in activity in the microbody fraction was balanced by a corresponding increase in activity of the supernatant.

Mitochondria Fraction—The designation, mitochondria or broken chloroplasts fraction, was arbitrary, as the initial centrifugal technique did not clearly separate these fractions. In the examples shown in Table II, 5 to 15% of the total enzymic activity associated with the peroxisome was initially separated with the mitochondria fraction. The mitochondria fraction also contained broken chloroplasts (about 20% of the total chlorophyll). The fraction designated as broken chloroplast also contained cytochrome *c* oxidase activity, which is attributed to mitochondria. Thus, the initial separation of the particles into fractions, designated as whole chloroplasts, broken chloroplasts, and mitochondria, as has generally been done, is arbitrary and for convenience. When using sucrose gradient centrifugation, the initial particle separation would not be necessary except to prevent overloading of the gradient.

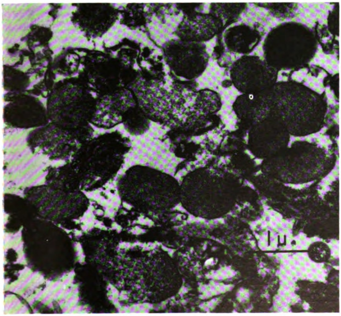


Fig. 2. Electron micrograph of a peroxisome-rich fraction. × 26,000.

When the mitochondria fraction was sedimented in the sucrose gradient, the peroxisomes also banded in 1.9 M sucrose as they did from the broken chloroplast fraction. There was no glycolate oxidase, catalase, or DPNH-glyoxylate reductase in the 1.5 M sucrose region of the gradient where mitochondria were located, as indicated by cytochrome *c* oxidase activity. Previous reports have indicated that a particulate fraction typical of plant mitochondria contained part of the leaf glycolate oxidase activity (8, 28). However, the investigators were unable to couple glycolate oxidation to phosphorylation. The presence of peroxisomes in these mitochondria preparations undoubtedly accounted for the glycolate oxidase activity.

DISCUSSION

The significance and function of peroxisomes in liver and kidney are unknown, but have been carefully considered by De Duve and Baudhuin (12). One possibility has been that these microbodies function as a site to dispose of cell excesses by the combined action of oxidases and catalase. This description of peroxisomes from spinach leaves establishes a wider distribution for these microbodies. A current survey indicated that peroxisomes are present in many different plants, although spinach has so far yielded the highest percentage of the glycolate oxidase activity in peroxisomes.[1]

In considering the function of leaf peroxisomes, two facets of the photosynthetic system provide clues which could not be studied with the liver and kidney. Glycolate is a major and universal product of CO_2 photosynthesis (for review see Reference 27). In the light, many leaves have an enhanced rate of respiration, which is called photorespiration and is attributed to glycolate oxidation (29–32). Thus, in contrast to the liver and kidney, in the leaf a major substrate for the peroxisomes is known. Further, in the leaf, photorespiration may be attributable to the peroxisomes, and photorespiration can be differentiated *in vivo* from mitochondria respiration. The present state of knowledge does not assign a clear function to the glycolate pathway and the peroxisomes. A working hypothesis is that during CO_2 photosynthesis, P-glycolate is synthesized by the chloroplasts and excreted as glycolate (27) by the aid of the specific P-glycolate phosphatase (25). Excess glycolate would be oxidized by the peroxisomes and account for photorespiration.

When glycolate-^{14}C has been fed to leaves, it is rapidly converted into sugars via glycine, serine, and glyceric acid, *i.e.* via the glycolate pathway (33). The glycolate-^{14}C was not converted into CO_2 and refixed photosynthetically. If all the initial oxidation of added glycolate-^{14}C were occurring in the peroxisomes, then the peroxisome function is not to destroy the glycolate by complete oxidation to CO_2.

H_2O_2 production from glycolate oxidase has been considered as a potential source of H_2O_2 for peroxidase metabolism. However, the absence of peroxidases in the peroxisomes suggest that glycolate oxidase and the peroxisomes do not function for synthesis or metabolism via peroxidases. The presence of 7000-fold more catalase activity in the peroxisomes than glycolate oxidase activity indicates that the H_2O_2 produced there is not used.

Zelitch has emphasized that the glycolate system could serve as a terminal oxidase for any pyridine nucleotide-linked dehydrogenase (5). Glyoxylate reductase would catalyze the oxidation of DPNH and the resynthesis of glycolate, which, in turn, would be reoxidized by oxygen as catalyzed by glycolate oxidase. The packaging of the DPNH-specific glyoxylate reductase in the peroxisomes, along with glycolate oxidase and catalase, is certainly convincing evidence that such a terminal oxidase system could function to dispose of excess DPNH. Oxidation of excess DPNH, then, is a possible function for peroxisomes.

In this paper, glycolate oxidase, glyoxylate reductase, and catalase are designated as constituents of the peroxisomes. Previously, these enzymes have been considered to be associated with the soluble fraction of the leaf homogenate. This error seems to have been caused by the fragility of the peroxisomes. Investigators isolating enzymes from leaves did not grind in 0.5 M sucrose, and even if they did so, when isolating chloroplasts, the peroxisomes were stable for only a few hours and were broken if the solutions were diluted. By use of techniques described here, it has been shown that 50% of the total glycolate oxidase in spinach leaves can be isolated in particulate fractions. We do not know whether the other half of the oxidase activity in the supernatant represents a soluble glycolate oxidase or whether it arose from fragmentation of the labile peroxisomes during their isolation.

Pierpoint (10) and Thompson and Whittingham (11), with the use of sucrose density gradient centrifugation to separate particles, concluded that glycolate oxidase activity was only in the soluble fraction from tobacco leaves. Pierpoint's data indicate that there was activity which moved into the sucrose gradient, but it was ignored. Thompson and Whittingham probably did not detect the peroxisomes because they ground the leaves in too dilute a solution of NaCl or sucrose and used only the whole chloroplast fraction. On the basis of the present work, glycolate oxidase can be found in peroxisome particles when a simple isolation procedure is followed.

Acknowledgments—Appreciation is expressed to G. Longo, C. Longo, and M. Jost of the Michigan State University-Atomic Energy Commission Plant Research Laboratory (AEC Contract AT (11-1)-1338) for the preparation and interpretation of the electron micrographs.

REFERENCES

1. Clagett, C. O., Tolbert, N. E., and Burris, R. H., *J. Biol. Chem.*, **178,** 977 (1949).
2. Tolbert, N. E., Clagett, C. O., and Burris, R. H., *J. Biol. Chem.*, **181,** 905 (1949).
3. Kenten, R. H., and Mann, P. J. G., *Biochem. J.*, **52,** 130 (1952).
4. Zelitch, I., and Ochoa, S., *J. Biol. Chem.*, **201,** 707 (1953).
5. Zelitch, I., *J. Biol. Chem.*, **216,** 553 (1955).
6. Tolbert, N. E., and Cohan, M. S., *J. Biol. Chem.*, **204,** 649 (1953).
7. Zelitch, I., and Barber, G. A., *Plant Physiol.*, **35,** 626 (1960).
8. Zelitch, I., and Barber, G. A., *Plant Physiol.*, **35,** 205 (1960).
9. Pierpoint, W. S., *Biochem. J.*, **75,** 511 (1960).
10. Pierpoint, W. S., *Biochem. J.*, **82,** 143 (1962).
11. Thompson, C. M., and Whittingham, C. P., *Biochim. Biophys. Acta*, **153,** 260 (1968).
12. De Duve, C., and Baudhuin, P., *Physiol. Rev.*, **46,** 323 (1966).
13. Mollenhauer, H. H., Morré, D. J., and Kelley, A. G., *Protoplasma*, **62,** 44 (1966).
14. Breidenbach, R. W., and Beevers, H., *Biochem. Biophys. Res. Commun.*, **27,** 462 (1967).
15. Hogg, J. F., and Kornberg, H. L., *Biochem. J.*, **86,** 462 (1963).
16. Muller, M., and Hogg, J. F., *Fed. Proc.*, **26,** 284 (1967).
17. Simon, E. W., *Biochem. J.*, **69,** 67 (1958).
18. Smith, Z., *Method. Biochem. Anal.*, **2,** 427 (1955).
19. Zelitch, I., *J. Biol. Chem.*, **201,** 719 (1953).
20. Zelitch, I., and Gotto, A. M., *Biochem. J.*, **84,** 541 (1962).
21. Luck, H., in H. U. Bergmeyer (Editor), *Methods of enzymatic analysis*, Academic Press, New York, 1965, p. 886.
22. Gregory, R. P. F., *Biochem. J.*, **101,** 582 (1966).
23. Anderson, D. E., and Tolbert, N. E., in W. A. Wood (Editor), *Methods in enzymology, Vol. IX*, Academic Press, New York, 1966, p. 646.
24. Arnon, D. I., *Plant Physiol.*, **24,** 1 (1942).
25. Richardson, K. E., and Tolbert, N. E., *J. Biol. Chem.*, **236,** 1280 (1961).
26. Plesnicar, M., Bonner, W. D., Jr., and Storey, B. T., *Plant Physiol.*, **42,** 366 (1967).
27. Tolbert, N. E., *Nat. Acad. Sci.—Nat. Res. Counc. Publication*, **1145,** 648 (1963).
28. Kuczmak, M., Ph.D. thesis, Michigan State University, 1961
29. Egle, K., and Fock, H., in T. W. Goodwin (Editor), *Biochemistry of chloroplasts, Vol. II*, Academic Press, New York, 1967, p. 79.
30. Goldsworthy, A., *Phytochemistry*, **5,** 1013 (1966).
31. Tregunna, E. B., Krotkov, G., and Nelson, C. D., *Can. J. Bot.*, **39,** 1045 (1961).
32. Zelitch, I., *Annu. Rev. Plant Physiol.*, **15,** 121 (1964).
33. Rabson, R., Tolbert, N. E., and Kearney, P. C., *Arch. Biochem. Biophys.*, **98,** 154 (1962).

---

[1] [illegible] unpublished data.