AMINOTRANSFERASES IN PEROXISOMES AND DISTRIBUTION OF PEROXISOMAL ENZYMES AMONG LEAF CELLS

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ABSTRACT

AMINOTRANSFERASES IN PEROXISOMES
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By

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Spinach leaf organelles were isolated by isopycnic centrifugation in sucrose gradients. The distribution of the organelles in the gradient was determined by measuring catalase for microbodies, cytochrome c oxidase for mitochondria and chlorophyll for chloroplasts. Four aminotransferase activities were assayed by spectrophotometric and radiochemical procedures. Serine:glyoxylate aminotransferase was found to be located exclusively in the peroxisomes. Most of the alanine:glyoxylate aminotransferase activity was also in the peroxisomes, but some activity which did not coincide with the mitochondria or broken chloroplast peaks was located at lower densities of sucrose. Aspartate aminotransferase activity was located in both the peroxisomes and mitochondria on the sucrose gradients. Although the broken chloroplasts on the sucrose gradients did not contain aspartate aminotransferase, whole chloroplasts, isolated by differential centrifugation, did contain this aminotransferase.

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The activities of serine:glyoxylate, alanine:glyoxylate and glutamate:glyoxylate aminotransferases from isolated peroxisomes were compared in various buffers at pH 7. Serine:glyoxylate aminotransferase was inhibited by phosphate buffer while the other two aminotransferase activities were unaffected. The phosphate inhibition of serine:glyoxylate aminotransferase is probably not of physiological significance since it was only 34% at 10 mM phosphate. Serine:glyoxylate aminotransferase was also inhibited by D-serine. In the presence of D-serine, the 1/velocity versus 1/[L-serine] plot was nonlinear.

The amino acid:glyoxylate aminotransferases of isolated peroxisomes were separated by ion exchange chromatography on triethylaminoethyl-cellulose columns. Serine:glyoxylate aminotransferase was eluted as one peak, but no activity of alanine:glyoxylate aminotransferase or glutamate:glyoxylate aminotransferase was detected. On isoelectric focusing columns, the serine:glyoxylate and glutamate:glyoxylate aminotransferases were separated. The alanine:glyoxylate aminotransferase activity had coincident peaks with each of the other two glyoxylate aminotransferases.

Serine:glyoxylate aminotransferase also catalyzed

a serine:pyruvate aminotrasferase reaction. Both of these activities peaked together during ion exchange chromatography and isoelectric focusing. Also, both the serine:glyoxylate and the serine:pyruvate aminotransferase activities were inhibited by D-serine and phosphate and both were equally sensitive to heat denaturation. At a serine concentration of 20 mM, the Km of the serine:glyoxylate aminotransferase for glyoxylate was 0.15 mM while for pyruvate it was 2.82 mM. At a concentration of 1 mM glyoxylate the Km for L-serine was 2.72.

The serine:glyoxylate aminotransferase was not inhibited by adenosine mono- di- and triphosphate, sodium nitrate, potassium nitrite, O-phospho-L-serine, D-glycerate or 3-phosphoglycerate. Ammonium sulfate caused some inhibition.

The serine:glyoxylate, glutamate:glyoxylate and alanine:glyoxylate aminotransferase reactions were not reversible. A very small glycine-glyoxylate exchange was detected with isolated peroxisomes.

Polyacrylamide disc gel electrophoresis of the aspartate aminotransferase from peroxisomes showed the presence of three isoenzymes. The chloroplasts and mitochondria each contained only one major band of

aspartate aminotransferase activity. Both the chloroplast and the mitochondrial enzymes had the same electrophoretic characteristics as the fastest moving isoenzyme from the peroxisomes. The three peroxisomal isoenzymes were separated both by ion exchange chromatography and by isoelectric focusing. None of the three aspartate aminotransferase isoenzymes appeared to coincide with the other aminotransferase activities of the peroxisomes.

In the spinach leaf peroxisomes, the specific activities of the aminotransferases were: glutamate: glyoxylate, 2.40 μmoles x min^{-1} x mg $protein^{-1}$; serine: glyoxylate, 1.54; alanine:glyoxylate, 0.87; and aspartate: α-ketoglutarate 0.15.

Peroxisomes and mitochondria were also isolated from rat liver and dog kidney tissues by isopycnic centrifugation in sucrose gradients. The mitochondria contained all of the particulate aspartate aminotransferase and the bulk of the particulate alanine:glyoxylate aminotransferase. However, the rat liver peroxisomes contained a significant amount of alanine:glyoxylate aminotransferase activity which could not be attributed to mitochondrial contamination. Serine:glyoxylate aminotransferase

appeared to be located only in the liver peroxisomes, and this activity was inhibited by phosphate. Nearly all of the glutamate:glyoxylate aminotransferase activity was in the soluble fraction. The dog kidney peroxisomes did not contain significant quantities of alanine:glyoxylate, glutamate:glyoxylate, serine:glyoxylate or aspartate: α-ketoglutarate aminotransferase activities. The major portion of these activities were in the mitochondria and the supernatant.

The distribution of the peroxisomal enzymes within the leaves of spinach, wheat, corn and sugarcane plants was determined. The peroxisomal enzymes, glycolate oxidase, hydroxypyruvate reductase and catalase were found to be located in both the bundle sheath cells and the mesophyll cells of corn and sugarcane. The bundle sheath enzymes were not completely extracted from the leaf tissue even after two minutes of homogenization in a Waring blendor whereas the mesophyll enzymes were extracted by this treatment. Breakage of the bundle sheath cells was accomplished with a mortar and pestle or a roller mill. The total activity of glycolate oxidase in corn and sugarcane, which do not lose carbon dioxide during photorespiration (without CO_2-photorespiration), was

one-third to one-half of that found in spinach and wheat which do exhibit CO_2-photorespiration. Both Atriplex patula, with CO_2-photorespiration and Atriplex rosea, without CO_2-photorespiration had similar levels of glycolate oxidase.

A differential grinding procedure, consisting of homogenization in a Waring blendor followed by grinding in a mortar, was used to obtain extracts of the mesophyll cells and the bundle sheath cells. The kinetic characteristics of glycolate oxidase and hydroxypyruvate reductase from the two types of cells were similar. Glycolate oxidase had a Km (glycolate) of 0.5 mM and hydroxypyruvate reductase had a Km (hydroxypyruvate) of 77 μM. Glycolate oxidase from corn did not reduce p-nitroblue tetrazolium in a phenazine methosulfate-nitroblue tetrazolium system whereas spinach glycolate oxidase readily reduced nitroblue tetrazolium in the same system. No isoenzymes of catalase or hydroxypyruvate reductase from corn leaf extracts were detected by electrophoresis.

The function of the peroxisomal aminotransferases in the operation and possible control of peroxisomal metabolism via the glycolate pathway is discussed. In addition, the levels of the glycolate pathway enzymes in

plant leaves is considered in relation to the process of photorespiration in plants.

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Dedicated to my wife, Claudette

and to our daughter, Shannon

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LIST OF ABBREVIATIONS

Bicine	N,N-Bis (2-hydroxyethyl) glycine
DCIP	2,6 Dichloroindophenol
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	2 (N-Morpholino) ethane sulfonic acid
NBT	p-Nithoblue tetrazolium
PMS	phenazine methosulfate
TEAE-	triethylaminoethyl-
Tris	tris (hydroxymethyl) aminomethane

INTRODUCTION

In the 1950's Calvin and coworkers (7, 26), elucidated the pathway of CO_2 fixation during photosynthesis and found the first product of CO_2 fixation to be 3-P-glycerate. The 3-P-glycerate was reduced to glyceraldehyde-3-phosphate, which was then converted to pentose and hexose, mono- and diphosphate esters and finally the CO_2 acceptor molecule, ribulose-1,5-diphosphate was regenerated. This reductive pentose pathway is considered to be the only photosynthetic pathway for net CO_2 fixation in higher plants and algae. During the past 5 years, other investigators such as Hatch and Slack (68, 70) have shown that many plants do not initially fix CO_2 into 3-P-glycerate, but that oxaloacetate, along with aspartate and malate are the first labeled products of $^{14}CO_2$ fixation. This system is called the C_4-dicarboxylic acid pathway or for brevity the C_4-pathway. This pathway appears to function for CO_2 trapping, storage and transport, but it does not result in net CO_2 fixation. Plants which possess a substantial amount of the C_4-pathway

activity are often called C_4-plants while plants with mainly the reductive pentose phosphate pathway are often called C_3-plants. However it must be kept in mind that even in C_4-plants, net CO_2-fixation occurs by the same process, namely the reductive photosynthetic carbon cycle or C_3-pathway.

During CO_2 fixation via the C_3-pathway considerable quantities of glycolate are formed (152). This glycolate is one of the early products of CO_2 fixation and is thought to come from one of the diphosphate esters in the reductive pentose pathway (152). The metabolism of glycolate has been mainly studied by Tolbert and coworkers (72, 80, 126). Glycolate is rapidly converted to glycine and serine and can be further metabolized to glycerate and on to sugars. This sequence of reactions from glycolate to sugars is known as the glycolate pathway and this pathway may carry as high as 50% of the total CO_2 fixed by the plant (176). Most of the enzymes of the glycolate pathway are located in a subcellular organelle called the peroxisome. The leaf peroxisomes which were first isolated from plants by Tolbert *et al.* (156) were similar to the rat liver peroxisomes previously described by de Duve and coworkers (42). The enzymes which have been found in the leaf peroxisomes include

catalase, glycolate oxidase, hydroxypyruvate reductase, malate dehydrogenase and at least four aminotransferase activities which are either associated with or are a part of the glycolate pathway (154).

During the operation of the glycolate pathway in leaf tissue, O_2 is consumed both during glycolate biosynthesis in the chloroplast and during its oxidation, by glycolate oxidase in the peroxisomes. Carbon dioxide is released in the conversion of glycine to serine in the mitochondria. This overall uptake of O_2 and formation of CO_2 in the light has been called photorespiration and the operation of the glycolate pathway will account for the total phenomenon (154). Photorespiration counteracts the net gain of photosynthesis in the plant and thus appears to be a wasteful process. It has been found that C_4-plants, which appear to grow faster than C_3-plants, do not exhibit photorespiration i.e., they do not lose CO_2 in the light (55). The C_4-plants have also been reported to contain lower levels of the glycolate pathway enzymes and thus may not be expected to show as much photorespiration (120, 157). However, Björkman and Gauhl (12) found that C_4-plants contained bundle sheath cells which did not readily break during homogenization, but if they were broken, they contained the enzymes of the

C_3-pathway of CO_2 fixation. Since the glycolate pathway and photorespiration were associated with the C_3-pathway, one might expect the glycolate pathway enzymes to be also located in the bundle sheath cells of C_4-plants.

The research reported in this thesis is divided into two chapters. Chapter I deals with the aminotransferase activities in peroxisomes of spinach leaves, dog kidney and rat liver. Of the four main aminotransferase activities in spinach leaf peroxisomes, aspartate aminotransferase and serine:pyruvate aminotransferase activities are catalyzed by separate proteins. A more difficult task has been to determine whether the other aminotransferase activities are also catalyzed by these two enzymes or if other separate proteins are present. In this chapter, some of the characteristics of the spinach peroxisomal aminotransferases and some preliminary studies of the aminotransferase activities in mammalian peroxisomes are reported. An understanding of the number and characteristics of the peroxisomal aminotransferases has helped elucidate the function of peroxisomes (154). Aminotransferases have important functions in the metabolism of cells, for they are involved in nitrogen metabolism and serve as a metabolic link between the amino acids or proteins and the carbohydrate pools.

The second chapter in this thesis describes research on the distribution of the peroxisomal enzymes

among the leaf cells of C_4- and C_3-plants. Previous studies had indicated that low levels of the glycolate pathway enzymes were present in C_4-plants (120, 157). However, bundle sheath cells of the C_4-plants were undoubtedly not broken in those studies so the results represented mesophyll cells and are invalid for the whole leaf. The purpose of the studies reported in this thesis was to determine if the bundle sheath cells of C_4-plants contained the enzymes of the glycolate pathway and, if so, how did the total amount of activity of these enzymes in the C_4-plants compare to the levels in the C_3-plants. Since activity of the glycolate pathway is manifested by photorespiration, the cellular distribution for enzymes of the glycolate pathway or peroxisomes in C_4-plants should contribute to an understanding of the function of photorespiration and its regulation. Photorespiration appears to inhibit plant growth and a knowledge of the mechanism and control of it may be of great significance to agricultural production. It is hoped that this work describing both the aminotransferases in peroxisomes and the unique distribution of peroxisomal enzymes in the bundle sheath cells of C_4-plants will help in the understanding of this subject area of plant biochemistry and thereby contribute someday, to the improvement of world food production.

LITERATURE REVIEW

CARBON DIOXIDE FIXATION PATHWAYS

In the fixation of CO_2 by the reductive pentose phosphate pathway, 3-P-glycerate is the first product formed (7). The acceptor molecule is ribulose-1,5-diphosphate and the fixation is catalyzed by ribulose-1, 5-diphosphate carboxylase, sometimes also designated as carboxydismutase. Although oxaloacetate, malate and aspartate are the first products of CO_2 fixation in the C_4-pathway, 3-P-glycerate is also labeled quite early in these C_4-plants (68). It has been shown that the carboxyl carbon of the 3-P-glycerate formed in these plants, was derived from the C-4 carbon of the dicarboxylic acids (81). Since the C_4-plants appeared at first to contain low levels of ribulose-1,5-diphosphate carboxylase, Hatch and Slack (68) proposed a transcarboxylation reaction to form 3-P-glycerate from the C_4 dicarboxylic acids. However, Björkman and Gauhl (12) and later Andrews and Hatch (3) found that the C_4-plants had levels of ribulose-1, 5-diphosphate carboxylase similar to those found in C_3-plants. They also showed that most of the ribulose-1, 5-diphosphate carboxylase was located in the bundle sheath cells of the C_4-plants. Plants which possess the

C_4-pathway have well developed bundle sheath cells surrounding the vascular tissue (48, 94) and these bundle sheath cells contain chloroplasts. In contrast, the bundle sheath cells in C_3-plants are few, small and without chloroplasts.

Details of the two CO_2 fixation reactions in C_4-plants have been summarized (69) and so only a brief description of the pathway of CO_2 fixation in C_4-plants will be given. Malate is formed in the mesophyll cells and is transported to the bundle sheath cells where it is decarboxylated by malic enzyme. The CO_2 is refixed by ribulose-1,5-diphosphate carboxylase, to form carboxyl labeled 3-P-glycerate. The pyruvate from the malic enzyme reaction moves back to the mesophyll cell and is phosphorylated by pyruvate phosphate dikinase. The PEP can then serve as an acceptor for another molecule of CO_2. The net fixation of CO_2 occurs via the C_3-pathway with the C_4-cycle serving as the initial fixation reaction. Thus the C_4-cycle may serve as a CO_2 concentrating or transport mechanism to the ribulose diphosphate carboxylase site. In C_3-plants, carbonic anhydrase may function in a similar capacity to facilitate the transport of CO_2 to the ribulose diphosphate carboxylase. It has been found that Chlorella grown at high CO_2 concentrations

could not readily fix CO_2 at lower CO_2 concentrations until after the level of carbonic anhydrase in the cells had increased (65). Likewise, inhibition of carbonic anhydrase inhibits photosynthesis (64). It should be noted that in plants with the C_4-pathway of CO_2 fixation, both the mesophyll cells and the bundle sheath cells are involved in the fixation of the CO_2. It is interesting that Rhoades and Carvalho in 1944 (131) suggested that the mesophyll cells of corn fixed the CO_2 while the bundle sheath cells stored the carbohydrate. They observed that in variegated corn leaf tissue, the normal bundle sheath cells which were next to mutant mesophyll cells did not accumulate starch whereas the normal bundle sheath cells lying next to normal mesophyll cells did accumulate starch. Moss and Rasmussen (111) found that in short term $^{14}CO_2$ fixation nearly all of the radioactivity in corn leaves was quickly transported into the bundle sheath cells. These and other experimenters have established that there is a rapid transport of photosynthetic products between the two types of cells (11, 121) but the mechanisms for these transport steps are not well elucidated.

Each of the two cell types in C_4-plants contain chloroplasts which are both morphologically and biochemically distinguishable. Laetsch and coworkers (92, 93, 94)

as well as other authors (15, 47) have shown that the bundle sheath chloroplasts are larger, contain more starch grains and in certain species contain no grana whereas the mesophyll chloroplasts are smaller, do not contain many starch grains and always contain grana. The mesophyll chloroplasts contain PEP carboxylase, NADP-malate dehydrogenase, pyruvate phosphate dikinase, adenylate kinase, pyrophosphatase and glycerate kinase (145, 146). The bundle sheath chloroplasts contain the enzymes of the C_3-pathway, including ribulose diphosphate carboxylase and ribulose-5-phosphate kinase. They also contain malic enzyme (145, 146). Plants which do not possess the C_4-pathway, i.e. C_3-plants, do not have well developed bundle sheath cells which contain chloroplasts (47). Instead, all chloroplasts are of one type and have the characteristics of the bundle sheath chloroplasts in the C_4-plants.

PHOTORESPIRATION

Photorespiration is defined as the uptake of O_2 and the release of CO_2 in the light. A distinguishing feature of C_4-plants is that they do not release CO_2 in the light. However, they do consume O_2 in the light and they also metabolize glycolate and glycine to CO_2 (86, 178)

and thus they do possess photorespiration (53, 77, 78). Krotokov, Nelson and coworkers (54, 55, 160) extensively studied photorespiration by measuring a CO_2 burst when leaves were transferred from light to dark. The burst was interpreted to be due to photorespired CO_2, which normally would be refixed in the light. These authors observed that leaves of corn, a C_4-plant, did not show a CO_2 burst while soybean and tobacco which are C_3-plants did release CO_2. Photorespiration has been shown to be a different process than dark or mitochondrial respiration. Krotkov and colleagues (55, 160) observed that at low O_2 concentrations photorespiration was severely inhibited but the mitochondrial respiration was not affected. Photorespiration was also found to respond differently to CO_2 concentrations and temperature (63, 74, 75, 82).

In the process of photorespiration, O_2 is consumed and CO_2 is released while in photosynthesis, O_2 is formed and CO_2 is fixed. Björkman *et al*. (13) vividly demonstrated the apparently wasteful process of photorespiration by growing beans and corn in an atmosphere of 5% O_2 where the rate of photorespiration would be very low. The beans grew twice as fast in the 5% O_2 as compared to beans grown in air. However, the growth of corn was not affected by the 5% O_2 atmosphere.

Another method of detecting photorespiration in plants is by measuring the CO_2 compensation point (54). The CO_2 compensation point is the concentration of CO_2 at which the rate of photosynthesis equals photorespiration in a closed system. Plants which have the C_3-pathway of CO_2 fixation have CO_2 compensation points of 40-60 ppm CO_2 to as high as 155 ppm CO_2 for leaves of trees, while C_4-plants have values of 0-5 ppm CO_2. Moss and coworkers (29, 108) used the low compensation point of corn to test plants for the presence of photorespiration. When a C_3-plant was placed in an illuminated closed chamber with corn, the C_3-plant died in 2-5 days. The corn leaf lowered the CO_2 concentration below the CO_2 compensation point of the C_3-plant, and the C_3-plant lost CO_2 through photorespiration while the corn grew on this CO_2. This closed chamber system was used to test 2500 soybean varieties and a couple of thousand wheat varieties looking for a natural mutant which did not possess photorespiration. The results were negative. Björkman and coworkers (14, 19) crossed a C_3-plant and a C_4-plant of the Atriplex family and observed that all of the F_1 and F_2 generations possessed photorespiration.

Zelitch (178), Moss (110) and Goldsworth (63) showed that recently fixed $^{14}CO_2$ was released into CO_2

free air at a faster rate in the light than in the dark. They also observed the α-hydroxypyridinemethane sulfonic acid inhibited the release of $^{14}CO_2$ in the light. This sulfonic acid was known to be an inhibitor of glycolate oxidase (175, 177) and this result was consistent with the hypothesis that glycolate was the substrate of photorespiration.

GLYCOLATE PATHWAY

Glycolate is one of the early products of photosynthesis and is probably formed from one of the sugar diphosphate intermediates of the C_3-pathway of CO_2 fixation (61, 152). The optimum conditions for the formation of glycolate are high light and a high $[O_2]/[CO_2]$ ratio (125, 152). Although the mechanism of glycolate biosynthesis is not known, it has recently been shown that spinach leaves incorporate exogenously supplied O_2 into the carboxyl group of glycine and serine which is derived from the carboxyl group of glycolate (Andrews, Lorimer and Tolbert, in manuscript). The reaction sequence of the glycolate pathway has been mainly elucidated by Tolbert and coworkers (72, 80, 126, 158). A specific P-glycolate phosphatase is located with the chloroplast and may be involved in the transport of glycolate out of the

chloroplast (132). The glycolate is oxidized to glyoxylate by the FMN dependent glycolate oxidase in the peroxisomes (181). The glyoxylate is converted to glycine by the irreversible glutamate:glyoxylate aminotransferase (85) and then two molecules of glycine can be converted to one serine plus CO_2 and ammonia. Hess and Tolbert (72) showed that in tobacco leaves the glycolate formed during short term $^{14}CO_2$ fixation was uniformly labeled, as were glycine and serine whereas 3-P-glycerate was carboxyl labeled. The serine is quite rapidly converted to glycerate and sugars (80, 126). The glycolate pathway is a gluconeogenic pathway, but 25% of the carbon is lost as CO_2 in the conversion of two glycines to one serine. This CO_2 loss has been postulated to be the source of the photorespired CO_2 (154).

PEROXISOMES

Tolbert *et al.* (156) established that part of the glycolate pathway enzymes were located in a distinct organelle, called the peroxisome. The term peroxisome was used because the leaf organelle was similar to the peroxisomes which were isolated earlier from rat liver by de Duve and coworkers (41, 42). Peroxisomes have been isolated from the leaves of spinach, wheat, Amaranthus, sunflower, Swiss

Chard, bean, pea and tobacco (157). Other sources are rat liver and kidney, chicken liver and kidney, frog liver and kidney, Tetrahymena, yeast (40) and sunflower cotyledons (Schnarrenberger, Oeser and Tolbert, in press).

Peroxisomes have a single membrane, a finely granular matrix and sometimes contain a crystalline core (41, 57, 67). The enzyme composition of the peroxisomes varies among species and among tissues within a species. All peroxisomes contain catalase and most contain flavin oxidases (154). de Duve and coworkers (42) first isolated peroxisomes from rat liver by isopycnic centrifugation in sucrose gradients. The mammalian peroxisomes contained catalase, D-amino acid oxidase, L-amino acid oxidase, urate oxidase and α-hydroxyacid oxidase (9). The spinach leaf peroxisomes were also isolated by glycolate oxidase (α-hydroxyacid oxidase) and NADH-hydroxypyruvate reductase (156). Frederick and Newcomb (56) and Vigil (167) have shown that the crystalline core, sometimes observed in plant peroxisomes, appears to be catalase. In addition to the enzymes mentioned above, spinach leaf peroxisomes have been found to contain NAD-malate dehydrogenase (173), NADP-isocitrate dehydrogenase (174), glutamate:glyoxylate aminotransferase, alanine:glyoxylate aminotransferase, serine:glyoxylate aminotransferase (85), serine:pyruvate

aminotransferase and aspartate:α-ketoglutarate amino-transferase (174).

Another plant organelle which has the same physical characteristics of the peroxisomes but contains the enzymes of the glyoxylate cycle is called the glyoxysome. This organelle was first isolated from castor bean endosperm by Breidenbach and Beevers (22). The glyoxysomes which have also been isolated from the seedlings of several other fat storing plants (154) contain all of the enzymes needed for the conversion of fatty acids to succinate and they also contain catalase and glycolate oxidase (33, 34).

The function of the glyoxysomes is to convert the fatty acids in the storage tissues of seedlings to carbo-hydrates. One of the functions of leaf peroxisomes is to form glycine which can then be converted to serine. The serine can go on to glycerate and then on to carbohydrates. Other possible functions of peroxisomes have been described in a recent review by Tolbert (154).

AMINOTRANSFERASES

The aminotransferase reaction was first observed in animal tissue in 1937 by Braunstein and Kritzmann (20), two Russian biochemists. In the early studies of amino-transferases, it was thought that only the transamination

reactions involving glutamate, aspartate and alanine were of physiological importance (106). As the understanding of amino acid metabolism developed, however, it was recognized that most, if not all, naturally occurring amino acids were involved in physiologically important transamination reactions.

Braunstein and Kritzmann preferred to call their enzymes aminopherases. The English writing authors, however, preferred transaminases or aminotransferases and these are now the officially recognized names (76). The naming of aminotransferases has varied over the years. Some authors used the two amino acid designation, i.e. glutamate:aspartate aminotransferase while others have used the amino acid:α-keto acid as in glutamate:oxaloacetate aminotransferase. One has to keep in mind that most, if not all, aminotransferase reactions are reversible and thus either designation seems adequate. The official designation (76) of naming aminotransferases is by the amino acid:α-keto acid reaction involved which permits two names. However, if α-ketoglutarate is involved it should be in the name rather than glutamate. Aspartate:α-ketoglutarate is accepted but glutamate:oxaloacetate is not acceptable. In this thesis, the selected designation is based upon the cited work by previous author(s).

EARLY STUDIES OF AMINOTRANSFERASES IN PLANTS

The first aminotransferase reaction observed in plant tissue was reported by Virtanen and Laine in 1938 (168). They observed aspartate:pyruvate aminotransferase activity in peas. Other early investigators of amino-transferases in plants included Kritzmann, Adler and co-workers, and Cedrangalo and Carancante (100). Albaum and Cohen (1) observed very active aspartate:α-ketoglutarate aminotransferase activity in oat seedlings. The pH optimum was 8.5.

Rautanen (127) reported the presence of glutamate: pyruvate and aspartate:pyruvate aminotransferase activities in green plants. These two activities had pH optima of 6.9, both had temperature optimum of 41 C and both reactions were reversible. In the same tissue, he also observed aspartate:α-ketoglutarate and valine:α-ketoglutarate amino-transferase activities. Twenty plants plus wheat germ were reported by Leonard and Burris (99) to have an active glutamate:oxaloacetate aminotransferase. Generally the highest specific activity, based upon nitrogen, was located in the roots of the plants. Wheat germ also contained alanine:α-ketoglutarate aminotransferase activity. In none of this work was it known whether one general aminotransferase or many specific enzymes were involved.

Wilson et al. (171) did an extensive study of transamination reactions in plants. Using lupine and barley, they showed that 17 amino acids could enzymatically donate their amino groups to α-ketoglutarate. Many of these activities were present in the particulate fractions of the plant extracts. Using corn radicles, pea, white and blue lupine, barley, oat and mung bean seedlings they detected transamination of alanine, aspartate, glycine, phenylalanine, valine, leucine, methionine and histidine to α-ketoglutarate although not all plant sources exhibited all activities. Wilson et al. (171) also observed a glutamate: glyoxylate aminotransferase reaction in tobacco leaf juice. This reaction was dependent upon glutamate and pyridoxal-5-phosphate.

The activities mentioned above described some of the earlier work with aminotransferases in plants. Many other activities have been observed and further information can be found in references 39, 71, 100 and 139.

GLYOXYLATE AMINOTRANSFERASES IN PLANTS

One of the first reports of a glyoxylate aminotransferase activity in plants was the glutamate:glyoxylate aminotransferase found in tobacco leaves by Wilson et al. (171). They observed this activity by incubating

glycolate-1-^{14}C, glutamate and pyridoxal-5-phosphate with the leaf extract. The glycolate was very likely oxidized to glyoxylate by glycolate oxidase which was known to be present in tobacco leaves (31). The glyoxylate was then converted to glycine with glutamate serving as the amino donor.

Cossins and Sinha (35) were the first to extensively study the glyoxylate aminotransferase activities in carrot tissue, corn coleoptile, pea leaves and sunflower cotyledons. The amino donor specificity was not great, being nearly equally active with glutamate and alanine and only slightly less active with serine and aspartate. The glutamate: glyoxylate aminotransferase activity in extracts of pea leaves had a broad pH spectrum with good activity from pH 4.6 to 8.5. It was thought that the aspartate:glyoxylate and alanine:glyoxylate aminotransferase activities were reversible. These were relatively long term experiments and the percentage of glycine-^{14}C converted to glyoxylate-^{14}C was 9.0% with oxaloacetate and 7.3% with pyruvate relative to a control value of 6.0% with no α-keto acid. In view of more recent data these results are probably not significant.

In crude homogenates of wheat leaves, King and Waygood (83) observed alanine:, aspartate:, glutamate: and

serine:glyoxylate aminotransferase activities as well as glutamate:pyruvate and aspartate:α-ketoglutarate aminotransferase activities. They partially purified the serine:glyoxylate aminotransferase and found it had a pH optimum of 8.2. The substrate dependent Km values (fixed substrate at 13 mM) were 0.9 mM for serine and 0.25 mM for glyoxylate. The enzyme would not use D-serine, L-phosphoserine, glycolate or glycoaldehyde. No pyridoxal-5-phosphate activation could be observed but phosphate activation was observed. In fact no serine:glyoxylate aminotransferase activity was observed in plant extracts made in water or Tris buffer until phosphate was added to the assay.

A more highly purified serine:glyoxylate aminotransferase was obtained from oat leaves by King and coworkers (23). The specificity of the enzyme varied according to the purification procedure used. One preparation which contained only a single protein band in disc gel electrophoresis showed serine:glyoxylate, alanine:glyoxylate and glutamate:glyoxylate aminotransferase activities. Using a different purification procedure, the alanine:glyoxylate and glutamate:glyoxylate aminotransferase activities were separated from the serine:glyoxylate aminotransferase. The substrate independent

Km values for the serine:glyoxylate aminotransferase were 2.88 mM for serine: and 0.508 mM for glyoxylate. The reaction kinetics showed that the mechanism of serine: glyoxylate aminotransferase was of the Ping Pong Bi Bi type of mechanism (45). Pyridoxal-5-phosphate activation was also observed with this enzyme. Kisaki and Tolbert (85) found several glyoxylate aminotransferase activities in the peroxisomes of spinach leaves. The most active, glutamate:glyoxylate aminotransferase had a pH optimum of 7 and Km values for glutamate and glyoxylate of 3.6 mM and 4.4 mM, respectively. No pyridoxal-5-phosphate activation was observed but isonicotinyl hydrazide inhibition was observed.

GLYOXYLATE AMINOTRANSFERASES FROM SOURCES OTHER THAN PLANTS

Braunstein and Kritzmann(20) who first discovered transaminase reactions reported glycine:α-ketoglutarate aminotransferase activity in some animal tissues. Cammarata and Cohen (27) found glycine:α-ketoglutarate activity in liver but none was observed in kidney. Ornithine:asparagine: and glutamine:glyoxylate aminotransferase activities were observed in rat liver by Meister and coworkers (105, 107). Silkworm larva have been found to contain glyoxylate aminotransferase activities with

alanine: glutamate, aspartate and cysteine serving as amino donors (60) while Pseudomanas aeruginosa has been reported to contain a glycine:α-ketoglutarate aminotransferase activity (6).

In 1964 Nakada (114) partially purified glutamate: glyoxylate aminotransferase from rat liver. He found the aminotransferase to be irreversible and not active towards the D-isomers of the amino acids. Likewise the enzyme was not active with serine or aspartate but was active with glutamate > alanine > glutamine. The pH optimum was 7.2 and the Km for glutamate was 4.6 mM and for glyoxylate it was 8.3 mM. A 91% inhibition was observed with 20 mM isonicotinic hydrazide.

Thompson and Richardson (150, 151) have characterized glutamate:glyoxylate aminotransferase and alanine: glyoxylate aminotransferase from human liver. The characteristics of the human liver glutamate:glyoxylate aminotransferase were similar to the rat liver enzyme. The pH optimum was 7.3 and the Km for both serine and glyoxylate was 2 mM. The relative rates of activity exhibited by the enzyme were glutamate-100, alanine-66, glutamine-39, methionine-17 and arginine-12, while serine, valine, aspartate, histidine, phenylalanine, tyrosine and isoleucine showed no activity. Thompson and Richardson

(150) also found the glutamate:glyoxylate aminotransferase to be irreversible. The human liver enzyme was not inhibited by 1 mM isonicotinic hydrazide but 1 mM Cu^{++} inhibited it 100%.

The alanine:glyoxylate aminotransferase purified from human liver had different characteristics than the glutamate:glyoxylate aminotransferase (151). The pH optimum was 8.4 and the Km for alanine was 1 mM at a fixed concentration of 20 mM glyoxylate. At pH 8.4 the relative rates with various amino donors were alanine-100, serine-84, arginine-13 and DL tryptophan-4. Eighteen other amino acids including aspartate, glutamate and D-alanine had no activity. The α-keto acceptor reactions with alanine were limited to glyoxylate-100 and hydroxypyruvate-6. Again the reaction was not reversible. Cu^{++} at 1 mM inhibited the alanine:glyoxylate aminotransferase only 16%. The observed serine:glyoxylate aminotransferase activity was attributed to the nonspecificity of the alanine:glyoxylate aminotransferase since the two activities did not separate upon purification and the reaction rate with both alanine and serine present was not equal to the sum of the two individual rates. Vandor and Tolbert (160) found that glutamate:glyoxylate aminotransferase activity was present in rat liver peroxisomes.

ASPARTATE:α-KETOGLUTARATE AMINOTRANSFERASE IN PLANTS

The aspartate:α-ketoglutarate aminotransferase or as it is commonly called aspartate aminotransferase, appears to be the most frequently observed aminotransferase. In spite of this, there are few reports of its characterization from plant tissue. Cruickshank and Isherwood (36) reported the glutamate:aspartate aminotransferase in wheat had a pH optimum of 8-8.5 and they found it to be inhibited 40% by 1 mM $AgNO_3$. Ellis and Davies (49) purified the enzyme from cauliflower florets and found that it did not react with γ-hydroxyglutamate, γ-methyleneglutamate, β-hydroxyaspartate, cysteate or cysteinesulfinate. The pH optimum was 7-8 and the Km values for glutamate, oxaloacetate, aspartate and α-ketoglutarate were 36mM, 0.08 mM, 7.2 mM and 0.66 mM respectively (37). They measured the specificity of the enzyme by observing amino acid inhibition of the aspartate + α-ketoglutarate reaction. No inhibition was found with 12 amino acids including DL-serine, glycine, D-alanine or L-alanine.

Verjee (165) determined the following substrate independent Km values for the aspartate:α-ketoglutarate aminotransferase from wheat germ: glutamate, 4.4 mM; oxaloacetate, 0.05 mM; aspartate, 0.8 mM and α-ketoglutarate, 0.5 mM. These values are lower, especially for

glutamate, than those reported by Davies and Ellis (37). The molecular weight of the wheat germ aspartate:α-ketoglutarate aminotransferase was reported to be 75,000 ± 5,000. The pH optimum was 8-8.5 and pyridoxal-5-phosphate activation was observed (166).

The particulate and soluble fractions in germinating pea cotyledons contained aspartate:α-ketoglutarate aminotransferase (172). Wong and Cossins (172) reported the soluble enzyme had a pH optimum of 8 whereas the particulate had a broad range with activity from 6.8 - 8.5. The soluble enzyme was not active with pyruvate, hydroxpyruvate or glyoxylate in place of α-ketoglutarate. Likewise, aspartate could not be replaced by D-aspartate, asparagine, serine, leucine, glutamine, γ-aminobutyrate, alanine or glycine. The apparent Km values for the particulate and soluble enzymes were similar, although the same concentration of fixed substrate was not used in the assays for both of the enzymes.

Characterization of aspartate:α-ketoglutarate aminotransferase from the particulate and soluble fractions of germinating pumpkin cotyledons has been reported by Splittstoesser (148). Both enzyme fractions had pH optimum of 8. The soluble enzyme fraction was inhibited very strongly by hydroxylamine and to a lesser extent by

p-hydroxymercuribenzoate and sodium bisulfite. The most recent report of aspartate:α-ketoglutarate aminotransferase activity in plants has been from Yamazaki and Tolbert (174). They studied the subcellular location of the enzyme and their work will be reviewed in the section on the subcellular location of aminotransferases.

ASPARTATE:α-KETOGLUTARATE AMINOTRANSFERASE FROM SOURCES OTHER THAN PLANTS

The aspartate:α-ketoglutarate aminotransferase appears to be ubiquitous and thus only a few references will be cited. Green et al. (66) showed the pig heart aspartate:α-ketoglutarate aminotransferase to be quite specific. Alanine, leucine, serine and methionine could not replace aspartate, and glutamine could not replace glutamate. In 1947 O'Kane and Gunsalus (119) separated glutamate:aspartate aminotransferase from glutamate:alanine aminotransferase, and proposed that the aspartate:alanine aminotransferase activity in crude homogenates was an artifact caused by the presence of the two glutamate aminotransferases.

Cammarata and Cohen (28) partially purified the glutamate:oxaloacetate aminotransferase from pig heart muscle and found that sixteen amino acids including glycine and glutamine would not react with α-ketoglutarate.

Boyd (17) found that mammalian tissues had two isoenzymes of aspartate aminotransferase and that they had different pH curves and Km values. The soluble form had a pH optimum of 8.5 while the mitochondrial form was active from pH 5-9. The soluble enzyme had Km values for α-ketoglutarate and aspartate of 0.2 mM and 2.1 mM respectively, whereas the mitochondrial enzyme had Km values of 1.0 mM and 0.47 mM for α-ketoglutarate and aspartate respectively. Similar Km values have been reported by several authors (52, 118).

Velick and Vavra (164) measured the Km values of all four substrates for the soluble pig heart enzyme and obtained the following results: Km (aspartate), 0.9 mM; Km (α-ketoglutarate), 0.1 mM; Km (glutamate), 4 mM and Km (oxaloacetate), 0.04 mM. The kinetic characteristics for both the soluble and mitochondrial isoenzymes from beef liver were reported by Morino et al. (109). The Km values of glutamate and oxaloacetate were similar for both isoenzymes but instrument sensitivity may have been a limiting factor in their determinations. The observed Km values (mM) for glutamate, α-ketoglutarate, aspartate and oxaloacetate were for the soluble enzyme 20, 2.0, 0.4 and 0.02 and for the mitochondrial enzyme, 20, 0.3, 5.0 and 0.05 respectively.

The kinetic characteristics of aspartate:α-ketoglutarate aminotransferase appear to be affected by ions. Boyde (18) observed that as the concentration of phosphate was increased, the Km of both aspartate and α-ketoglutarate increased. This trend was observed with both isoenzymes but the affect varied between the isoenzymes. Nisselbaum (117) reported that phosphate and sulfate inhibit the rat liver mitochondrial aspartate aminotransferase but increased the activity of the soluble enzyme. Cl^- increased the activity of the mitochondrial enzyme but had no affect on the soluble enzyme. Other authors have reported anion and cation affects on aspartate aminotransferase (10, 161, 169). It has been reported that phosphate and other anions interfere with the reconstitution of apoaspartate aminotransferase with pyridoxal-5-phosphate (50, 143).

SUBCELLULAR LOCATION OF AMINOTRANSFERASES

In 1960, Bone and Fowden (16) reported asparate:α-ketoglutarate and alanine:α-ketoglutarate aminotransferase activities in mung bean mitochondria. Particulate aspartate:α-ketoglutarate has also been found in pumpkins (149) and pea cotyledons (172). Mukerji and Ting (112) reported that glutamate:oxaloacetate aminotransferase was

located in nonaqueously prepared chloroplasts from cactus phylloclades. They also reported the aminotransferase was present in the mitochondria and chloroplasts prepared on sucrose gradients. However, NADH-malate dehydrogenase had the same distribution as glutamate:oxaloacetate and it has since been shown that chloroplasts do not contain NADH-malate dehydrogenase (133, 173) and so the presence of the aminotransferase in chloroplasts was not definitely established.

Tolbert and Yamazaki (158) first reported that aspartate:α-ketoglutarate aminotransferase was located in the peroxisomes, mitochondria and chloroplasts of spinach leaves. They also found that the peroxisomes contained three isoenzymes of the aminotransferase (174). It was thought that one of these isoenzymes was due to mitochondrial contamination. Cooper and Beevers (33) also found that aspartate aminotransferase was located in both the glyoxysomes and the mitochondria of castor bean endosperm. Breidenbach (21) reported D-aspartate aminotransferase activity in glyoxysomes. Mathieu (104) reported glutamate: pyruvate and glutamate:oxaloacetate aminotransferase in nonaqueous prepared chloroplasts from Kalanchoë. However, mitochondria and possibly other subcellular organelles are not well separated from chloroplasts by such a

procedure. Santarius and Stocking (137) also using non-aqueous isolation procedures found glutamate:oxaloacetate aminotransferase was located both inside and outside the chloroplasts.

Yamazaki and Tolbert (174) found serine:pyruvate aminotransferase activity in spinach leaf peroxisomes. Earlier, Kisaki and Tolbert (85) had reported spinach peroxisomes contained several glyoxylate aminotransferases with glutamate, alanine and serine being the best amino donors. The particulate serine:glyoxylate aminotransferase from spinach leaves was first shown to be located only in the peroxisomes (129, this thesis). Serine:glyoxylate, glutamate:glyoxylate and aspartate:α-ketoglutarate aminotransferases have been found in the microbodies of castor bean endosperm and sunflower cotyledons (Schnarrenberger, Oeser and Tolbert, in press). Alanine:glyoxylate and glutamate:glyoxylate aminotransferase have been found in both the supernatant and particulate fractions of sunflower cotyledons (35).

The cationic and anionic isoenzymes of aspartate aminotransferases from mammalian tissue were identified as soluble and mitochondrial forms by Boyd (17). These two isoenzymes have been found in many tissues and have been extensively studied as was described previously in

the section Aspartate:α-Ketoglutarate Aminotransferase From Sources Other Than Plants. de Duve and coworkers (8) and Muller (113) reported that aspartate aminotransferase and alanine aminotransferase were not located in the peroxisomes of rat liver but were located in the mitochondria. However, with the use of electron microscopy, Papadimitriou and VanDuijn (124) observed aspartate aminotransferase activity on the peroxisome membrane as well as in the mitochondria, and on the plasma membranes of many cells including erythrocytes and bacteria. Lee (96) found that some of the kidney aspartate:α-ketoglutarate aminotransferase was located in the mitochondria but the enzyme was concentrated in the subapical vesicles and suggested it may be involved in ammonia excretion.

The mitochondrial aspartate aminotransferase has been reported to be located both in the matrix and on the mitochondrial membranes (103, 142). These differences in possible location of the aminotransferase may be related to the sticking of aspartate aminotransferase to membranes under certain ionic conditions (130). Vandor and Tolbert (162) found glutamate:glyoxylate aminotransferase activity in the peroxisomes of rat liver, but de Duve (40) has reported that it was not in the peroxisomes.

It has recently been reported in an abstract that

most of the alanine:glyoxylate aminotransferase activity of rat liver was located in the mitochondria (147). In Neurospora, the alanine:glyoxylate aminotransferase was a soluble enzyme (32).

Several excellent reviews covering some of the topics pertinent to this thesis have recently been published and are listed below. Microbodies-Peroxisomes and Glyoxysomes (154); Photosynthetic CO_2-Fixation Pathways (69); The Peroxisomes: a New Cytoplasmic Organelle (41); Photorespiration (78) and Nitrogen Metabolism of Amino Acids (136).

CHAPTER I

AMINOTRANSFERASES IN PEROXISOMES

Leaf peroxisomes were first isolated by Tolbert et al. (156) and were found to contain most of the enzymes associated with the glycolate pathway (154, 156). In the conversion of glycolate to glycerate, two transamination reactions occur: glyoxylate is converted to glycine and the serine is converted to hydroxpyruvate. Four major aminotransferase activities have been found in spinach leaf peroxisomes. Kisaki and Tolbert (85) found glutamate:glyoxylate and alanine:glyoxylate aminotransferase activities. The aminotransferase activities with glutamate:glyoxylate and alanine:glyoxylate were suggested to be from one enzyme since mixed substrate assays were not additive. Aspartate aminotransferase activity was also detected by Kisaki and Tolbert (85) but they thought it was due to the nonspecificity of the glutamate:glyoxylate aminotransferase.

Yamazaki and Tolbert (174) found spinach peroxisomes contained a serine:pyruvate aminotransferase activity. These authors also concluded that the peroxisomal aspartate

aminotransferase activity could not be accounted for by mitochondrial contamination and it could not be due to the nonspecificity of the glutamate:glyoxylate aminotransferase since the specific activity of the aspartate aminotransferase was higher than the glutamate:glyoxylate aminotransferase. With the use of isoelectric focusing, Yamazaki and Tolbert (174) showed that serine:pyruvate aminotransferase in peroxisomes was a different protein than the aspartate aminotransferase. They also showed that after electrophoresis, the aspartate aminotransferase activity from peroxisomes was present as three isoenzyme bands on the polyacrylamide gels. However, one of these may have been due to mitochondrial contamination.

Of the four major aminotransferase activities reported in peroxisomes, it was shown that serine:pyruvate and aspartate:α-ketoglutarate aminotransferase were different proteins. Whether these two proteins also exhibited glutamate:glyoxylate and alanine:glyoxylate aminotransferase activities was not known. Also it was not known if the three isoenzymes of aspartate:α-ketoglutarate aminotransferase were specific for aspartate and α-ketoglutarate or if other aminotransferases such as the serine:pyruvate aminotransferase were exhibiting some activity towards aspartate and α-ketoglutarate.

The purpose of this study was to determine how

many different enzymes were involved in the peroxisomal aminotransferase activities and to determine some of the characteristics of the enzymes.

MATERIAL AND METHODS

PLANTS

Spinach was either purchased at a local market or grown in growth chambers at 20 C and a light period of 11 hr. Spinach was grown in a soil-peat moss mixture and watered with Hoagland's solution at least three times a week.

ANIMALS

Female Spraque-Dawley rats, weighing 200-300 g, were purchased commercially and fed Purina Laboratory chow and watered ad libitum. Kidneys from dogs (species unknown) were furnished by the staff in the Department of Human Development. From an anesthetized animal, the kidney was perfused with saline solution and immediately used.

ISOLATION OF SPINACH LEAF PEROXISOMES

Small Zonal Rotor Procedure

Peroxisomes and mitochondria were obtained by three procedures. The first procedure was used for intermediate size preparations of subcellular organelles and involved the use of the International Zonal Centrifuge. Washed and deribbed spinach leaves weighing 250 g were

homogenized in a Waring blendor for 7-10 sec at high speed. The grinding medium was 30% (w/w) sucrose in 20 mM glycylglycine at pH 7.5. Unless stated otherwise, all sucrose solutions which were used for grinding media or gradients were made in 20 mM glycylglycine at pH 7.5 and the percentages of sucrose are based upon w/w. The spinach homogenate was squeezed through 8 layers of cheesecloth. Cellular debris and most whole chloroplasts were removed by centrifugation at 650 g for 5 min. The homogenate was then placed directly in the center of a B-30 rotor. The operation of the zonal centrifuge will not be discussed here but further information can be found in references 2, 46, 163. The gradient which was found to result in the best separation of subcellular organelles from spinach leaves was composed of the following sucrose solutions: 50 ml of 56% sucrose, 20 ml each of 53%, 52.5%, 52%, 51%, 50%, 49%, 44%, 43%, 42%, 41%, 40%, and 10 ml each of 35%, 30%, and 25% (46). Starting with the least dense sucrose solution, the solutions were loaded into the zonal rotor from the edge. After the gradient was in the rotor, the remaining volume was filled with 56% sucrose. The small zonal rotor (B-30) would hold approximately 560 ml. Up to 260 ml of spinach homogenate was added to the rotor through the core orifices. Centrifugation at 30,000 rpm for 2 hr separated the mitochondria and peroxisomes. The

rotor was unloaded by pumping water into the core and collecting 10 ml fractions from the edge of the rotor.

The small zonal rotor was also used for processing up to 1 kg of spinach. The spinach was homogenized in 250 g batches and applied to the zonal rotor in the following manner. The homogenate from the first 250 g of spinach leaves was placed in the rotor as previously described. Instead of centrifuging at 30,000 rpm for 2 hr, the rotor was spun at 30,000 rpm for 15 min to move the particles just into the gradient. The rotor was slowed to 3,000 rpm and 56% sucrose was used to push the supernatant out through the core. The next batch of 250 g of spinach was loaded through the core and the process repeated. After the last spinach homogenate was added, the 2 hr centrifugation was run to move the organelles to their isopycnic point.

Large Zonal Rotor Procedure

The second procedure was the same as the first except that the large zonal rotor (B-29) with a volume of 1500 ml was used. The gradient was generally the same except that 50 ml of each sucrose solution was used. The 250 g batches of spinach tissue were prepared in the same manner. Spinach extracts equal to 500 g of spinach leaves

could be placed in the rotor at one time.

By using the process of applying one spinach homogenate, centrifuging a short time, removing the supernatant and applying the second homogenate, etc., 4 kg of spinach were processed in one experiment.

Simulated Zonal Procedure

The third procedure was performed in the SW 25.2 swinging bucket rotor with a Beckman L-2 Ultracentrifuge and was similar to that described by Tolbert (153). The gradient was composed of 2 ml of each of the same sucrose solutions that were used in the small zonal rotor procedure. The homogenate from 20 g of spinach tissue was applied to the gradient and centrifuged for 2.5 hr at 25,000 rpm. Fractions of 1 ml were collected by puncturing the bottom of the tube and draining.

It was found that a better yield of peroxisomes was obtained from 20 g of spinach tissue by grinding the tissue with a mortar and pestle rather than a mechanical blender. The tissue was first placed in a beaker containing 30% sucrose grinding media and cut into very small pieces with a scissors. The tissue was kept immersed in the grinding media during the cutting. The finely cut tissue in the grinding media was then placed in a mortar. A small

amount of sand was added and then the tissue was ground until no pieces of tissue remained. The homogenate was squeezed through 4 layers of cheesecloth, centrifuged at 650 g for 5 min to remove cell debris and most of the whole chloroplasts, and then placed on the sucrose gradient. The mortar and pestle procedure resulted in a little higher percentage recovery of peroxisomal enzymes in particulate form and about twice the yield that was obtained from a Sorvall Omni-mixer.

ISOLATION OF CHLOROPLASTS

Most of the intact chloroplasts were pelleted by centrifuging the spinach homogenate at 650 g for 5 min. This pellet was resuspended in the grinding medium (30% sucrose) and layered over 15 ml of 30% sucrose in a centrifuge tube. The chloroplasts were pelleted through this sucrose by centrifugation at 270 g for 10 min while the smaller particles remained in the supernatant. The pelleted chloroplasts were resuspended in buffer and either used directly or were centrifuged at 100,000 g for 1 hr to remove most of the chlorophyll and then the nearly colorless supernatant was used.

ISOLATION OF MAMMALIAN PEROXISOMES

Female rats were injected with Triton WR 1339 (Ruger Chemical Co., New Jersey) 3.5 days before sacrificing. The rats were decapitated and the livers were perfused with either water or 7% sucrose solution before removing. Livers from 3-10 rats were immersed in 7% sucrose at 0 C, minced with either a scissor or a razor blade and homogenized in a motor drived Potter-Elvehem homogenizer for only one stroke down and back up. The homogenate was filtered through 4 layers of cheesecloth. Debris from the homogenate was removed by either centrifuging at 270 g for 5 min or by the differential centrifugation procedure of de Duve et al. (43). The homogenate was applied to the small zonal rotor (B-30) filled with the following sucrose gradient: 40 ml, 56%; 12 ml each of 49%, 48%, 47.5%, 47%, 46.5%, 46%, 45%, 40%, 38%, 36%, 33%, 30%, 25%, 20% and 15%. Centrifugation was at 30-35,000 rpm for only 35 min. Fractions of 5 ml each were collected from the edge of the rotor.

Medula and cortex tissue from a dog kidney was minced with either a meat grinder, mortar and pestle or a tissue grinder-press. Homogenization in 25% sucrose was achieved with a motor drived Potter-Elvehem homogenizer. The extract was filtered through 4 layers of cheesecloth,

and centrifuged for 5 min at 480 g. The supernatant was either placed directly in the zonal rotor or was first centrifuged at 39,000 g for 30 min and the pellet, resuspended in grinding media, was placed in the zonal rotor. The velocity of the zonal rotor was increased in increments of 10,000 rpm for 15 min, 20,000 rpm for 15 min and finally held at 30,000 rpm for 2 hr. The sucrose gradient was similar to the one used for the rat liver preparation. Fractions were collected from the edge of the rotor.

ASSAYS

All spectrophotometric assays were conducted with a Gilford recording spectrophotometer at 25 C. The radiochemical assays on plant tissue were at 25 C while the mammalian tissues were assayed at 36 C. The pH of all reagents was adjusted to the pH of the assay. Unless stated otherwise, all amino acids were of the L form. A unit of enzyme activity is defined as that amount of enzyme which will form 1 μmole of product in 1 min.

Serine:Glyoxylate Aminotransferase (EC 2.6.1.-)

The spectrophotometric procedure of measuring the activity of serine:glyoxylate aminotransferase was a modification of the linked enzyme assay described by Brock et al. (23). The hydroxypyruvate formed in the

transamination reaction was enzymatically reduced by hydroxypyruvate reductase and the oxidation of NADH was measured at 340 mμ. The assay mixture contained 0.7 ml of 0.1 M HEPES at pH 7, 0.04 ml of 4.2 mM NADH, 0.02 ml of 5 mM pyridoxal-5-phosphate, 0.05 ml of 20 mM sodium glyoxylate, 0.05 units of crystalline spinach glyoxylate reductase (Sigma), 0.05 ml of 0.4 M L-serine and 0.12 ml of enzyme plus water. The addition of NADH was used to start the measurement of the endogenous rate and the transamination reaction was started with L-serine. An extinction coefficient of 6.2×10^3 $cm^{-1} \times M^{-1}$ for NADH was used. All rates were corrected for the endogenous rate of glyoxylate reduction by the hydroxypyruvate reductase.

The serine:pyruvate aminotransferase activity was measured using the same protocol as in the serine:glyoxylate aminotransferase assay with the following changes: 0.02 ml of 4.2 mM NADH, 0.03 ml of 0.1 M sodium pyruvate and 0.16 ml of enzyme plus water. The serine:pyruvate aminotransferase procedure is a modification of the assay used by Yamazaki and Tolbert (174).

Alanine:Glyoxylate Aminotransferase (EC 2.6.1.12)

The alanine:glyoxylate aminotransferase activity was measured in a manner similar to the serine:glyoxylate

aminotransferase and was described by Brock et al. (23). Lactate dehydrogenase was used to reduce the pyruvate and the oxidation of NADH was followed at 340 mμ. The protocol for the reaction mixture was the same as for the serine: glyoxylate aminotransferase except that 0.03 units of lactate dehydrogenase (Sigma) and 0.05 ml of 0.4 M L-alanine were used.

Glutamate:Glyoxylate Aminotransferase (EC 2.6.1.4)

The glutamate:glyoxylate aminotransferase activity was measured by the method of Kisaki and Tolbert (85). The assay mixture contained 0.7 ml of 0.1 M phosphate or cacadylate at pH 7, 0.02 ml of 5 mM pyridoxal-5-phosphate, 0.08 ml of 0.4 M L-glutamate and 0.180 ml of enzyme plus water. The reaction was started with the addition of 0.02 ml of 0.25 M glyoxylate-U-^{14}C. The reaction mixtures were kept in ice until the glyoxylate was added and then they were placed in a water bath of 25 or 36 C. The reaction mixture reached 25 C within 60 sec. The reaction was terminated by placing the test tube in boiling water for 3 min. The reaction mixture was cooled and placed on a Dowex-1 acetate column (0.6 x 2 cm) to remove excess glyoxylate-U-^{14}C. The effluent was collected directly in a glass scintillation vial. The reaction tube and

Dowex column were washed twice with 1 ml of water and the column was then blown dry. Control columns showed 99.2% of the glyoxylate-U-^{14}C stayed on the column and 100% of added glycine-^{14}C was eluted from the column.

After adding 17 ml of scintillator fluid [50 mg POPOP (phenyl-oxazolylphenyl-oxazolylphenyl), 4 g PPO (2,5-diphenyloxazole), 1000 ml toluene, 1000 ml Triton X-100] to each vial, the samples were counted in a Packard Scintillation Counter. Quenching was observed by the channel ratio method. The same volume of glyoxylate-U-^{14}C that was used in each assay, was added directly to a counting vial containing 3 ml of water. This standard had the same degree of quenching as the reaction mixtures and was used to calculate the specific activity of the glyoxylate-U-^{14}C. The specific activity of glyoxylate-U-^{14}C varied between experiments.

Other Radiochemical Aminotransferase Assays

Serine:glyoxylate aminotransferase and alanine:glyoxylate aminotransferase were also assayed by a radiochemical procedure. The same protocol as in the glutamate:glyoxylate aminotransferase assay was used except that L-serine or L-alanine was substituted for the L-glutamate.

The radiochemical assay was also conducted using

^{14}C labeled amino acids. In this case, the reaction was started with the amino acid. The reaction was stopped by the addition of 0.35 ml of 10% trichloroacetic acid. The reaction mixtures were placed on Dowex 50-H^+ columns to remove the excess amino acid and the column washed twice with 1 ml of 10 mM HCl. The rest of the procedure was the same as previously described. In a control experiment 100% of added glyoxylate-U-^{14}C was recovered in the effluent. Exchange reactions between an amino acid and its corresponding α-keto acid were conducted using the same basic radiochemical protocol of glutamate: glyoxylate aminotransferase. The assay mix contained a final concentration of 0.8 mM ^{14}C labeled amino acid and 8 mM of the corresponding α-keto acid. The ^{14}C labeled α-keto acids were eluted from the Dowex 50-H^+ column as described above.

Aspartate:α-Ketoglutarate Aminotransferase (EC 2.6.1.1)

Aspartate aminotransferase activity was measured as described by Yamazaki and Tolbert (174). The oxaloacetate formed in the transamination reaction was reduced by NADH and malate dehydrogenase, and the change in concentration of NADH was measured at 340 mμ. The assay mixture contained 0.7 ml of 0.1 M HEPES at pH 7, 0.03 ml

of 0.1 M α-ketoglutarate, 0.02 ml of 5 mM pyridoxal-5-phosphate, 0.02 ml of 4.2 mM NADH, 2 units of malate dehydrogenase (Sigma), 0.16 ml of enzyme plus water and 0.05 ml of 0.4 M L-aspartate. The reaction was started with the L-aspartate.

Other Assays

Catalase was measured by following the decrease in the absorbance of H_2O_2 at 240 mμ (102). An extinction coefficient of 42 $cm^{-1} \times M^{-1}$ for H_2O_2 was used. Cytochrome c oxidase activity was measured as described by Tolbert et al. (156) and an extinction coefficient of 21.1 $\times 10^3$ $cm^{-1} \times M^{-1}$ for the reduced cytochrome c was used. The cytochrome c was reduced by sodium dithionite until the A_{550}/A_{565} ratio was equal to 6-10. Malate dehydrogenase and hydroxypyruvate reductase were assayed by measuring the oxidation of NADH (156). Chlorophyll was determined by Arnon's procedure (4). Protein was assayed by the procedure of Lowry et al. (101) with crystalline bovine serum albumin as the standard protein.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Three stock solutions for the preparation of polyacrylamide gels were prepared according to the procedure of Davis (38). Solution A consisted of 18.3 g of

Tris, 0.11 ml of TEMED, 24 ml of 1 N HCl and water to 50 ml (pH 8.9); solution B was composed of 14 g of acrylamide, 0.367 g of BIS and water to 50 ml; solution C was 0.07 g of ammonium persulfate dissolved in 50 ml of water. Stock solution A and B were stored for up to one month but solution C was prepared freshly once a week. The gels were prepared by mixing 1 part solution A, 2 parts solution B, 1 part water and 4 parts solution C. The gel mixture was placed in glass tubes to a height of 7 cm and then a layer of distilled water was carefully layered on top of the gel. The gels were set in 30-45 min and then they were placed in the cold room for at least 1 hr prior to adding the sample. Up to 0.1 ml samples, containing at least 10% (w/w) sucrose, were layered onto the top of the gels. Samples directly from the sucrose gradients gave the same electrophoretic results as samples which were treated by osmotic shock or detergent to break the organelles prior to electrophoresis. In the case of the samples from the TEAE-cellulose columns, solid sucrose was added to the sample before it was applied to the gel. The electrode buffer solution was composed of 3 g Tris, 14.4 g glycine and water to 5 l (pH 8.3). Electrophoresis was generally run for 2 hr at 2-5 ma per gel. Tracking dye (Bromo Phenol blue) was

either mixed with one sample or placed on a separate gel which had no enzyme sample.

STARCH GEL

A method similar to that of Fine and Costello (51) was used to prepare the starch gel. The starch mixture, consisting of 36 g hydrolyzed starch (Connaught Medical Research Lab., Toronto, Canada) and 300 ml of 5 mM Tris-38 mM glycine at pH 8.3 was stirred continuously and heated until it reached 70-80 C. The hot mixture was quickly poured into a 2 l suction flask which was sitting in hot tap water and a vacuum was applied until very few bubbles remained. The gel was then poured into a Plexiglas form. A mold for sample wells was placed on the gel. The gel was left at room temperature until firm (about 30 min), covered with Saran Wrap and placed in the cold room. Gels were left in the cold room 1-12 hr before use. Approximately 0.1 ml of sample was placed in each sample well. The electrode buffer was the same as that used in the gel. Sponge wicks were placed on top of each end of the gel to form the bridge to the buffer tanks. Electrophoresis was conducted at 400-500 volts, for 10-16 hr.

STAINING GELS FOR ASPARTATE AMINOTRANSFERASE

A stain specific for oxaloacetate has been reported (5) and was used to stain electrophoretic gels for aspartate aminotransferase (24). The staining solution was a modification of the one used by Yamazaki and Tolbert (174) and consisted of 0.05 ml of 0.4 M L-aspartate, 0.05 ml of 0.1 M α-ketoglutarate, 0.02 ml of 5 mM pyridoxal-5-phosphate, 0.8 ml 0.1 M TES at pH 7 and 0.08 ml of water containing 1 mg of Fast Violet B. The dye was added to the rest of the staining solution just before applying the staining solution to the gels. The staining solution is not sensitive to light, however, it did turn reddish-brown with time, and it would not stain the enzyme if it was mixed too early before use. The polyacrylamide gels were immersed in staining solution in test tubes. Starch gels were sliced in half, and a piece of filter paper was placed on the exposed surface to absorb some of the moisture. The first filter paper was removed and a second filter paper which had been soaked with the staining solution was placed on the gel surface. The purple stains in both types of gels were fixed by treatment with 5% acetic acid.

TEAE-CELLULOSE COLUMNS

TEAE-cellulose was prepared by washing with 0.5 N KOH until the effluent was colorless, rinsing with water until neutral, washing with 0.5 N HCl until washes were colorless and again rinsing with water until neutral. The TEAE-cellulose was mixed with 5 mM Tris-HCl at pH 8.3 and poured into a column. The column was equilibrated overnight with 5 mM Tris-HCl at pH 8.3. The sample which was applied to the TEAE-cellulose column was a high speed supernatant of broken peroxisomes. Spinach leaf peroxisomes isolated on sucrose gradients, were broken by dilution or dialysis and the broken peroxisomes were centrifuged at 100,000 g for 1 hr to remove the membranes. The peroxisomal membranes were used by R. Donaldson for other assays and their removal was probably not necessary for my work. The supernatant (150-220 ml) was applied to the TEAE-cellulose column. The column was washed with approximately two void volumes to remove the glycolate oxidase. The aminotransferases were eluted with a 200 ml linear gradient of 0 to 0.3 M KCl in 5 mM Tris-HCl at pH 8.3.

ISOELECTRIC FOCUSING

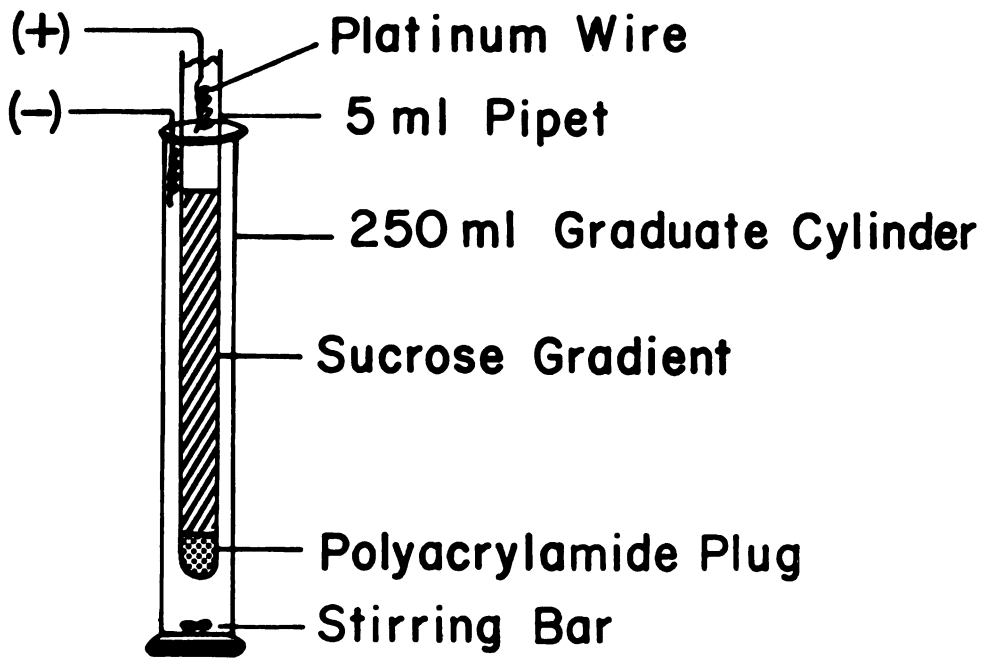
G. N. Godson (62) described a procedure for running isoelectric focusing in small columns and a modified column, most of which was constructed by G. Lorimer, was used in the present studies (Figure 1). The column was a 5 ml pipet with the tip cut off. Polyacrylamide gel was used to form a plug in the bottom of the column. Layered onto the polyacrylamide plug was 1 ml of cathode solution (5% ethanolamine in water) containing 35% (w/w) sucrose. A linear gradient of 10-30 (w/w) sucrose containing up to 5 mg of peroxisomal protein and 1.14% of either pH 3-10 or pH 5-8 Ampholine (LKB Produkter AB) was placed in the column. Anode solution (5% H_3PO_4 in water) was added to fill the column. All of the weight of the solution was on the polyacrylamide plug, and it may be advantageous to make a slight constriction at the cut off end of the pipet so the polyacrylamide plug does not slip out of the column.

A 250 ml graduated cylinder filled with cathode solution was placed around the column. If the cylinder is placed around the column before the column is filled, the polyacrylamide plug will tend to move up into the column. The cathode solution was stirred to help dissipate the heat from the column. Platinum electrodes were immersed in the two electrode solutions. Unless stated otherwise,

Figure 1

Isoelectric Focusing Apparatus

A 5 ml pipet with the tip cut off served as the column. A polyacrylamide plug kept the sucrose gradient in the column. The one electrode solution was layered on top of the sucrose gradient. The other electrode solution was in the 250 ml graduated cyliner but a portion of it was also placed on top of the polyacrylamide plug. The solution in the graduated cylinder was stirred to help dissipate the heat.


(+)
(–)
Platinum Wire
5 ml Pipet
250 ml Graduate Cylinder
Sucrose Gradient
Polyacrylamide Plug
Stirring Bar

the isoelectric focusing was run at 500 volts, at <5 ma for 9 hr in the cold room. To drain the column, a capillary tubing was carefully forced through the polyacrylamide plug and approximately 0.1 ml fractions were collected.

RESULTS

The aminotransferase activities of peroxisomes isolated from three different biological sources have been examined. Most of the studies were done with spinach leaf peroxisomes but other sources of peroxisomes were checked to determine whether all microbodies contained the aminotransferases. The four aminotransferase activities to be considered are: glutamate:glyoxylate, serine:glyoxylate, alanine:glyoxylate and aspartate:α-ketoglutarate.

SERINE:GLYOXYLATE AMINOTRANSFERASE ASSAY

In the serine:glyoxylate aminotransferase reaction, hydroxypyruvate and glycine are the products. The activity was assayed spectrophotometrically by linking the reaction with hydroxypyruvate reductase which, however, also reduces glyoxylate. The glyoxylate concentration was kept at 1 mM which was low enough that the endogenous rate of the glyoxylate reduction by hydroxypyruvate reductase was low and could be subtracted. This concentration of

glyoxylate was, however, higher than the Km (glyoxylate) value of 0.15 mM for the aminotransferase. The NADH-hydroxypyruvate reductase from spinach has a Km for glyoxylate of 50 mM and a Km for hydroxypyruvate of 0.05 mM (89). The difference in these Km values permitted hydroxypyruvate reductase to be used for measuring the serine:glyoxylate aminotransferase activity. The assay was linear with both time and enzyme concentration (Figure 2). The reaction was always started with L-serine so the endogenous rate of glyoxylate reduction could be measured. The reaction was dependent upon enzyme, L-serine and glyoxylate.

Serine:glyoxylate aminotransferase activity was also measured by a radiochemical procedure. Measuring the formation of glycine-^{14}C from glyoxylate-U-^{14}C or the formation of hydroxypyruvate-^{14}C from serine-U-^{14}C gave similar results. The reaction was dependent upon enzyme, L-serine and glyoxylate. The reaction was linear with protein and appeared to be linear with time although the line did not go through zero (Figure 3). The reason for this is not known. Since the reaction was linear with protein up to the formation of 1.2 μmoles of glycine-^{14}C, substrate concentrations were probably not limiting.

Glyoxylate is quite easily converted to glycine

Figure 2

Serine:Glyoxylate Aminotransferase
Spectrophotometric Assay

Spinach leaf peroxisomes, isolated on a sucrose gradient, were used. The assay mixture contained 70 μmoles of HEPES at pH 7, 0.17 μmoles of NADH, 0.1 μmoles of pyridoxal-5-phosphate, 1 μmole of glyoxylate, 0.05 units of glyoxylate reductase, 20 μmoles of L-serine and enzyme in a total volume of 1 ml.

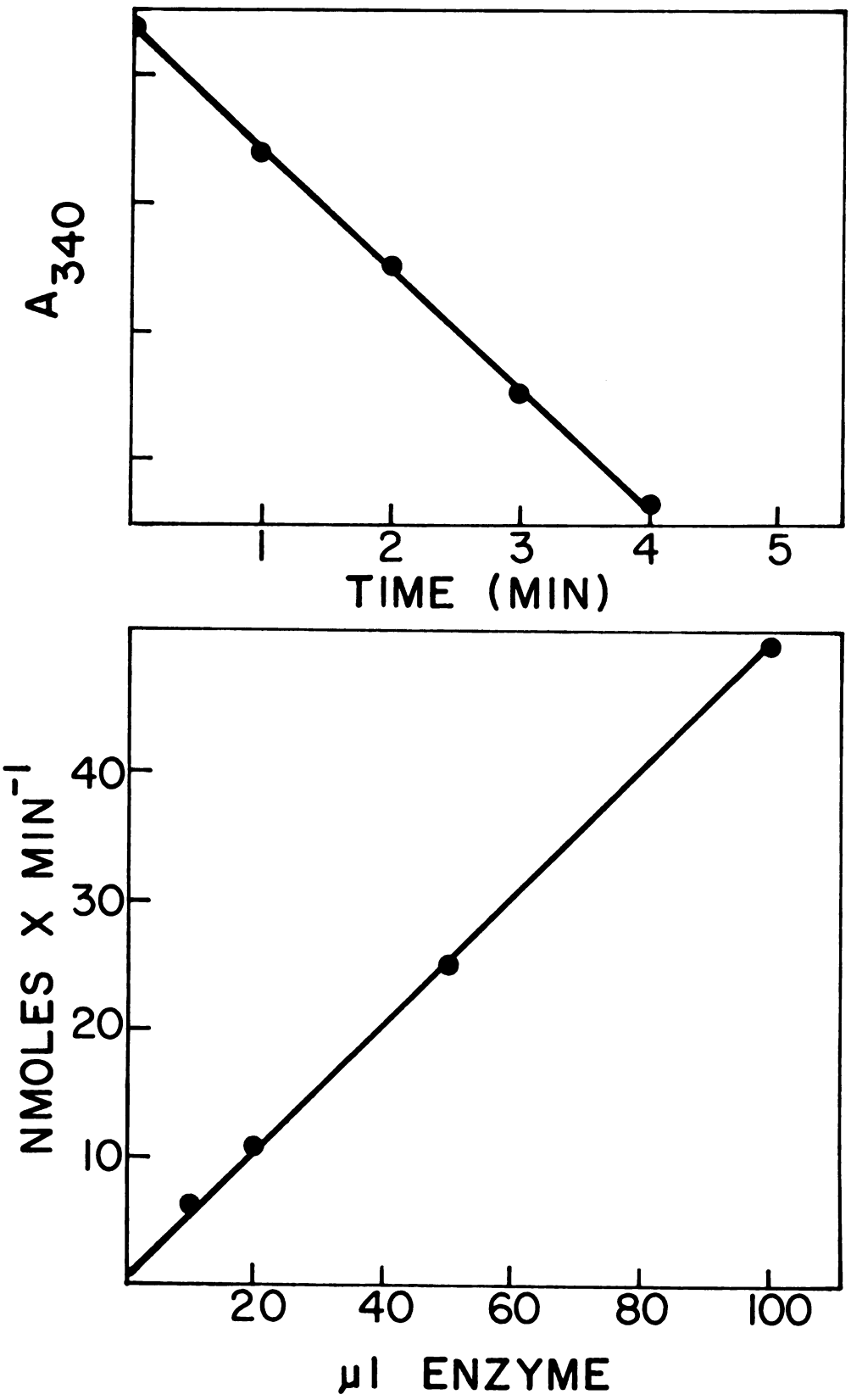
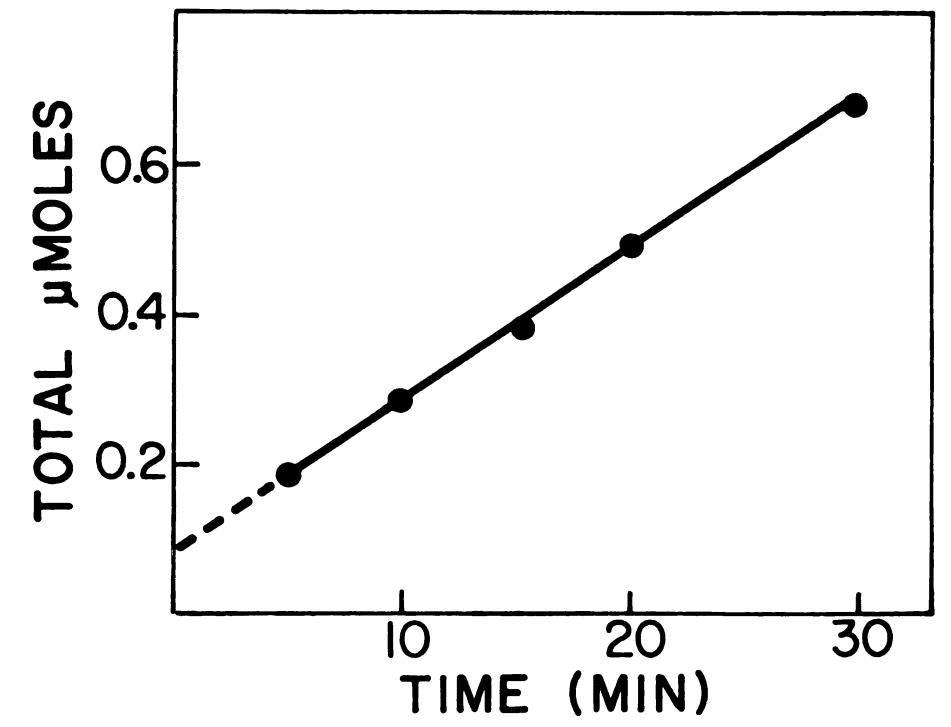
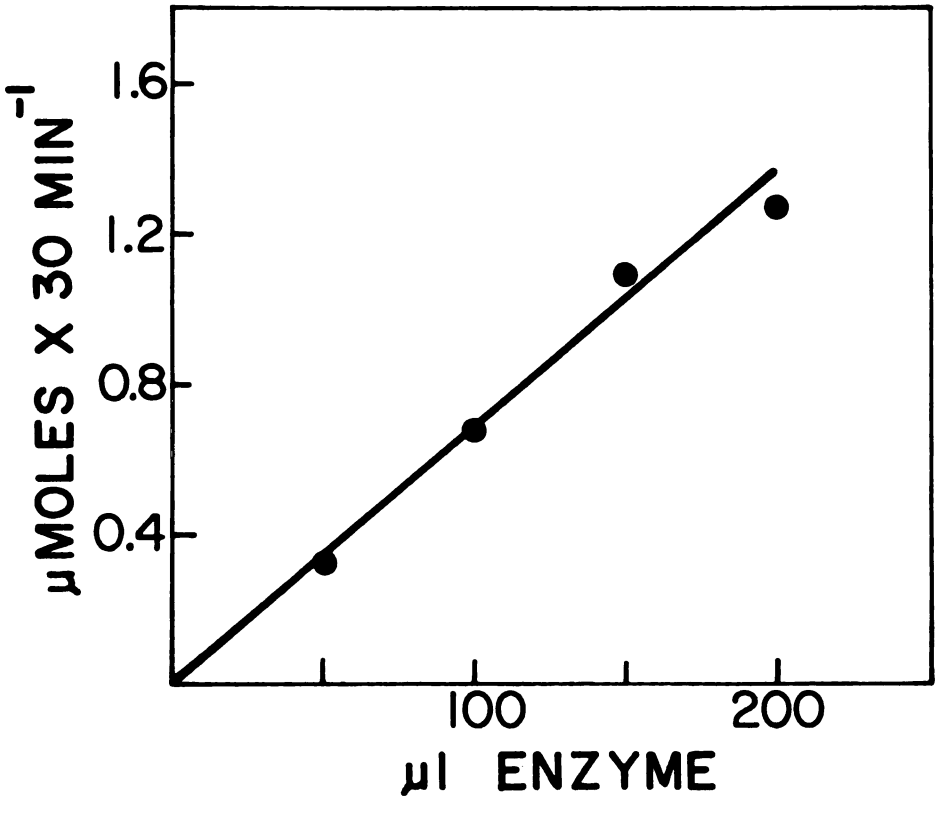

A340
1
2
3
4
5
TIME (MIN)
NMOLES X MIN-1
10
20
30
40
20
40
60
80
100
µl ENZYME

Figure 3

Serine:Glyoxylate Aminotransferase
Radiochemical Assay

Isolated spinach leaf peroxisomes were used for the assays. The reaction mixture contained 70 μmoles of either phosphate or cacodylate at pH 7, 0.1 μmoles of pyridoxal-5-phosphate, 32 μmoles of L-serine, 5 μmoles of glyoxylate-U-^{14}C and enzyme in a total volume of 1 ml.


TOTAL µMOLES
0.6
0.4
0.2
10
20
30
TIME (MIN)


µMOLES X 30 MIN⁻¹
1.6
1.2
0.8
0.4
100
200
µl ENZYME

by a nonenzymatic reaction (115). The rate of this nonenzymatic reaction is dependent upon the amino donor, with glutamate being most active. However, even heat denatured enzyme can somehow serve as an amino donor for an endogenous rate (Kisaki and Tolbert, unpublished). In all radiochemical assays, a control containing either no enzyme or heat denatured enzyme was used to correct for the nonenzymatic transamination reaction. The two types of controls had similar rates. The observed rate of serine:glyoxylate aminotransferase activity was similar with either the radiochemical assay or the spectrophotometric assay. Generally the spectrophotometric procedure was used.

Details of the other assays will not be described since the other assays were either similar to the serine: glyoxylate aminotransferase assay or have been described in detail in the literature. Controls were run on all assays to show the dependency of enzyme and substrates.

SUBCELLULAR LOCATION OF AMINOTRANSFERASES IN SPINACH LEAVES

Peroxisomes from spinach leaf tissue were isolated on sucrose gradients. It had previously been shown that peroxisomes contained catalase, glycolate oxidase, NADH-hydroxypyruvate reductase, NAD-malate dehydrogenase and

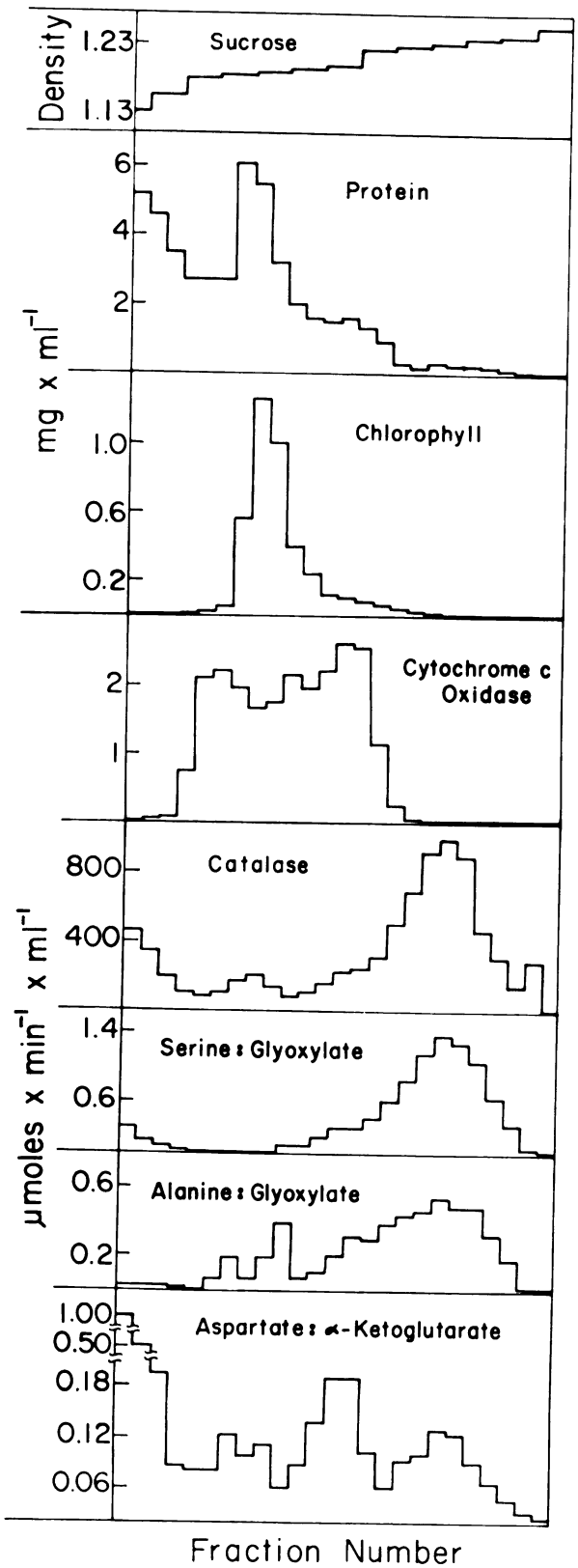
several other enzymes (154). In the present studies, catalase was used as the marker enzyme for peroxisomes while cytochrome c oxidase served as the mitochondrial marker and chlorophyll as the marker for chloroplasts. A typical enzyme distribution pattern of a sucrose gradient is shown in Figure 4. The catalase peak was at a sucrose density of 1.24 g x cc^{-1} and the peroxisomes were well separated from the mitochondria and chloroplasts. There were two peaks of cytochrome c oxidase; the major mitochondrial peak was at the density of 1.20 g x cc^{-1} while some activity was in a peak at a lower sucrose density which was characteristic of microsomes (46). The chlorophyll peak contained mostly broken chloroplasts. The intact chloroplast band was at the same sucrose density as the mitochondria but there were only a few percent of the chlorophyll in this fraction (133). This distribution of organelles was similar whether the small zonal, large zonal or simulated zonal procedure was used.

The distribution of serine:glyoxylate aminotransferase on the sucrose gradient was the same as for catalase (Figure 4). There was no peak of activity with the mitochondria or broken chloroplasts. The serine:glyoxylate aminotransferase appears to be located exclusively in the peroxisome. Similarly glutamate:glyoxylate aminotransferase

Figure 4

Distribution of Particulate Enzymes
From Spinach Leaves on a Sucrose Gradient

The homogenate from 20 g of spinach leaves, after centrifugation at 650 g for 5 min, was placed on the sucrose gradient and centrifuged for 2.5 hr.


Density
1.23
1.13
Sucrose
6
4
2
Protein
mg x ml⁻¹
1.0
0.6
0.2
Chlorophyll
2
1
Cytochrome c Oxidase
800
400
Catalase
μmoles x min⁻¹ x ml⁻¹
1.4
0.6
Serine : Glyoxylate
0.6
0.2
Alanine : Glyoxylate
1.00
0.50
0.18
0.12
0.06
Aspartate : α-Ketoglutarate
Fraction Number

activity has been reported to be located exclusively in the peroxisomes (85). Thus these two aminotransferase activities in spinach leaves are distinctive peroxisomal enzymes. Most of the alanine:glyoxylate aminotransferase activity was also located in the peroxisomes, but there was some alanine:glyoxylate aminotransferase activity at lower sucrose densities that did not correspond with either the mitochondria or broken chloroplast fractions. It is not known whether this means that some of the alanine: glyoxylate aminotransferase activity is in another particle or whether it just represents broken peroxisomes. It can be concluded that the three aminotransferase reactions with glyoxylate are peroxisomal and this is consistent with glyoxylate formation only in the peroxisomes during glycolate oxidation.

Tolbert and Yamazaki (158) were the first to report that aspartate aminotransferase was located in the peroxisomes, mitochondria and chloroplasts of spinach leaves. Mukerji and Ting (112) reported the activity to be in the chloroplasts and in the mitochondria in cactus. The data presented in this thesis confirms that the aspartate aminotransferase is located in all three organelles. The peroxisomal and mitochondrial sucrose gradient fractions both contained substantial aspartate aminotransferase

activity, but the broken chloroplast fraction contained very little activity. However, washed intact chloroplasts contained considerable aspartate aminotransferase activity (Table 1). In Table 1 the percentage of the total aspartate aminotransferase activity in each of the organelle fractions which could be attributed to contamination by the other organelles has been calculated. These calculations are based upon the percent of activity of the marker enzymes in each of the organelle fractions. An example of this calculation is:

$$\left[\frac{\text{catalase in chloroplasts}}{\text{catalase in peroxisomes}}\right]\left[\frac{\text{transaminase in peroxisomes}}{\text{transaminase in chloroplasts}}\right] \times 100$$

$$= \text{\% of total transaminase activity in chloroplasts attributable to peroxisomes.}$$

Of the total aspartate aminotransferase activity in the peroxisomes, only 15% could be attributed to mitochondrial and chloroplast contamination. Likewise, most of the activity in the mitochondrial fraction was not due to contamination. However, all of the aspartate aminotransferase activity in the broken chloroplast fraction was accounted for by mitochondrial and peroxisomal contamination. On the other hand, of the aspartate aminotransferase activity present in intact chloroplasts isolated by differential centrifugation, only 13% could be attributed to mitochondrial and peroxisomal contamination.

Table 1. Aspartate Aminotransferase Activity in Organelle Fractions

The whole chloroplasts were isolated by differential centrifugation and the other organelles by a sucrose gradient. Markers for the organelles were: catalase, peroxisomes; cytochrome c oxidase, mitochondria; chlorophyll, chloroplasts. The percentages of contamination were based upon the amount of each marker enzyme in each of the organelle fractions. See text for a sample calculation.

Organelle Fraction	Specific Activity	Peroxisomal Activity in Fractions	Mitochondrial Activity in Fractions	Broken Chloroplast Activity in Fractions	Whole Chloroplast Activity in Fractions
	nmoles x min^{-1} x mg $protein^{-1}$	%	%	%	%
Peroxisomes	194	85	4	-	11
Mitochondria	96	14	56	-	30
Broken Chloroplasts	18	15	160	0	-
Whole Chloroplasts	50	6	6	-	88

Thus aspartate:α-ketoglutarate aminotransferase is located in all three subcellular organelles while only the peroxisomes contain serine:glyoxylate, alanine:glyoxylate and glutamate:glyoxylate aminotransferase activities. It is not known whether all of the *in vivo* activity of these aminotransferases is located in the organelles or if the cytosol contains some soluble activities.

CHARACTERISTICS OF THE PEROXISOMAL GLYOXYLATE AMINOTRANSFERASES

Buffer and pH Effects

Both serine:glyoxylate and alanine:glyoxylate aminotransferases had maximum activities around pH 7 under the assay conditions described (Table 2). The observed rate of the serine:glyoxylate aminotransferase activity was nearly the same at pH 7 in HEPES, cacodylate or Bicine buffer. The activity was also nearly maximum in Bicine at pH 8 but declined at pH 6 in cacodylate. Phosphate buffer severely inhibited the serine:glyoxylate aminotransferase activity. However, alanine:glyoxylate aminotransferase activity was not affected by phosphate buffer and this can be a test to distinguish between the two enzymes. The alanine:glyoxylate aminotransferase had nearly the same activity in HEPES, cacodylate, phosphate and Bicine buffers at pH 7. The rate was about 50% less

Table 2. Buffer and pH Effects on Serine:Glyoxylate and Alanine:Glyoxylate Aminotransferase Activity

Peroxisomes were isolated using the small zonal rotor and broken by dilution with buffer. A 30-60% $(NH_4)_2SO_4$ precipitate of the broken peroxisomes was resuspended and passed through a Sephadex G-25 column. This protein fraction was used for the assays. Activity units are nmoles x min^{-1} x ml^{-1}.

Buffer		Serine:Glyoxylate Aminotransferase		Alanine:Glyoxylate Aminotransferase	
		Activity	Relative Rate	Activity	Relative Rate
HEPES	pH 7	1290	100	645	100
Cacodylate	pH 6	645	50	354	55
	pH 7	1130	88	677	105
Phosphate	pH 6	129	10	516	80
	pH 7	258	20	580	90
Bicine	pH 7	1220	95	774	120
	pH 8	1130	88	354	55

at pH 6 in cacodylate and at pH 8 in Bicine. The glutamate:glyoxylate aminotransferase activity in the spinach leaf peroxisomes had the same activity in cacodylate and phosphate buffers at pH 7 (data not shown). Kisaki and Tolbert (85) reported that the glutamate:glyoxylate aminotransferase in spinach leaf peroxisomes had a pH optimum of 7 and this is the same pH optimum reported for both the rat liver and human liver enzymes (114, 150). King and Waygood (83), using phosphate buffer, reported a pH optimum of 8.2 for serine:glyoxylate aminotransferase from wheat leaves.

Of the three glyoxylate aminotransferases assayed in spinach peroxisomes, only serine:glyoxylate aminotransferase was affected by phosphate. This difference in the effect of phosphate suggests the serine:glyoxylate aminotransferase reaction may be catalyzed by a different protein than the glutamate:glyoxylate and alanine:glyoxylate aminotransferase reactions.

D-Serine Inhibition

D-serine at 40 mM was found to inhibit the serine:glyoxylate aminotransferase 85%, whereas the alanine:glyoxylate aminotransferase was inhibited only 35% (Table 3). However, D-alanine had little affect on the

Table 3. D-Serine Inhibition of Glyoxylate Aminotransferases

Spinach leaf peroxisomes were isolated on a sucrose gradient and assayed by the spectrophotometric procedure. Specific activity is in nmoles x min^{-1} x mg $protein^{-1}$. The D-amino acids were at a final concentration of 40 mM.

Addition to Assay	Serine:Glyoxylate Aminotransferase		Alanine:Glyoxylate Aminotransferase	
	Specific Activity	Percent Inhibition	Specific Activity	Percent Inhibition
None	630	-	510	-
D-Serine	97	85	330	35
D-Alanine	530	16	390	23

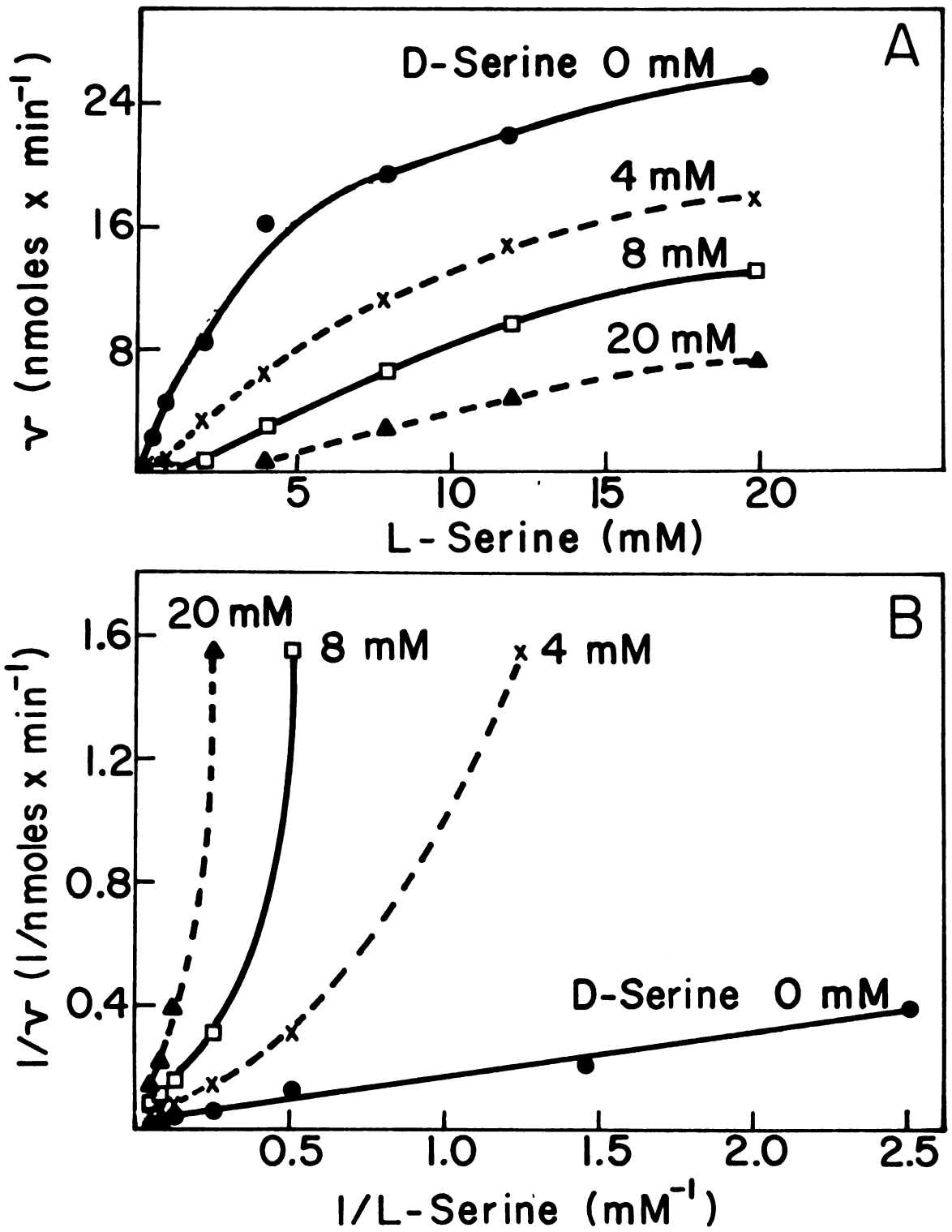
alanine:glyoxylate or on the serine:glyoxylate aminotransferase activities. The D-serine inhibition of serine:glyoxylate aminotransferase did not appear to be purely competitive or noncompetitive (Figure 5). The Km of L-serine appeared to be affected by the D-serine. The 1/velocity versus 1/[L-serine] plots at various concentrations of D-serine were nonlinear (Figure 5). These kinetics are characteristic of allosteric enzymes. However, since the aminotransferase reaction probably occurs by the Ping Pong Bi Bi mechanism, the D-serine may interact differently with the various enzyme-coenzyme-substrate complexes which occur in the aminotransferase reaction and thus display the complex kinetics. The enzyme may not have an allosteric site. The D-serine inhibition was probably not caused by some contaminant in the D-serine. Sigma quality control reports that lot 108B-1860 of D-serine which was used in the assay had only one ninhydrin positive spot or one iodine positive spot after thin layer chromatography in three different solvent systems. The experimentally determined nitrogen content was 13.54% while 13.33% is the theoretical value. Heavy metals were less than 10 ppm. To the authors knowledge this is the first report of D-amino acid inhibition of an L-amino acid aminotransferase. Aspartate

Figure 5

D-Serine Inhibition of Serine:Glyoxylate Aminotransferase

Spinach peroxisomes were isolated on a sucrose gradient. The spectrophotometric procedure was used and the glyoxylate concentration was at 1 mM. The D-serine was added to the assay mix before the enzyme. Incubation of the enzyme with D-serine for two minutes gave the same results as with no incubation period. The concentration of D-serine was either 0,4,8 or 20 mM.

A. Velocity versus Concentration of L-Serine
B. Lineweaver-Burk Plot at Various Concentrations of D-Serine


A
D-Serine 0 mM
4 mM
8 mM
20 mM
v (nmoles x min⁻¹)
24
16
8
5
10
15
20
L-Serine (mM)
B
20 mM
8 mM
4 mM
D-Serine 0 mM
1/v (1/nmoles x min⁻¹)
1.6
1.2
0.8
0.4
0.5
1.0
1.5
2.0
2.5
1/L-Serine (mM⁻¹)

aminotransferase from spinach peroxisomes was not effected by D-aspartate (data not shown) and as shown above, D-alanine had little effect on the alanine:glyoxylate aminotransferase. Kisaki and Tolbert (85) reported that in spinach peroxisomes D-glutamate was more active than L-glutamate in the transamination of glyoxylate.

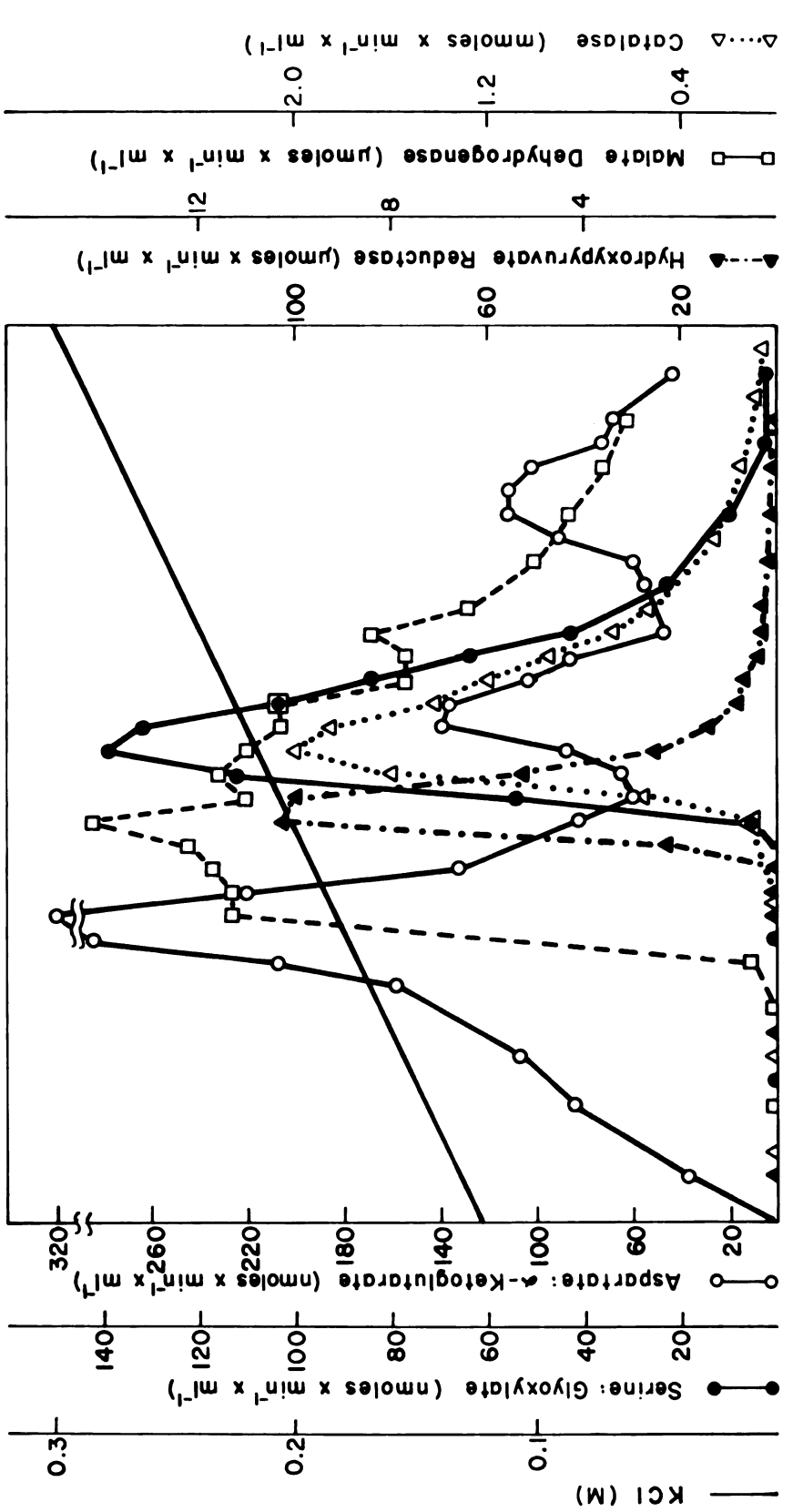
SEPARATION OF GLYOXYLATE AMINOTRANSFERASE ACTIVITIES USING ION EXCHANGE CHROMATOGRAPHY

The serine:glyoxylate aminotransferase was inhibited by D-serine and phosphate whereas the alanine:glyoxylate and glutamate:glyoxylate aminotransferase activities were not inhibited and these differences suggested that separate proteins may catalyze these reactions. Broken peroxisomes from spinach leaf tissue were placed on a TEAE-cellulose column and eluted with a linear gradient of 0 to 0.3 M KCl. The profile of the elution is shown in Figure 6. Serine:glyoxylate aminotransferase eluted at a KCl concentration of approximately 0.2 M. Only one peak of activity was observed but the recovery was only 50%. This peak had coincident activity with either glyoxylate or pyruvate as the amino acceptor and the data shown is that for serine:pyruvate aminotransferase activity. It will be shown in subsequent results that serine:glyoxylate and serine:pyruvate aminotransferase

Figure 6

Separation of Peroxisomal Enzymes by
TEAE-Cellulose Column Chromatography

Peroxisomes, isolated on a sucrose gradient were broken by osmotic shock and the membranes were removed by high speed centrifugation. The supernatant was placed on a TEAE-Cellulose column. The enzymes were eluted with a 0 to 0.3 M KCl gradient. O———O, aspartate aminotransferase; ●———●, serine:glyoxylate aminotransferase; □-----□ ; malate dehydrogenase; Δ·····Δ, catalase; and ▲-·-·-·-▲, hydroxypyruvate reductase. The serine: glyoxylate aminotransferase was assayed by the serine:pyruvate aminotransferase protocol. The values given are for serine: pyruvate aminotransferase activity and the serine:glyoxylate aminotransferase activity would be approximately 10 times higher.


KCl (M)
0.1
0.2
0.3
Serine: Glyoxylate (nmoles x min⁻¹ x ml⁻¹)
20
40
60
80
100
120
140
Aspartate: α-Ketoglutarate (nmoles x min⁻¹ x ml⁻¹)
20
60
100
140
180
220
260
320
Hydroxypyruvate Reductase (μmoles x min⁻¹ x ml⁻¹)
4
8
12
Malate Dehydrogenase (μmoles x min⁻¹ x ml⁻¹)
0.4
1.2
2.0
Catalase (mmoles x min⁻¹ x ml⁻¹)
20
60
100
Fraction Number

activities are very likely catalyzed by the same enzyme.

The column fractions were also assayed for glutamate: glyoxylate aminotransferase and alanine:glyoxylate aminotransferase but no activity was detected. Washing the column with 1.0 M KCl still did not elute the glutamate: glyoxylate aminotransferase activity. However, the glutamate:glyoxylate aminotransferase was active in an aliquot of the original solution which had been applied to the column. These results suggest that peroxisomes contain a serine:glyoxylate aminotransferase which does not catalyze the glutamate:glyoxylate or alanine:glyoxylate aminotransferase reactions. Brock et al. (23) also reported that upon partial purification of serine:glyoxylate aminotransferase from oat leaves, the glutamate:glyoxylate and alanine:glyoxylate aminotransferase activities were separated from the serine:glyoxylate aminotransferase. The spinach peroxisomal catalase peak coincided exactly with the serine:glyoxylate aminotransferase (Figure 6). Other peroxisomal proteins such as malate dehydrogenase and hydroxypyruvate reductase were partially separated from the serine:glyoxylate aminotransferase. The aspartate aminotransferase data will be discussed later. However, it should be noted that the serine:glyoxylate aminotransferase peak does not correspond to any of the

aspartate aminotransferase peaks of activity.

SEPARATION OF THE PEROXISOMAL GLYOXYLATE AMINOTRANSFERASES USING ISOELECTRIC FOCUSING

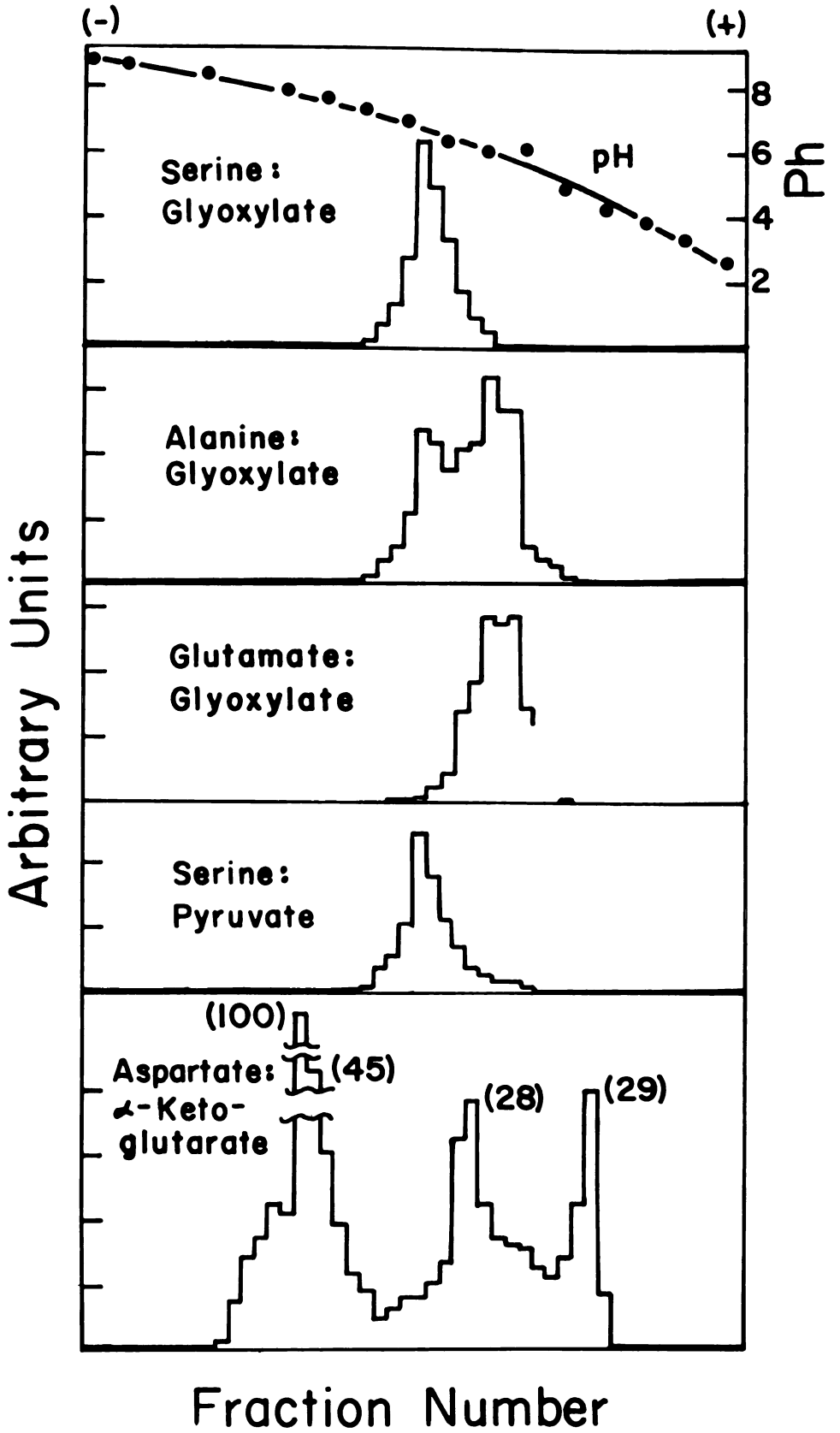
An isoelectric focusing column with a total volume of 5 ml was used to separate the aminotransferases. The distribution of the enzymes in the pH 3-10 gradient showed only one peak of serine:glyoxylate aminotransferase (Figure 7). The recovery of the serine:glyoxylate aminotransferase activity from the column was 92%. One peak of glutamate:glyoxylate aminotransferase and two peaks of alanine:glyoxylate aminotransferase were also observed on the column. The serine:glyoxylate aminotransferase was separated from the glutamate:glyoxylate aminotransferase but both enzyme peaks had an alanine:glyoxylate aminotransferase activity. The pI values of the serine:glyoxylate aminotransferase and the glutamate:glyoxylate aminotransferase were 6.7 and 5.8 respectively.

In the peak of the serine:glyoxylate aminotransferase, the serine:glyoxylate and alanine:glyoxylate aminotransferase activities were not additive and likewise in the glutamate:glyoxylate aminotransferase peak the glutamate:glyoxylate and alanine:glyoxylate activities were not additive (data not shown). This indicates that the alanine:glyoxylate aminotransferase activity is due to

Figure 7

pH 3-10 Isoelectric Focusing of
Peroxisomal Aminotransferases

Peroxisomes were isolated on a sucrose gradient, broken by osmotic shock, centrifuged to remove the traces of chlorophyll and placed on a pH 3-10 ampholyte isoelectric focusing column. The column was run at 500 volts for 9 hr. Enzyme activities are in arbitrary units per ml. Values in parenthesis for aspartate:α-ketoglutarate aminotransferase are relative values of the peak fractions. Specific activities of the peroxisomal enzymes are given in Table 9.


(-)
(+)
8
6
4
2
pH
Ph
Serine:
Glyoxylate
Alanine:
Glyoxylate
Glutamate:
Glyoxylate
Serine:
Pyruvate
(100)
Aspartate:
(45)
α-Keto-
glutarate
(28)
(29)
Arbitrary Units
Fraction Number

nonspecificity of the other two aminotransferases.

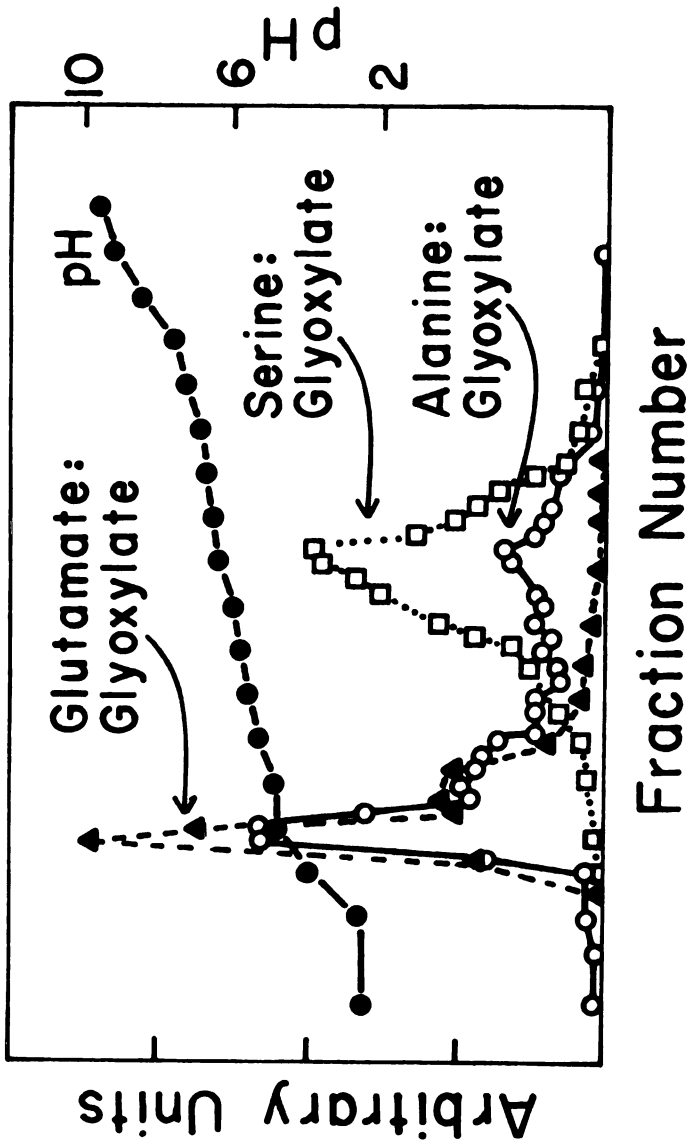
The spinach peroxisomal serine:glyoxylate and glutamate:glyoxylate aminotransferase reactions were catalyzed by two separate proteins. Separate enzymes for these two aminotransferase activities appear to also be present in rat liver, since partially purified glutamate: glyoxylate aminotransferase did not have any activity with serine (114). However, the rat liver enzyme did react with alanine just as the spinach peroxisomal enzyme appears to do. Human liver glutamate:glyoxylate aminotransferase was also not active with serine but again did react with alanine (150). Human liver also contains an alanine:glyoxylate aminotransferase which does not react with glutamate but does have activity with serine (151).

Since on the pH 3-10 isoelectric focusing column the serine:glyoxylate and glutamate:glyoxylate aminotransferase peaks were relatively close, an isoelectric focusing column of pH 5-8 was used to obtain a better resolution of these two enzymes. The activity in the column fractions was low but a general separation of the two aminotransferases was achieved (Figure 8). The alanine: glyoxylate aminotransferase activity was smeared throughout the column with a peak corresponding with the glutamate:glyoxylate aminotransferase and a slight peak

Figure 8

pH 5-8 Isoelectric Focusing of Peroxisomal Aminotransferases

The procedure was the same as given in Figure 7 except that the voltage on the pH 5-8 column was increased over a period of 5 hr to 700 volts and maintained there for an additional 22 hr. O———O, alanine:glyoxylate aminotransferase; ▲-----▲, glutamate: glyoxylate aminotransferase and □····□ , serine:glyoxylate aminotransferase.


pH
10
6
2
Glutamate:
Glyoxylate
Serine:
Glyoxylate
Alanine:
Glyoxylate
Arbitrary Units
Fraction Number

corresponding with the serine:glyoxylate aminotransferase. The enzymes were probably not completely focused since the peaks were very broad and the pI of glutamate:glyoxylate aminotransferase was 4.8 in this column as compared to 5.8 in the pH 3-10 column.

Studies were conducted on the two peaks of alanine: glyoxylate aminotransferase to determine if they had the same characteristics (Table 4). The alanine:glyoxylate aminotransferase activity in peak 2 (the serine:glyoxylate aminotransferase peak) was most active at pH 8 in borate buffer and it was least active in phosphate buffer at pH 7. The alanine:glyoxylate aminotransferase activity in peak 1 had about the same activity under all assay conditions. These different ratios of activity are consistent with the idea that the two peaks of alanine:glyoxylate aminotransferase are different proteins.

The question remains whether the alanine:glyoxylate aminotransferase activities are separate enzymes or whether these reactions are nonspecific activities of the glutamate:glyoxylate and serine:glyoxylate aminotransferases. Although the results presented here are consistant with the alanine:glyoxylate aminotransferase activity being due to the other two aminotransferases, no definite conclusion can be drawn until further experiments

Table 4. Characteristics of Alanine:Glyoxylate Aminotransferase Peaks From a pH 5-8 Isoelectric Focusing Column

Fractions were assayed by the spectrophotometric procedure. Peak 1 contained glutamate:glyoxylate aminotransferase and peak 2 contained serine:glyoxylate aminotransferase (Figure 8).

Buffer		Alanine:Glyoxylate Aminotransferase	
		Peak 1	Peak 2
		μmoles x min^{-1} x ml^{-1}	
Cacodylate	pH 7	1.15	0.44
Borate	pH 8	1.11	1.52
Phosphate	pH 7	1.25	0.30

are conducted. Preferably, future experiments would involve purification of the aminotransferases to homogeneity and then definite characteristics could be established.

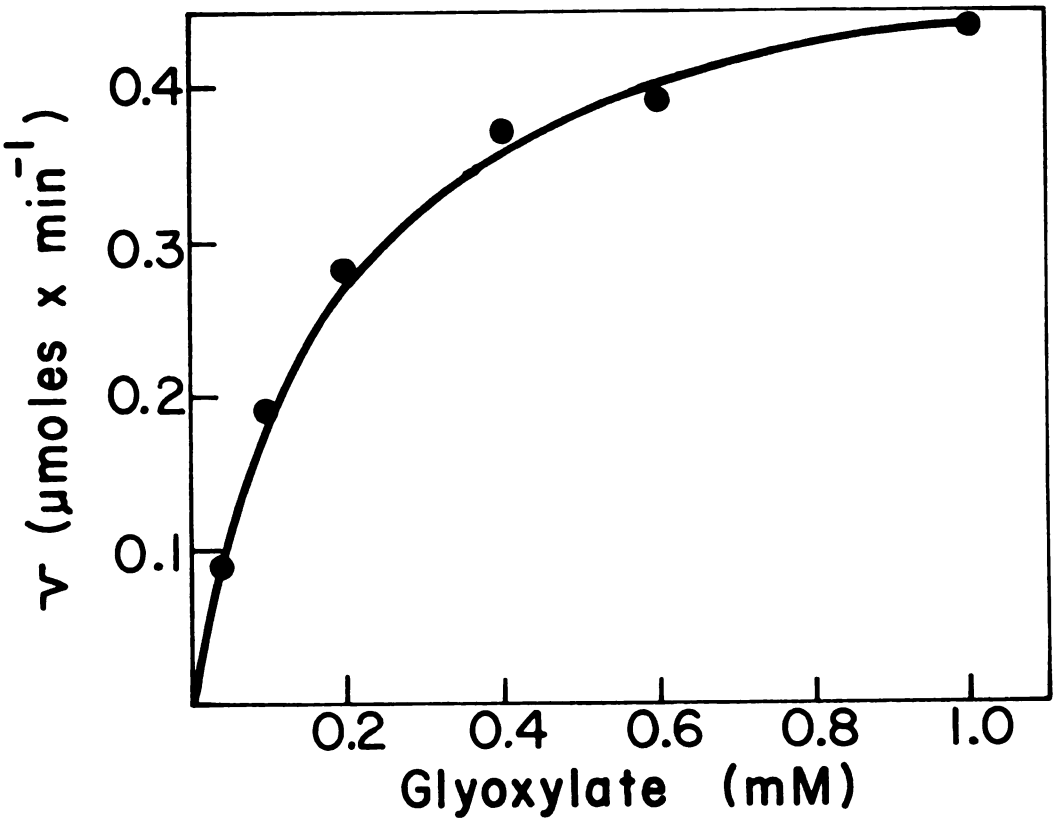
ADDITIONAL STUDIES OF SERINE:GLYOXYLATE AMINOTRANSFERASE

The serine:glyoxylate aminotransferase also catalyzed a serine:pyruvate aminotransferase activity. Yamazaki and Tolbert (174) had reported earlier that a serine:pyruvate aminotransferase was located in spinach leaf peroxisomes. Osmond and Harris (122) found that Atriplex and sorghum contained a serine:glyoxylate aminotransferase activity. The extracts of these plants also catalyzed a serine:pyruvate aminotransferase reaction but at a slower rate than the serine:glyoxylate aminotransferase reaction. Sallach and coworkers (30, 170) have reported that the green leaves of several plants contain alanine:hydroxypyruvate aminotransferase activity. In spinach peroxisomes, the enzyme was more active with glyoxylate than pyruvate and thus the enzyme has been designated as serine:glyoxylate aminotransferase. At a fixed serine concentration of 20 mM, the Km for glyoxylate was 0.15 mM while for pyruvate it was 2.82 mM (Figures 9 and 10). These Km values were determined using the

Figure 9

Reaction Kinetics for Serine:Glyoxylate Aminotransferase With Varying Glyoxylate Concentrations

Isolated spinach leaf peroxisomes and the spectrophotometric assay procedure were used. The serine concentration was 20 mM for all assays.


0.4
0.3
0.2
0.1
v (μmoles x min⁻¹)
0.2
0.4
0.6
0.8
1.0
Glyoxylate (mM)

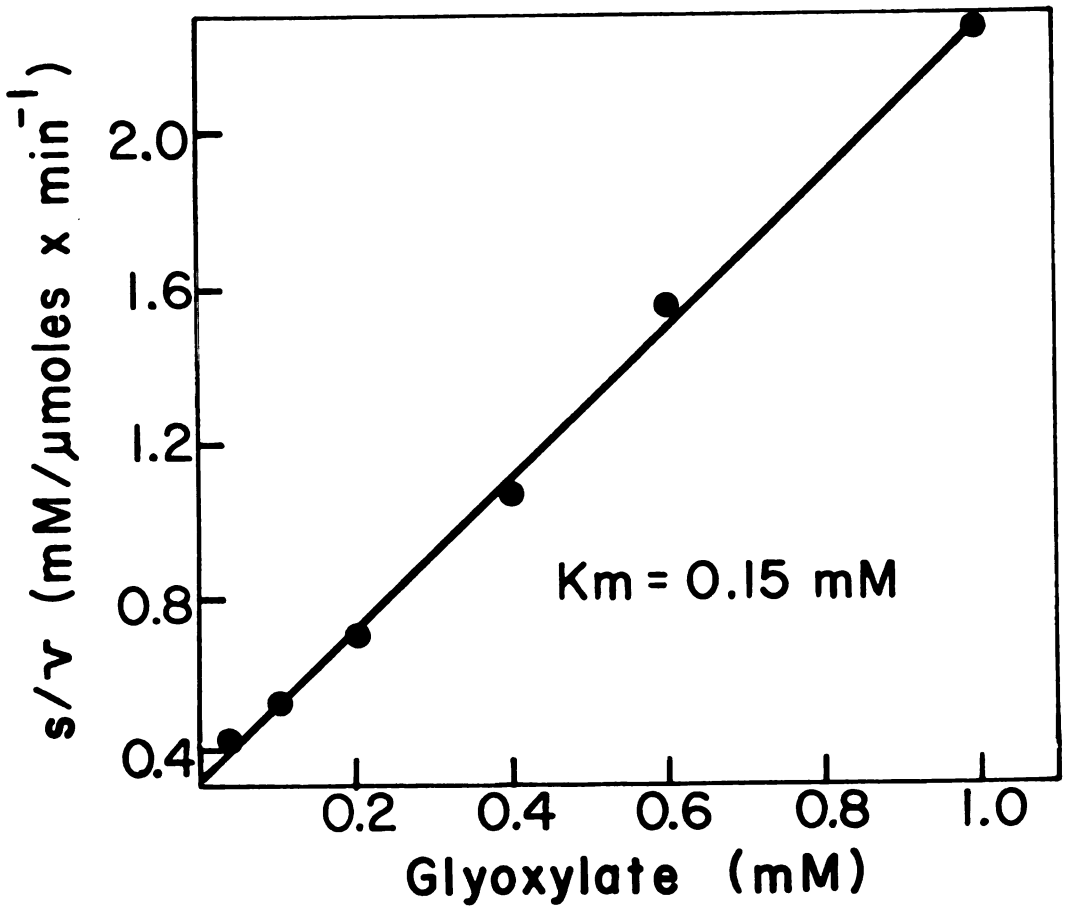
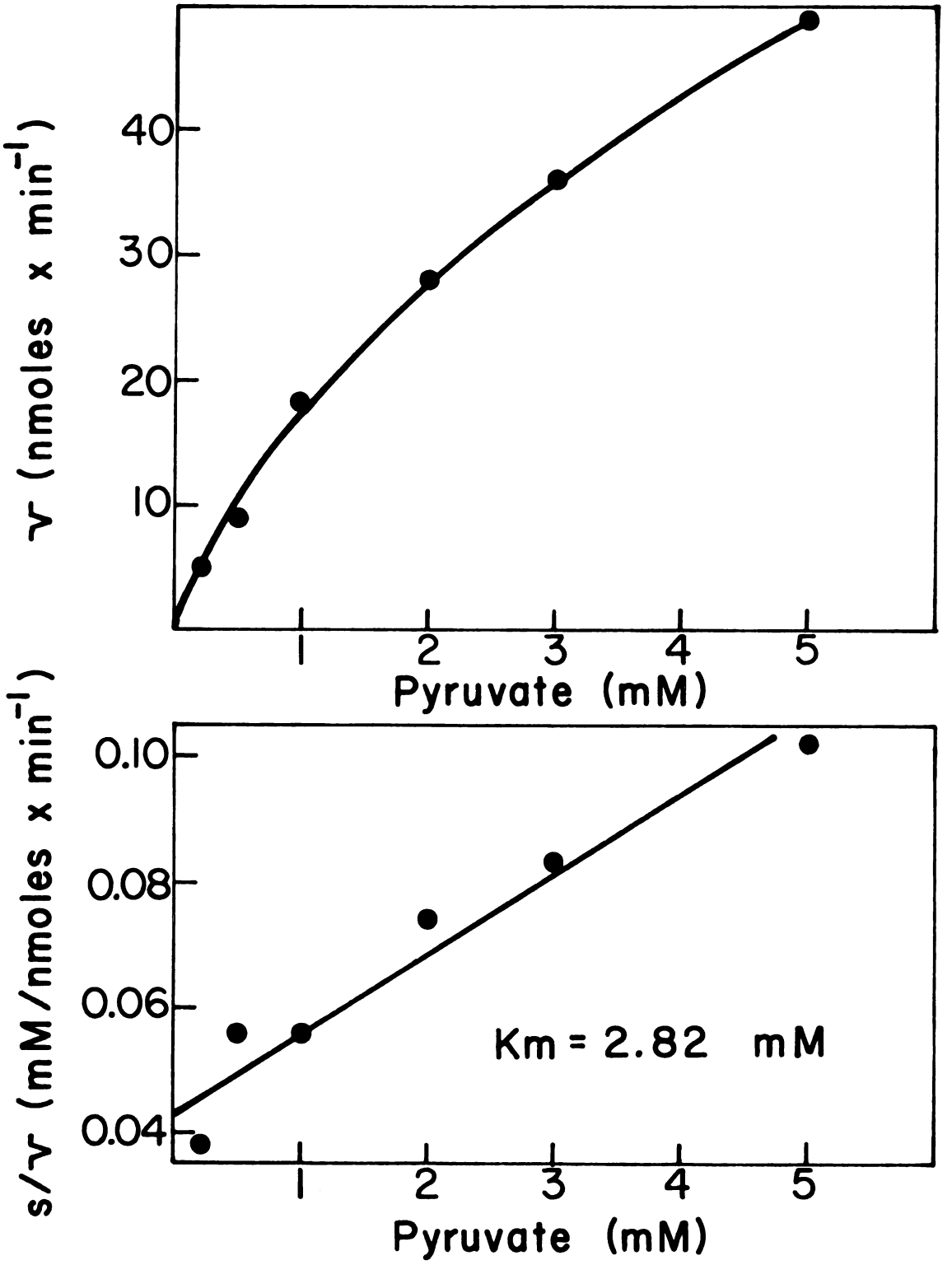

2.0
1.6
1.2
0.8
0.4
s/v (mM/μmoles x min⁻¹)
Km = 0.15 mM
0.2
0.4
0.6
0.8
1.0
Glyoxylate (mM)

Figure 10

Reaction Kinetics for Serine:Pyruvate Aminotransferase Activity With Varying Pyruvate Concentration

Isolated spinach leaf peroxisomes were assayed by the spectrophotometric procedure. The serine concentration was held constant at 20 mM.


v (nmoles x min⁻¹)
40
30
20
10
1
2
3
4
5
Pyruvate (mM)
s/v (mM/nmoles x min⁻¹)
0.10
0.08
0.06
0.04
Km = 2.82 mM
1
2
3
4
5
Pyruvate (mM)

spectrophotometric assay. Even though the linking enzyme in the assay (hydroxypyruvate reductase) acts upon the the glyoxylate, the glyoxylate concentration did not change significantly. In fact at the low glyoxylate concentrations, no endogenous rate was observed because of the high Km that hydroxypyruvate reductase has for glyoxylate (89). The Km for serine was 2.72 mM at a fixed concentration of 1 mM glyoxylate (Figure 11). It appears to be a general characteristic of aminotransferases that the Km for the amino acid is higher than that of the α-keto acid.

Several experiments were performed to try and show that the serine:glyoxylate and serine:pyruvate aminotransferase activities were due to one enzyme. The two activities peaked together on TEAE-cellulose columns and on a pH 3-10 isoelectric focusing column (Figure 7). The recoveries from the isoelectric focusing column were 92% for the serine:glyoxylate aminotransferase and 103% for the serine:pyruvate aminotransferase activity. Both enzyme activities were inhibited by phosphate and D-serine and both activities were equally sensitive to heat denaturation of the protein (Table 5). The serine:pyruvate aminotransferase activity was not inhibited by the D-serine to the same extent as was the serine:glyoxylate aminotransferase activity. This may indicate that D-serine also

Figure 11

Reaction Kinetics for Serine:Glyoxylate Aminotransferase With Varying L-Serine Concentrations

Spinach leaf peroxisomes were isolated on a sucrose gradient and assayed by the spectrophotometric procedure. The glyoxylate concentration was 1 mM for all assays.

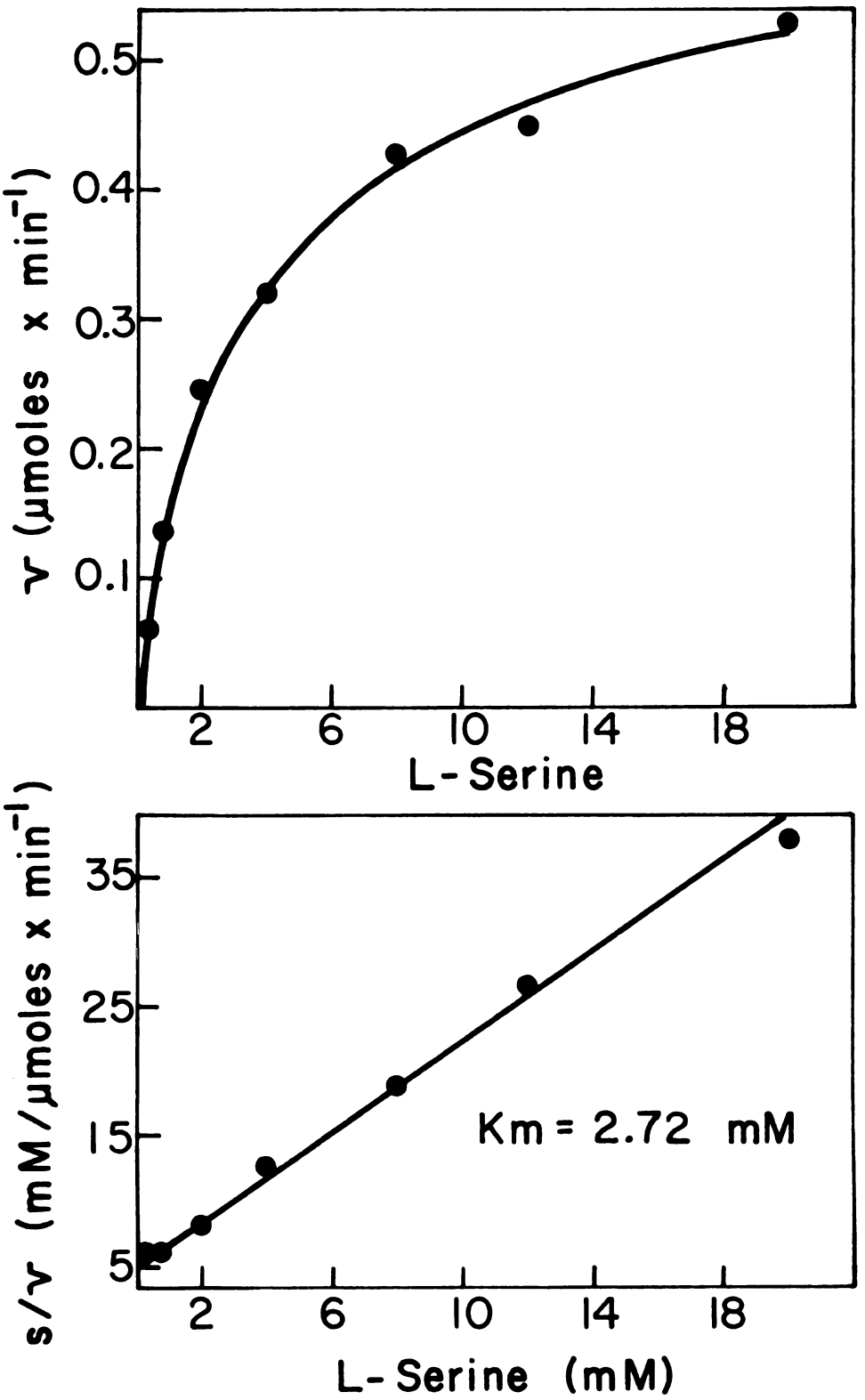

0.5
0.4
0.3
0.2
0.1
v (μmoles x min⁻¹)
2
6
10
14
18
L-Serine
35
25
15
5
s/v (mM/μmoles x min⁻¹)
Km = 2.72 mM
2
6
10
14
18
L-Serine (mM)

Table 5. Comparison of Serine:Glyoxylate and Serine: Pyruvate Aminotransferase Activities

Peroxisomes which were isolated by isopycnic centrifugation in different experiments were used for the various treatments. All assays were by the spectrophotometric procedure. The D- and L-serine were each at 20 mM.

Treatment	Serine:Glyoxylate Aminotransferase	Serine:Pyruvate Aminotransferase	Ratio
	nmoles x min^{-1} x ml^{-1}		
Heated at 50 C			
0 min	955	90	10.6
5	439	46	9.5
15	310	31	10.0
25	284	28	10.1
35	232	26	9.0
Buffer			
HEPES	419	53	8.0
Phosphate	97	0	-
Serine			
L-Serine	232	22	10.6
D-Serine	0	0	-
D- + L-Serine	64	17	3.8

affects the binding of the α-keto acid to the serine: glyoxylate aminotransferase. As shown previously (Figure 5), the D-serine appeared to affect the binding of the L-serine. While these experiments do not prove that only one enzyme is catalyzing both the serine: pyruvate and serine:glyoxylate aminotransferase reactions, they do strongly suggest that only one enzyme is involved.

The serine:glyoxylate aminotransferase was checked for inhibition by other compounds which may be involved in the possible regulation of the enzyme (Table 6). ATP, ADP, AMP, O-phospho-L-serine, 3-P-glycerate and D-glycerate did not significantly inhibit the enzyme. The phosphate inhibition is probably not of physiological importance since at 10 mM the inhibition was only 34%. Because a portion of the glycolate pathway involves nitrogen metabolism the effect of nitrate, nitrite and ammonium ions was checked. Ammonium sulfate at 12 mM was inhibitory but as with phosphate it is probably not of physiological importance. In fact it may have been the sulfate that was causing the inhibition.

REVERSIBILITY OF GLYOXYLATE AMINOTRANSFERASES

Some reports have shown an aminotransferase reaction using glycine as the amino donor (27, 35, 171).

Table 6. Effect of Various Compounds on Peroxisomal Serine:Glyoxylate Aminotransferase

Isolated peroxisomes were assayed by the spectrophotometric procedure. Unless state otherwise, all compounds were at a final concentration of 10 mM.

Compound	Relative Rate
--	100
ATP	71
ADP	86
AMP	83
O-Phospho-L-Serine	76
3-P-Glycerate	79
D-Glycerate	62
$NaNO_3$	77
KNO_2	77
$(NH_4)_2SO_4$ 12 mM	34
K_3PO_4	66
K_3PO_4 70 mM	23
D-Serine 20 mM	31

The authors who observed this activity have generally used crude homogenates and reaction times of 1 hr. There are some reports that glycine:α-keto acid aminotransferase activity could not be detected using partially purified enzymes (23, 150, 151). In spinach leaf peroxisomes, Kisaki and Tolbert (85) did not detect the reverse reaction of glutamate:glyoxylate aminotransferase. In the present studies with isolated peroxisomes, the reverse reactions for glutamate:glyoxylate, serine:glyoxylate and alanine: glyoxylate aminotransferases could not be detected (Table 7 - Lower part). The reason for the apparent lack of the reverse reaction of amino acid:glyoxylate aminotransferase may be because the equilibrium lies so far towards glycine formation that the reverse reaction can not be detected. If the glyoxylate aminotransferases function by a Ping Pong Bi Bi mechanism, as reports suggest (23), then one would expect to observe an isotope exchange between glyoxylate and glycine. Using whole peroxisomes, some glyoxylate-glycine exchange was observed (Table 7 - Upper part) but this exchange was very small. Kisaki and Tolbert (85) also reported a little glyoxylate-glycine exchange. Whether all of the peroxisomal glyoxylate aminotransferases catalyzed this exchange is not known. Thompson and Richardson (151) reported that purified

Table 7. Amino Acid:α-Keto Acid Exchange Reactions and Glycine:α-Keto Acid Aminotransferase in Spinach Peroxisomes

Peroxisomes were isolated by isopynic centrifugation. For the exchange reactions, radioactive amino acid and the corresponding unlabeled α-keto acid (alanine-^{14}C + pyruvate) were used in the radiochemical assay procedure. The formation of radioactive α-keto acid was measured. In the glycine:α-keto acid aminotransferase reaction, the transamination reaction between glycine-^{14}C and α-keto acid was measured by the radiochemical procedure.

Exchange Reactions

^{14}C Amino Acid	α-Keto Acid	^{14}C α-Keto Acid
		nmoles x 30 min^{-1}
Alanine	Pyruvate	1300
Glutamate	α-Ketoglutarate	2000
Aspartate	Oxaloacetate	81
Serine	Hydroxypyruvate	1510
Glycine	Glyoxylate	25

Glycine:α-Keto Acid Aminotransferase

α-Keto Acid	^{14}C Glyoxylate
	nmoles x 30 min^{-1}
Hydroxypyruvate	0
Pyruvate	0
Oxaloacetate	0
α-Ketoglutarate	0

alanine:glyoxylate aminotransferase from human liver did not catalyze a glyoxylate-glycine exchange.

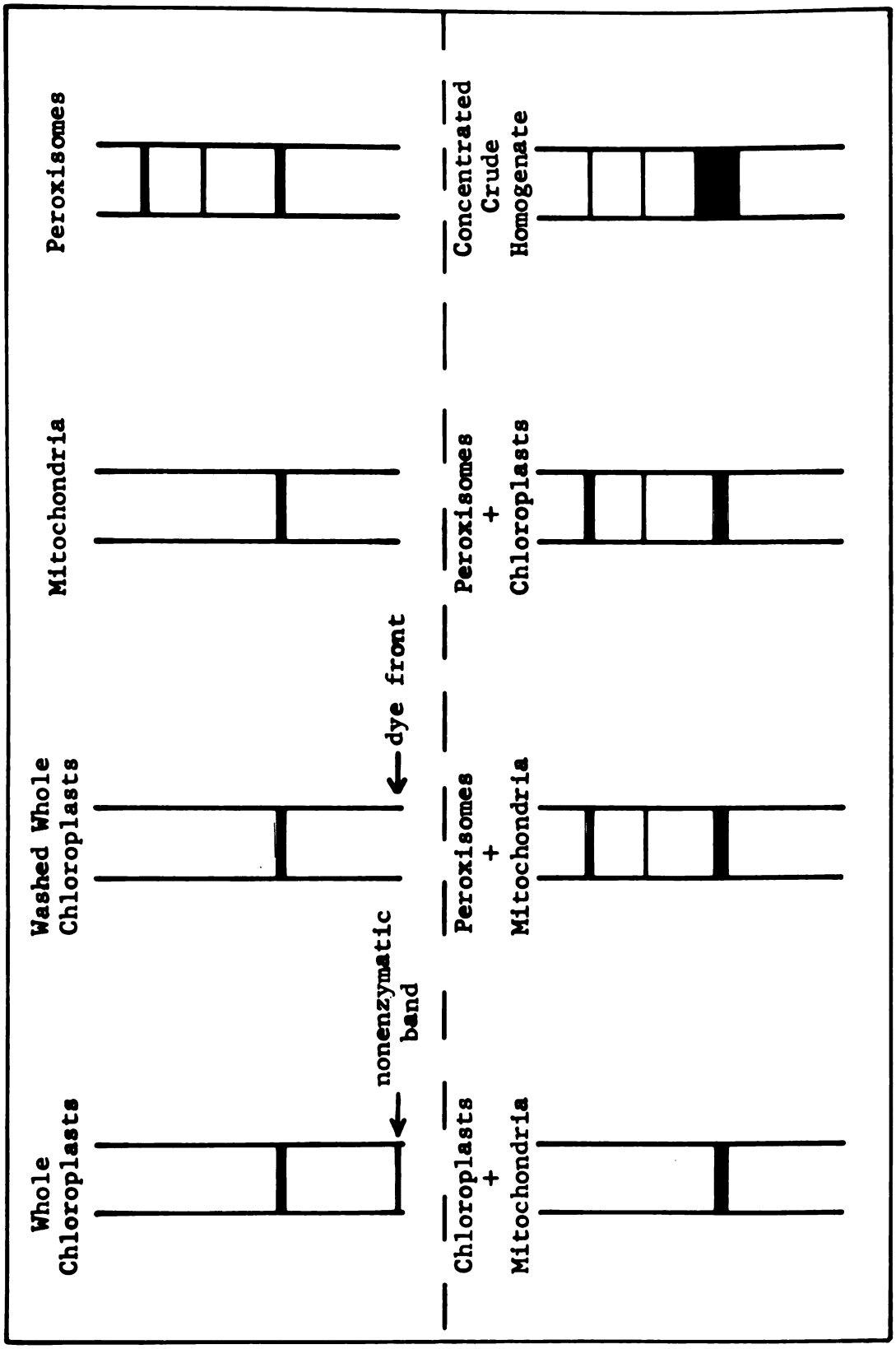
SPINACH LEAF ASPARTATE:α-KETOGLUTARATE AMINOTRANSFERASE

Yamazaki and Tolbert (174) reported this enzyme to be present in chloroplasts, mitochondria and peroxisomes and that the peroxisomes contained 3 isoenzymes. Additional studies on the electrophoretic properties of the aspartate aminotransferases in spinach leaf fractions is reported here. Both chloroplasts and mitochondria had a single protein band that had the same electrophoretic mobility (Figure 12). Mixtures of chloroplastic and mitochondrial fractions likewise, resulted in only one band of enzyme activity. The whole chloroplasts (i.e. the pellet of an initial low speed centrifugation of spinach homogenate) had a band that moved with the dye front and stained in the assay in the absence of aspartate. If the chloroplasts were resuspended and centrifuged through a 30% (w/w) sucrose layer, the nonenzymatic band was not detected. The nature of this nonenzymatic band is unknown, but the report by Yamazaki and Tolbert (174) that there might be another chloroplastic isoenzyme of aspartate aminotransferase is in error. Crude chloroplast fractions sometimes contained one of the peroxisomal

Figure 12

Aspartate Aminotransferase Activities
On Polyacrylamide Gels

The peroxisomes and mitochondria were isolated on a sucrose gradient. The fraction labelled whole chloroplasts was the pellet from centrifuging spinach homogenate at 650 g. The washed whole chloroplast fraction was the pellet obtained by centrifuging the resuspended whole chloroplast fraction through a layer of 30% sucrose. Concentrated homogenate was prepared by using less buffer during homogenization of the leaf tissue.


Whole Chloroplasts
Washed Whole Chloroplasts
Mitochondria
Peroxisomes
nonenzymatic band
dye front
Chloroplasts + Mitochondria
Peroxisomes + Mitochondria
Peroxisomes + Chloroplasts
Concentrated Crude Homogenate

isoenzymes, but this was not consistently seen.

Two slower moving bands of aspartate:α-ketoglutarate aminotransferase on the polyacrylamide gels were distinctly peroxisomal (Figure 12). A third fast moving enzyme band in the peroxisomes had the same mobility as the enzyme in the mitochondria and chloroplasts. Mixtures of the samples showed that the fast moving band in all three organelles was the same enzyme. The peroxisomal bands were not observed in dilute leaf homogenates, but in concentrated homogenates the two peroxisomal bands were detectable. It should be noted that in whole leaf homogenates of spinach leaves there were only the three isoenzyme bands and every band corresponded to one of the particulate isoenzymes. In other words, there was no specific soluble isoenzyme for the cytosol. It has been reported many times that mammalian tissues contain a soluble and a mitochondrial form of aspartate aminotransferase (136).

The spinach leaf isoenzymes of aspartate aminotransferase were also examined by starch gel electrophoresis (Figure 13). A single isoenzyme in the chloroplastic and mitochondrial fractions again had identical mobility. The peroxisomal activity was in two bands: a dark band of low mobility and a trace of activity located

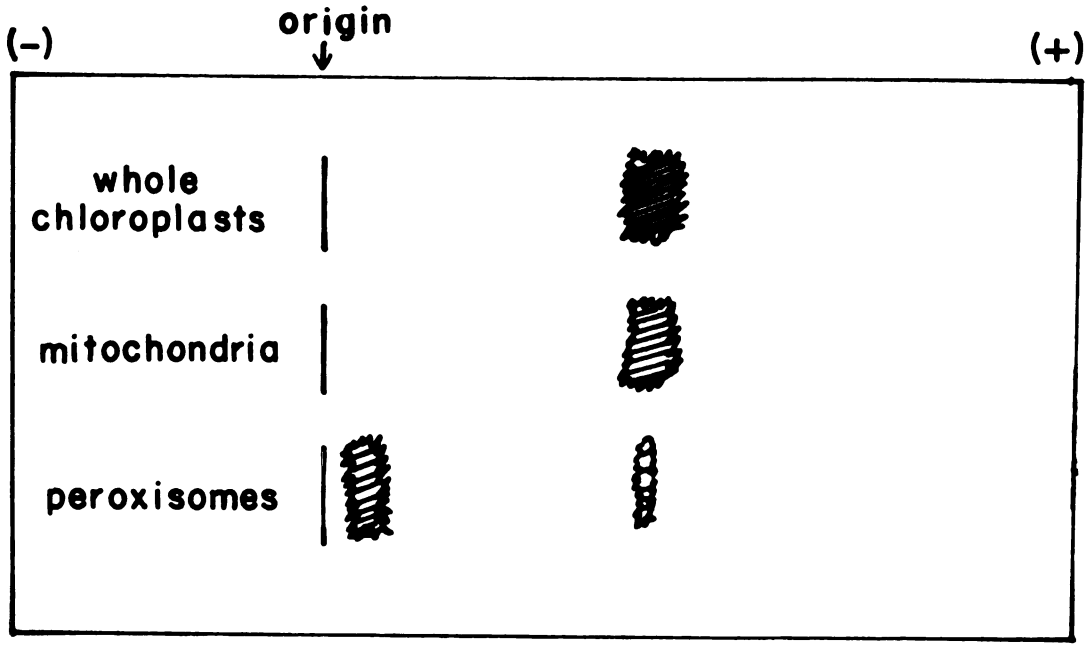
at the same R_F as the mitochondrial chloroplastic isoenzyme (Figure 13). This data on the peroxisomal forms of the enzyme was thus considerably different from the results with polyacrylamide gel separation but support the idea that the peroxisomes do not contain the same enzyme as the mitochondria and chloroplast.

To determine if any of the three peroxisomal aspartate aminotransferase isoenzyme bands were due to some nonspecificity of the glyoxylate aminotransferases, the proteins in isolated peroxisomes were separated on a TEAE-cellulose column. Upon elution with a linear KCl gradient, three peaks of aspartate aminotransferase were observed (Figure 6). None of the three peaks coincided with the serine:glyoxylate aminotransferase. As mentioned previously, the glutamate:glyoxylate and alanine:glyoxylate aminotransferase activities could not be detected on TEAE-cellulose columns. In a fraction where the aspartate aminotransferase and serine:glyoxylate aminotransferase overlapped, assays containing all four substrates were the sum of the individual aminotransferase activities (data not shown). These results strongly suggest the aspartate aminotransferase isoenzymes in the peroxisomes are not the result of nonspecificity of the glyoxylate aminotransferases.

Figure 13

Starch Gel Electrophoresis of Aspartate Aminotransferase Activities

The whole chloroplasts were prepared by differential centrifugation while the peroxisome and mitochondria were isolated by isopynic centrifugation in sucrose gradients. Both the gel and the electrode tanks contained 5 mM Tris-glycine at pH 8.3. Electrophoresis was at 400 volts for 10 hr.


(-)
origin
(+)
whole chloroplasts
mitochondria
peroxisomes

Each of the three peaks of aspartate aminotransferase from a TEAE-cellulose column was subjected to polyacrylamide electrophoresis. Each peak contained a different isoenzyme (Figure 14). The peak, which eluted first off of the TEAE-cellulose column, was called peak 1 and contained the slowest moving electrophoretic band, labelled band 1. Peak 2 contained the central electrophoretic band, and peak 3 the fastest moving band. There was some cross contamination, as could be expected by the TEAE-cellulose elution pattern. The electrophoretic band 1 from the TEAE-cellulose columns was not one distinct band, but it appeared to be 2 or 3 closely spaced bands. This phenomena was observed only on samples from the TEAE columns. Removal of the KCl in peak 1 by dialysis did not change the electrophoretic pattern.

In some earlier experiemnts, the aspartate aminotransferase activity in the mitochondrial fraction of a sucrose gradient appeared to be inhibited by phosphate buffer more than the activity in the peroxisomal fraction. Therefore assays on the three peaks of aspartate aminotransferase from the TEAE-cellulose column were run in different buffers (Table 8). The activity in peak 3 was inhibited by phosphate buffer similarly to the mitochondrial form of the enzyme. As mentioned previously, peak 3

Figure 14

Separation of the Aspartate Aminotransferase Isoenzymes by Ion Exchange Chromatography and Their Electrophoretic Patterns

The peroxisomal aspartate aminotransferase activity was eluted from a TEAE-cellulose column with a linear KCl gradient as in Figure 6. An aliquot from each peak was then run on polyacrylamide gel electrophoresis and stained for aspartate aminotransferase activity.

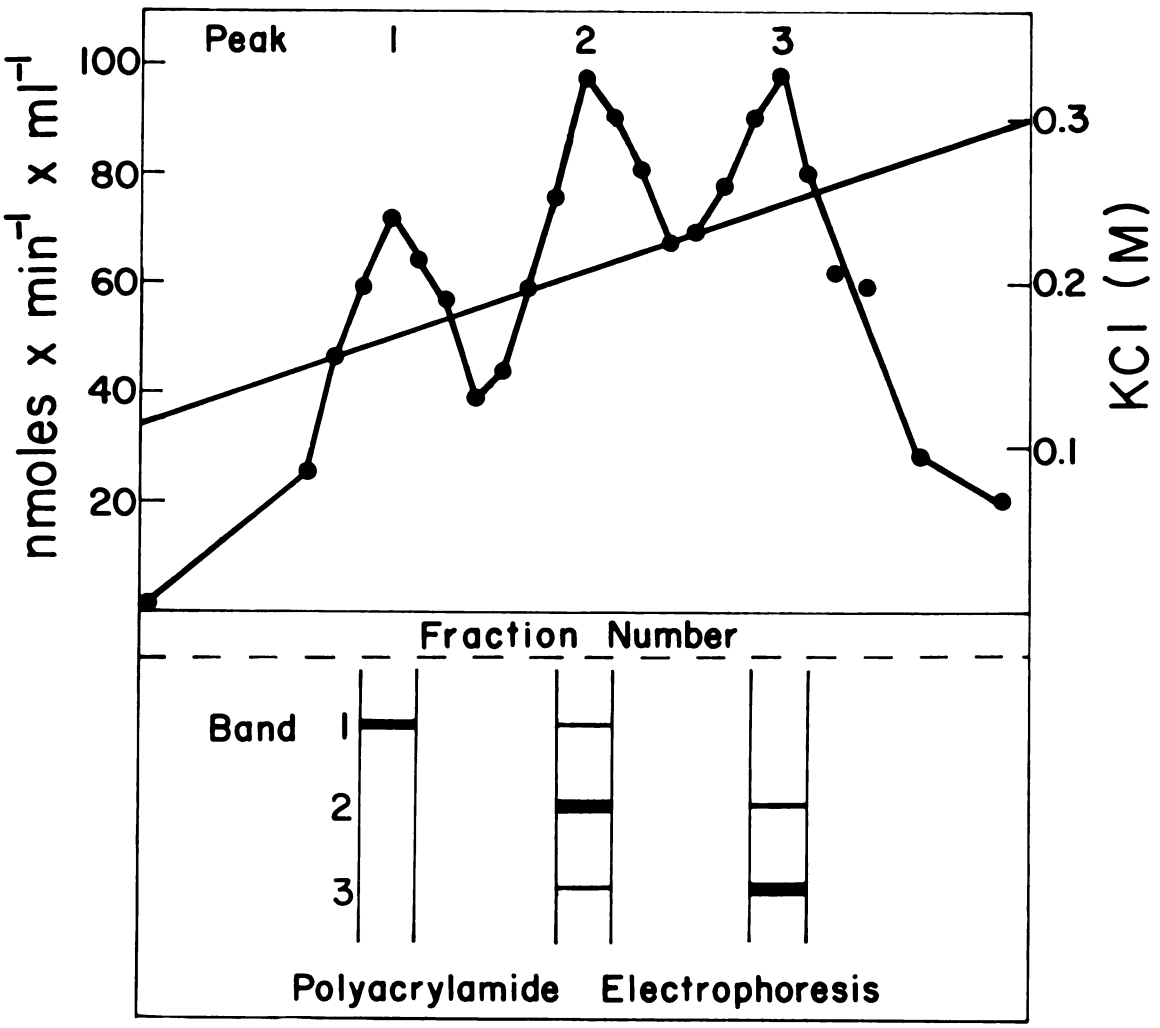

Peak
1
2
3
100
80
60
40
20
nmoles x min⁻¹ x ml⁻¹
0.3
0.2
0.1
KCl (M)
Fraction Number
Band
1
2
3
Polyacrylamide Electrophoresis

Table 8. Characteristics of Aspartate Aminotransferase Peaks From a TEAE-Cellulose Column

The fractions from a TEAE-cellulose column (Figure 14) were assayed by the spectrophotometric procedure.

Buffer		Peak		
		1	2	3
		Relative Rates		
MES	pH 7.4	100	100	100
MES	pH 6.7	80	84	41
Phosphate	pH 7.1	86	97	68

contained the fastest moving isoenzyme, which on the polyacrylamide gels had the same mobility as the form of the enzyme in the mitochondria. This isoenzyme was also more inhibited by MES buffer slightly below the pH optimum than the other two distinctly peroxisomal isoenzymes.

The separation of the 3 peroxisomal aspartate aminotransferase activities was also achieved on a pH 3-10 isoelectric focusing column (Figure 7). It should be noted that none of the aspartate:α-ketoglutarate aminotransferase peaks correspond exactly to any of the glyoxylate aminotransferases. These data confirm the results of the TEAE-cellulose column, that the aspartate aminotransferase peaks were not caused by any of the three glyoxylate aminotransferases. Boiled controls of the aspartate aminotransferase peaks had no activity and the recovery from the column was 100%. Yamazaki and Tolbert (174) reported finding only one peak of aspartate aminotransferase on an isoelectric focusing column. No explanation for this difference in the results is known.

It is not clear whether peroxisomes contain two isoenzymes or three isoenzymes of aspartate aminotransferase. The fastest moving isoenzyme band in the peroxisomes has the same characteristics as the mitochondria-chloroplast isoenzyme. However, this does not

mean the fast moving band is not a peroxisomal enzyme. Based upon the intensity of the stain in the polyacrylamide gels, the fastest moving band in the peroxisomes seemed to comprise 40-50% of the total activity. It is estimated that, of the total peroxisomal aspartate aminotransferase activity, only 15% was due to contamination by the other organelles (Table 1). Thus the fastest moving band may also be a peroxisomal enzyme. In the starch gel electrophoresis, the aspartate aminotransferase activity in the peroxisomes did not form three bands, but was present as one major band of low mobility and a little activity corresponding to the mitochondria-chloroplast isoenzyme. This data suggests that most of the aspartate aminotransferase activity in the peroxisomes was not separated by starch gel electrophoresis, but that a small amount of contamination by other organelles was present and moved separately.

SUMMARY OF SPINACH PEROXISOMAL AMINOTRANSFERASES

All aminotransferase activities with glyoxylate were located exclusively in the peroxisomes, where the glyoxylate is formed. These reactions were irreversible under the experimental conditions employed. Spinach peroxisomes contain a serine:glyoxylate aminotransferase

which appears to have some activity as a serine:pyruvate aminotransferase and possibly also some alanine:glyoxylate aminotransferase activity. A glutamate:glyoxylate aminotransferase is a separate peroxisomal protein and this enzyme appears to have alanine:glyoxylate aminotransferase activity. The specific activities of the peroxisomal aminotransferases show glutamate:glyoxylate to be the most active (Table 9). The descending order of the remaining specific activities are serine:glyoxylate aminotransferase, alanine:glyoxylate aminotransferase, aspartate:α-ketoglutarate aminotransferase and the activity for the serine:pyruvate aminotransferase reaction.

Kisaki and Tolbert (85) reported that in spinach leaf peroxisomes the most active aminotransferase was glutamate:glyoxylate aminotransferase, followed by alanine:glyoxylate and serine:glyoxylate aminotransferase activities. Probably the reason these authors observed less serine:glyoxylate aminotransferase activity is that they used phosphate buffer and DL-serine in their assays and as it has been shown, both phosphate and D-serine inhibit serine:glyoxylate aminotransferase.

Spinach peroxisomes also contain an aspartate:α-ketoglutarate aminotransferase which forms 3 isoenzyme

Table 9. Specific Activities of Aminotransferase Activities in Isolated Spinach Leaf Peroxisomes

Enzyme	μmoles x min^{-1} x mg $protein^{-1}$
Glutamate:Glyoxylate	2.40
Serine:Glyoxylate	1.54
Alanine:Glyoxylate	0.87
Aspartate:α-Ketoglutarate	0.15
Serine:Pyruvate	0.03

bands on polyacrylamide gels. This result is in agreement with the work of Yamazaki and Tolbert (174).

PRELIMINARY STUDIES ON THE SUBCELLULAR LOCATION OF AMINOTRANSFERASES IN MAMMALIAN TISSUES

Rat Liver

Mammalian tissues are reported to contain a mitochondrial and a soluble form of aspartate aminotransferase. de Duve and coworkers (8) and Müller (113) looked for aspartate aminotransferase in rat liver peroxisomes but concluded it was not a peroxisomal enzyme. Likewise, de Duve (40) reported that rat liver peroxisomes did not contain glutamate:glyoxylate aminotransferase. However, Vandor and Tolbert (162) did observe glutamate: glyoxylate aminotransferase activity in the rat liver peroxisomes. The subcellular location of alanine:glyoxylate aminotransferase in rat liver was recently reported in an abstract by Snell *et al*. (147), who concluded that a large portion of this aminotransferase activity was located in the mitochondria.

In a group effort in the laboratory, rat liver peroxisomes were isolated in sucrose gradients using a procedure similar to that described by de Duve *et al*. (43). I assayed fractions for aminotransferase activities (Table 10). All glyoxylate aminotransferase assays were carried

Table 10. Distribution of Aminotransferase Activities Among Particles From Rat Liver

Subcellular organelles were isolated on a sucrose gradient in a zonal rotor. All aminotransferase activities were assayed by the radiochemical procedure except for aspartate:α-ketoglutarate aminotransferase which was assayed by the spectrophotometric procedure.

Aminotransferase	Peroxisomes	Mitochondria	Supernatant	Mitochondrial Activity in Peroxisomal Fraction[a]	Peroxisomal Activity in Mitochondrial Fraction[b]
	nmoles x min^{-1} x ml^{-1}			%	%
Alanine: Glyoxylate	121	508	127	6	10
Glutamate: Glyoxylate	9	0	155	-	-
Serine: Glyoxylate	87	39	43	0.9	70
Aspartate: α-Ketoglutarate	310	16,800	2,840	120	0.5

[a]Values are the percentage of the aminotransferase activity in the peroxisomal fraction which can be attributed to mitochondrial contamination of the peroxisomal fraction (see Table 1).

[b]Values are the percentage of the aminotransferase activity in the mitochondrial fraction which can be attributed to peroxisomal contamination of the mitochondrial fraction (see Table 1).

out using the radiochemical procedure because the mammalian tissues had a high endogenous rate in the spectrophotometric procedure.

Aspartate:α-ketoglutarate aminotransferase was located in the mitochondria and all of the activity that was in the peroxisomal fraction could be attributed to mitochondrial contamination (Table 10). The rat liver peroxisomes differ in this respect from the peroxisomes of Tetrahymena pyriformis and spinach leaves as well as the glyoxysomes of castor bean endosperm, all of which do contain aspartate aminotransferase (40).

The rat liver peroxisomes contained a serine:glyoxylate aminotransferase activity. The mitochondria also contained some serine:glyoxylate aminotransferase activity but most of it appeared to be from peroxisomal contamination. Most of the alanine:glyoxylate aminotransferase activity was located in the mitochondria as previously reported by Snell et al. (147). However, the peroxisomes also contained a significant amount of this activity. Mitochondrial contamination of the peroxisomes could account for only 6% of this peroxisomal alanine:glyoxylate aminotransferase activity.

The serine:glyoxylate, alanine:glyoxylate and aspartate:α-ketoglutarate aminotransferase activities were

also present in the supernatant fraction. It has often been reported that mammalian tissues have a distinct soluble aspartate aminotransferase isoenzyme (17) and apparently this isoenzyme is not a peroxisomal enzyme. Snell et al. (147) reported that the soluble alanine: glyoxylate aminotransferase had a different Km value and pH curve than the mitochondrial enzyme. It is possible that this "soluble form" is actually due to broken peroxisomes. Likewise the soluble serine:glyoxylate aminotransferase may be due to the breakage of peroxisomes. Most of the glutamate:glyoxylate aminotransferase activity was located in the supernatant fraction from the rat liver homogenate. A very small amount of activity observed in the peroxisomes is probably not of significance.

Since in spinach leaves the serine:glyoxylate aminotransferase was inhibited by phosphate, the rat liver glyoxylate aminotransferases were also tested for phosphate inhibition (Table 11). As in spinach leaves, the serine:glyoxylate aminotransferase in rat liver peroxisomes was more sensitive to phosphate inhibition than was the alanine:glyoxylate aminotransferase activity. All three glyoxylate aminotransferases, particularly glutamate: glyoxylate aminotransferase, were inhibited by Triton X-100 treatment. In the case of the very low levels of glutamate:glyoxylate aminotransferase, the phosphate appeared to protect the activity against the Triton X-100 inhibition.

Table 11. The Effect of Phosphate and Triton X-100 on Rat Liver Peroxisomal Glyoxylate Aminotransferases

Rat liver peroxisomes were isolated on a sucrose gradient and assayed by the radiochemical procedure. Buffers were at pH 7.

Buffer	Serine: Glyoxylate	Alanine: Glyoxylate	Glutamate: Glyoxylate
	Relative Rates		
Cacodylate	100	100	100
Cacodylate + Triton X-100	73	84	0
Phosphate + Triton X-100	39	85	73

Dog Kidney

Aminotransferase activity of isolated kidney peroxisomes has not been reported. Dog kidney peroxisomes were isolated in a sucrose gradient in a zonal rotor and assayed for four aminotransferases listed in Table 12. The major portion of all four aminotransferases activities was located in the mitochondrial fraction. The peroxisomes appeared to contain small amounts of all of the aminotransferase activities except for glutamate:glyoxylate aminotransferase which was not detected in the peroxisomes. Mitochondrial contamination accounted for 71% of the peroxisomal aspartate aminotransferase activity and therefore this enzyme may not be in dog kidney peroxisomes. This 71% contamination was calculated on the basis of the total activity of aspartate aminotransferase observed in the mitochondrial fraction with Triton X-100 present in the assays. Triton X-100 treatment of the mitochondria was found to release considerable latent aspartate aminotransferase activity. The glyoxylate aminotransferase assays did not contain Triton X-100 as it was inhibitory to these reactions in the rat liver peroxisomes. The glyoxylate aminotransferase activities observed in the peroxisomes are probably near the total activity present in the organelles since peroxisomes did not exhibit much

Table 12. Distribution of Aminotransferase Activities Among Particles From Dog Kidney

Subcellular organelles were isolated on a sucrose gradient in a zonal rotor. All aminotransferase activities were assayed by the radiochemical procedure except for aspartate:α-ketoglutarate aminotransferase which was assayed by the spectrophotometric procedure.

Aminotransferase	Peroxisomes	Mitochondria	Supernatant	Mitochondrial Activity in Peroxisomal Fraction[a]	Peroxisomal Activity in Mitochondrial Fraction[b]
	nmoles x min^{-1} x ml^{-1}			%	%
Alanine: Glyoxylate	34	1242	56	32	2
Glutamate: Glyoxylate	0	522	197	-	-
Serine: Glyoxylate	34	423	70	26	3
Aspartate: α-Ketoglutarate	64	555	15	71	2

[a]Values are the percentage of the aminotransferase activity in the peroxisomal fraction which can be attributed to mitochondrial contamination of the peroxisomal fraction (see Table 1).

[b]Values are the percentage of the aminotransferase activity in the mitochondrial fraction which can be attributed to peroxisomal contamination of the mitochondrial fraction (see Table 1).

latency with aspartate aminotransferase. Since mitochondria did exhibit latency, the true glyoxylate aminotransferase activities in the mitochondria may be 2-3 times higher than reported in Table 12. If this would be the case, then nearly all of the observed activity in the peroxisomal fraction would be attributable to mitochondrial contamination. Data from the Triton X-100 treatment of mitochondria may be very difficult to interpret if both latency and inhibition of the enzymes occur.

The amount of the aminotransferase activities in the kidney peroxisomal fraction was very small and it is likely that these activities are insignificant. Lee (96) observed in electron micrographs of kidney that the aspartate aminotransferase was concentrated in the subapical vesicles, which may be physically similar to peroxisomes.

DISCUSSION

Spinach leaf peroxisomes were found to contain a serine:glyoxylate aminotransferase, an alanine:glyoxylate aminotransferase and an aspartate:α-ketoglutarate aminotransferase was located only in the peroxisomes. This distribution agrees with Yamazaki and Tolbert (174) who measured the enzyme by its serine:pyruvate aminotransferase

reaction. Recent studies have also shown serine:glyoxylate and glutamate:glyoxylate aminotransferase activities are present in the microbodies of castor bean endosperm and sunflower cotyledons (Schnarrenberger, Oeser and Tolbert, in press). Cooper and Beevers (33) had reported earlier that castor bean glyoxysomes did not contain glutamate:glyoxylate aminotransferase. In the C_4-plant, Atriplex spongioso, Osmond and Harris (7) found the serine:glyoxylate aminotransferase is mainly located in the bundle sheath cells. The significance of this observation will become evident in the next chapter of this thesis.

The spinach serine:glyoxylate aminotransferase was inhibited by phosphate. The nature of this inhibition is not known, but Severin and Dixon (143) have reported phosphate interferes with the recombination of pyridoxal-5-phosphate with apoaspartate aminotransferase. However, the serine:glyoxylate aminotransferase activity in isolated spinach peroxisomes did not show a requirement for exogenous pyridoxal-5-phosphate. The significance of phosphate inhibition of the serine:glyoxylate aminotransferase activity is unknown. Peroxisomal metabolism does not utilize phosphorylated substrates or form ATP or phosphorylated products. Thus phosphates might be important

regulants upon peroxisomal activity.

The kinetic studies of the D-serine inhibition of serine:glyoxylate aminotransferase indicate the D-serine affects the binding of the L-serine. In the presence of D-serine, the Lineweaver-Burk plot, with L-serine as the variable substrate, was nonlinear. These results are similar to those reported for malic enzyme from E. coli which showed substrate cooperativity only in the presence of an allosteric inhibitor of the enzyme (138). Aspartate aminotransferase from rat liver has recently been shown to be inhibited by DL-glyceraldehyde-3-phosphate (90, 91). However, the Lineweaver-Burk plots at various concentrations of DL-glyceraldehyde-3-phosphate were linear. The serine:glyoxylate aminotransferase was not active with D-serine. D-amino acid aminotransferases have been found in microorganisms (136). Breidenbach reported the glyoxysomes in castor bean endosperm contained D-aspartate:α-ketoglutarate aminotransferase activity (21). D-aspartate did not significantly inhibit the peroxisomal aspartate aminotransferase nor did D-alanine inhibit the alanine:glyoxylate aminotransferase activity. Glutamate: glyoxylate aminotransferase has also been reported not to be inhibited by D-glutamate (79).

The serine:glyoxylate inhibition by phosphate may

not be of physiological significance because mM concentrations were required. However, phosphate concentration in plant cells is often quite substantial. However, another compound or compounds of similar nature may be found which would be the physiological regulator of this enzyme. It may be that the D configuration and/or phosphate are important for binding to the enzyme, and if so a phosphorylated carbohydrate or amino acid may be the in vivo regulator of this transaminase.

The aspartate:α-ketoglutarate aminotransferase activity was found to be present in the chloroplasts, mitochondria and peroxisomes, as Yamazaki and Tolbert (174) had previously reported. The chloroplastic and mitochondrial enzymes had the same electrophoretic characteristic and thus appear to be the same enzyme. One could speculate that the enzyme is coded by the same nuclear gene. The peroxisomes had an isoenzyme which had the characteristics of the chloroplastic-mitochondrial enzyme. In addition, the peroxisomes had two other isoenzymes of aspartate aminotransferase. None of the three isoenzymes of aspartate aminotransferase activity were due to the nonspecific activity of the glyoxylate aminotransferases present in the peroxisomes. However, other aminotransferases may be present in the peroxisomes and if they had

some activity towards aspartate and α-ketoglutarate, they could explain the reason for the three isoenzymes of aspartate aminotransferase. Another possible explanation for the observed isoenzymes is that different amounts of another protein such as malate dehydrogenase are stuck to the aspartate aminotransferase. It has been reported that partially purified aspartate aminotransferase from cottonseeds had three bands of activity on electrophoretic gels and the bands also had malate dehydrogenase activity (50). Likewise aspartate aminotransferase and malate dehydrogenase from Neurospora tended to stick together (88). Malate dehydrogenase sticking to aspartate aminotransferase is probably not the reason for the observed peroxisomal isoenzymes in spinach. Even though the malate dehydrogenase smeared and appeared to stick to the other proteins during ion exchange chromatography, its elution pattern did not follow the aspartate aminotransferase peaks. Also, mitochondria contain malate dehydrogenase and yet only one band of aspartate aminotransferase was observed in the polyacrylamide gels.

Yamazaki and Tolbert (174) reported that of the aminotransferases present in spinach peroxisomes, aspartate aminotransferase had the highest specific activity while glutamate:glyoxylate aminotransferase had the lowest

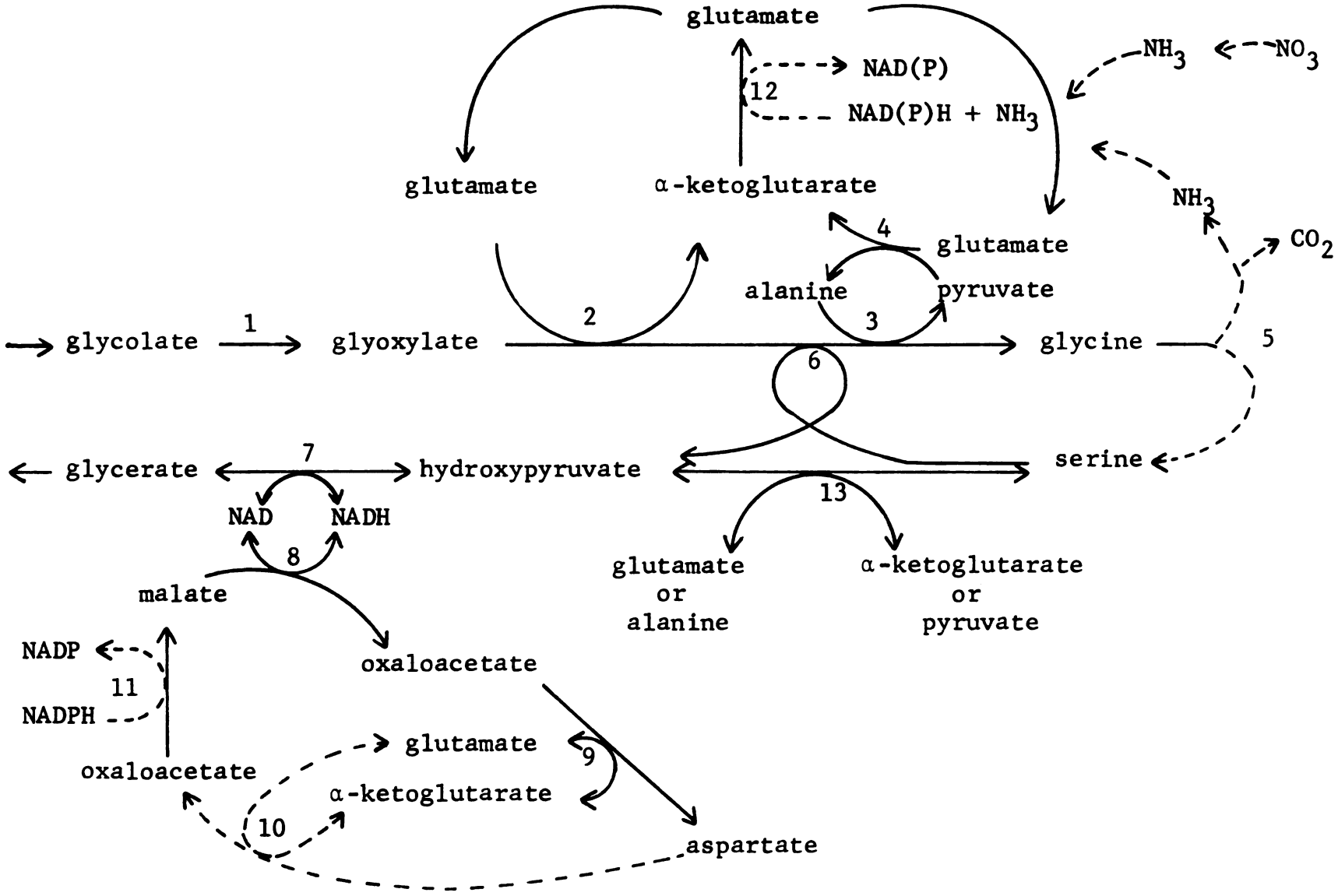
specific activity. The present findings give an opposite conclusion. A portion of the higher aspartate aminotransferase activity previously observed in the peroxisomes may have been due to more mitochondrial contamination. Kisaki and Tolbert (85) observed that the glutamate:glyoxylate aminotransferase activity in spinach peroxisomes was five times more active than the glutamate:oxaloacetate aminotransferase activity. Several authors (1, 16, 37) have reported that the glutamate plus oxaloacetate reaction is 2-3 times faster than the reverse reaction, but other authors (33) report aspartate plus α-ketoglutarate is 2-5 times faster. In either case, the aspartate:α-ketoglutarate aminotransferase does not appear to be more active than the glutamate:glyoxylate aminotransferase in spinach leaf peroxisomes.

The two spinach peroxisomal aminotransferases with the highest specific activities are postulated to function in the main pathway of glycolate metabolism as depicted in Figure 15. The numbers in the figure refer to the reactions discussed in this text. In reaction 1 glycolate, which is synthesized in the chloroplast, is oxidized in the peroxisome to glyoxylate by glycolate oxidase. Reaction 2 is the formation of glycine by glutamate:glyoxylate aminotransferase. This formation of glycine

Figure 15

Proposed Sequence of Reactions Occurring
During the Operation of the Glycolate Pathway

Reactions in solid lines occur in the leaf peroxisomes while reactions of dashed lines occur outside of the peroxisomes. The numbers refer to reactions which are discussed in the text.


glutamate
NAD(P)
12
NAD(P)H + NH3
NH3
NO3
glutamate
α-ketoglutarate
NH3
4
glutamate
CO2
alanine
pyruvate
2
3
glycolate
1
glyoxylate
glycine
5
6
glycerate
7
hydroxypyruvate
serine
NAD
NADH
13
8
glutamate
or
alanine
α-ketoglutarate
or
pyruvate
malate
NADP
oxaloacetate
11
NADPH
glutamate
9
oxaloacetate
α-ketoglutarate
10
aspartate

can also occur with alanine serving as the amino donor (reaction 3). This alanine:glyoxylate aminotransferase reaction is probably also catalyzed by the glutamate: glyoxylate aminotransferase. The regeneration of alanine can occur with glutamate serving as the amino donor (reaction 4). The glutamate:glyoxylate aminotransferase may catalyze this glutamate:pyruvate aminotransferase reaction since mixed substrate studies indicate they were the same enzyme (85). Thus the glutamate:glyoxylate aminotransferase functions in the conversion of glyoxylate to glycine whether glutamate or alanine serves as the amino donor. The regeneration of glutamate from α-ketoglutarate in reaction 2 and 4 will be discussed later.

The glycine to serine conversion apparently occurs in the mitochondria. Bruin (25) and Kisaki and Tolbert (85) have reported the mitochondrial fraction of sucrose gradients could catalyze the glycine to serine conversion. Recently, Kisaki *et al*. (84, 87) further investigated the mitochondria as the subcellular location of this activity. The serine enters the peroxisome and is converted to hydroxypyruvate by serine:glyoxylate aminotransferase (reaction 6). This conversion was previously postulated to occur with pyruvate serving as the amino acceptor (158). The serine:glyoxylate aminotransferase

reaction eliminates the necessity of alanine and pyruvate being intermediates and as such permits the pathway to operate more independently. The serine:glyoxylate aminotransferase had a Km (glyoxylate) of 0.15 mM while the glutamate:glyoxylate aminotransferase reportedly had a Km (glyoxylate) of 4.4 mM (85). These values would suggest that any serine present in the peroxisome would preferentially be used as the amino donor for glyoxylate and thus keep the glyoxylate pathway functioning to form glycerate. If the glutamate was used preferentially, serine would tend to accumulate. The pool size of serine may be controlled by regulators acting upon the serine: glyoxylate aminotransferase. The formation of glycerate from hydroxypyruvate (reaction 7) is catalyzed by hydroxypyruvate reductase. Reducing power is shuttled into the peroxisome via malate dehydrogenase and the oxaloacetate product is converted to aspartate by the aspartate aminotransferases of the peroxisome (reactions 8 and 9). The aspartate could then move or diffuse to the chloroplast, where it would be converted to oxaloacetate by the chloroplast aspartate aminotransferase (reaction 10). The chloroplast NADP-malate dehydrogenase would reduce the oxaloacetate to malate (reaction 11) to complete a cycle for the transport of reducing capacity into

the peroxisomes. It should be noted that no net nitrogen or ammonia source is needed for this shuttle if the glutamate and α-ketoglutarate move between chloroplast and peroxisome.

The regeneration of glutamate for reactions 2 and 4 is shown as occurring in two possible ways. The α-ketoglutarate could undergo reductive amination by NADP-glutamate dehydrogenase which is known to be in the chloroplast (reaction 12) (97). This pathway would be a means for the plant to transfer and utilize the ammonia formed from the reduction of NO_3. However, no net input of ammonia is needed to operate the glycolate pathway if a second method of forming glutamate were used (reaction 12). In this latter scheme, the ammonia released in the conversion of glycine to serine is used to form glutamate. Since the glycine to serine conversion appears to occur in the mitochondria, the mitochondrial NAD-glutamate dehydrogenase would be expected to catalyze this reaction. However, it is interesting to note that Leech and Kirk (97) have suggested that the mitochondrial NAD-glutamate dehydrogenase functions in the direction of oxidation of glutamate and that it is the NADP-glutamate dehydrogenase in the chloroplasts which synthesizes glutamate.

Although the glycolate pathway normally is

considered to function for the conversion of glycolate to glycerate, a portion of the pathway may be reversed. The peroxisomal glycerate-hydroxypyruvate interconversion is an anaerobic reversible metabolic pathway, but in the oxidative direction it occurs readily only at high pH (135). The conversion of hydroxypyruvate to serine (reaction 13) may occur with glutamate as the amino donor. Kisaki and Tolbert (85) observed significant rates for glutamate:hydroxypyruvate aminotransferase. The peroxisomal serine:glyoxylate aminotransferase may also catalyze the hydroxypyruvate to serine conversion, but probably not with glycine as the amino donor. The serine:pyruvate aminotransferase activity of this enzyme is very low and unless the reverse alanine:hydroxypyruvate aminotransferase reaction is considerably faster, the serine:glyoxylate aminotransferase probably would not significantly contribute in the conversion of hydroxypyruvate to serine.

The glutamate:glyoxylate aminotransferase is postulated to function in the formation of glycine which can then be converted to serine. The glycine and serine can be used for porphyrin and protein synthesis or used to form C_1 moieties for nucleic acid and cell wall biosynthesis in the leaf. The glutamate:glyoxylate aminotransferase was found to greatly increase in activity in

response to light in germinating sunflower cotyledons suggesting it may be important for the formation of glycine during the greening of leaf tissue (Schnarrenberger, Oeser and Tolbert, in press).

If the leaf tissue over produces glycine and serine, as during photorespiration, the serine:glyoxylate aminotransferase can direct the carbon compounds to glycerate and back into sugars. Since this aminotransferase catalyzes the first step in the conversion of serine to glycerate it may well be under metabolic regulation. The inhibition by D-serine indicates this serine:glyoxylate aminotransferase is an atypical aminotransferase and warrants further study.

The function of the peroxisomal aspartate:α-ketoglutarate aminotransferase is postulated to be in the malate-aspartate shuttle of reducing power into the organelle. This function for aspartate aminotransferase has been proposed previously (95, 98). The peroxisomal isoenzymes of aspartate aminotransferase may have different kinetic characteristics which would favor one direction of the reaction. The peroxisomal malate dehydrogenase was found to be different than the mitochondrial enzyme and was postulated to function in the direction of malate oxidation (173).

The aminotransferase content of the mammalian peroxisomes was different than that in spinach leaf peroxisomes. The rat liver peroxisomes did not contain aspartate:α-ketoglutarate aminotransferase nor significant levels of glutamate:glyoxylate aminotransferase. However, they did appear to contain serine:glyoxylate and alanine:glyoxylate aminotransferase activities. Rowsell and coworkers (134) have suggested that alanine:glyoxylate aminotransferase in rat liver is directly correlated with the gluconeogenic capabilities of the liver since it increased in activity with glucagon treatment. The alanine:glyoxylate aminotransferase is located in both the mitochondria and peroxisomes of rat liver. Because of previous speculation on the gluconeogenic function of peroxisomes (40, 154), it is tempting to speculate that the increased alanine:glyoxylate aminotransferase in liver occurred in the peroxisomes. Peroxisomes seem to have a correlation with gluconeogenesis in that they are found in significant amounts only in those tissues capable of gluconeogenesis. It should be pointed out that anyone isolating and purifying alanine:glyoxylate aminotransferase from rat liver by the classical enzyme isolation procedures would likely end up with the mitochondrial enzyme since it has the most activity.

The diversification of the enzyme composition of peroxisomes is evident with the dog kidney peroxisomes. These microbodies contained very low amounts of those aminotransferases which were assayed. In the kidney, the mitochondria contained most of the aminotransferase activities. In the present studies the kidney cortex and medulla tissues were not separated before isolation of the peroxisomes. It may very well be the two types of tissues would contain peroxisomes with different enzyme compositions.

The enzymatic composition of microbodies is known to vary depending upon the source of the organelles (40). This variation probably reflects the different functions of the microbodies. The glyoxysomes in castor bean endosperm and sunflower cotyledons function to form succinate from fatty acids. Numerous functions for peroxisomes in spinach leaves have been described (154), but glycine and serine synthesis from glycolate is certainly well established. The function of liver and kidney peroxisomes is not known. The liver peroxisomes do not contain aspartate aminotransferase or malate dehydrogenase and if these enzymes function in the shuttling of reducing power, as has been postulated, then liver peroxisomes may not be associated with the oxidation-reduction of NAD.

CHAPTER II

DISTRIBUTION OF PEROXISOMAL ENZYMES AMONG LEAF CELLS

The plants which fix CO_2 only by the reductive pentose phosphate cycle are called C_3-plants. These plants exhibit photorespiration, that is, they take up O_2 and form CO_2 in the light. It has been suggested that the glycolate pathway is the source of the photorespired CO_2. In contrast to the C_3-plants, the C_4-plants do not lose CO_2 by photorespiration. The C_4-plants contain PEP-carboxylase and initially fix CO_2 into oxaloacetate. In the earlier studies of C_4-plants, the RuDP carboxylase activity was reported to be lower than that found in the C_3-plants and could not account for the *in vivo* photosynthetic rates observed in the C_4-plants. Since the glycolate pathway has always been closely associated with the C_3-cycle, it was also not surprising to find low levels of the glycolate pathway enzymes in C_4-plants. It was thus suggested that low levels of activity for the glycolate pathway may be the reason C_4-plants did not photorespire (120).

Björkman and Gauhl (12) and Andrews and Hatch (3) found that exhaustive grinding of the tissues of C_4-plants resulted in recoveries of much higher values of RuDP carboxylase than previously reported. Björkman showed that "gentle" grinding broke mesophyll cells but did not release the RuDP carboxylase. After very harsh grinding to break the bundle sheath cells, most of the RuDP carboxylase activity was solubilized. This was interpreted to mean that RuDP carboxylase was located in the bundle sheath cells. The mesophyll cells, which were broken easily, contained most of the PEP-carboxylase. Thus it appeared that in C_4-plants the mesophyll cells contained the C_4-cycle enzymes while the bundle sheath cells contained the C_3-cycle enzymes. If the glycolate pathway were associated with the C_3-cycle within C_4-plants, then the glycolate pathway enzymes would also be expected to be located in the bundle sheath cells.

Thus the purpose of this study was to determine the cellular location and some of the characteristics of the glycolate pathway enzymes in C_4-plants.

MATERIALS AND METHODS

Most of the materials and methods used in these studies have been published and can be found in the

reprint in Appendix A.

The preparation of the starch gel for starch gel electrophoresis was the same as that described in Chapter I. The same buffer was used in both the starch gel and the electrode tanks. For catalase, the buffer was 6 mM Tris-50 mM HEPES at pH 6.8. The buffer system for hydroxypyruvate reductase was 10 mM Tris-HCl at pH 8.3, while for glycolate oxidase the buffer was 12 mM Tris-3 mM citrate at pH 7.0.

RESULTS

LOCATION OF GLYCOLATE PATHWAY ENZYMES

The major portion of this work has been published (129) and a reprint is attached in the appendix. Thus the results will only be summarized here.

Björkman and Gauhl (12) used a differential grinding technique to establish that the bundle sheath cells of C_4-plants contained most of the RuDP carboxylase, whereas the mesophyll cells contained most of the PEP carboxylase. The differential grinding procedure was used to determine the distribution of some of the enzymes of the glycolate pathway in C_3- and C_4-plants. With corn leaf tissue, only 60% of the glycolate oxidase and 40% of the P-glycolate phosphatase were extracted after 2 min of homogenization in

a Waring blendor (Figure 1) in Appendix A. The rest of these activities were obtained by harsh grinding of the bundle sheath cells with a roller mill. 3-P-Glycerate phosphatase was completely extracted by the 2 min of homogenization and thus was used as a marker enzyme for mesophyll cells.

The peroxisomal enzymes were readily solubilized from spinach and wheat by homogenization in a Waring blendor (Table 1) in Appendix A. This was to be expected since these plants do not contain bundle sheath cells. In corn and sugarcane, a 30 sec homogenization solubilized 30-50% of the glycolate oxidase and hydroxypyruvate reductase activities. The remaining 50-70% of these activities were solubilized by grinding with the roller mill. Since 30% of the 3-P-glycerate phosphatase activity was in the extract from the roller mill, some mesophyll cells were probably contributing to the activities observed in the extract from the roller. The fact that the peroxisomal enzymes; catalase, hydroxypyruvate reductase and glycolate oxidase; varied in their distribution may indicate that the peroxisomes in the two types of cells have different enzyme compositions. The varied distribution could also be an artifact caused by the differential solubilization of the peroxisomal enzymes. Fredrick and Newcomb (58)

found the peroxisomes in the bundle sheath cells of corn and sudan grass stained darker for catalase activity than did the mesophyll cell peroxisomes. They also reported that each bundle sheath cell of corn contained three to four times as many microbodies as did the mesophyll cell.

The activity of glycolate oxidase found in corn and sugarcane was two to three times higher than previously reported (157), but these C_4-plants still contained about half the activity found in the C_3-plants, spinach and wheat. However, the lack of CO_2-photorespiration in C_4-plants can not be attributed to the lack of glycolate pathway enzymes. This is particularly evident in the Atriplex. Atriplex rosea, a C_4-plant contained nearly the same amount of glycolate oxidase as Atriplex patula a C_3-plant.

Further support of the location of the glycolate pathway enzymes was sought in variegated corn mutants. If only one cell type (bundle sheath or mesophyll) was affected in the mutation, and if the chlorophyll-less cells would contain lower levels of glycolate oxidase, as in etiolated tissue, then assays of the mutants should reveal in which cell the glycolate oxidase was located. Microscopic observations on many variegated mutants of corn were made but none was found in which only one type of cell

contained all of the chlorophyll. Likewise the glycolate oxidase distribution was relatively constant among all of the mutants. Thus no additional support for the location of the glycolate pathway enzymes in the C_4-plants was obtained by studies with the available mutants.

The peroxisomal enzyme distributions suggested that both the mesophyll and the bundle sheath cells may contain the glycolate pathway enzymes. This idea is supported by the observations of Fredrick and Newcomb (58) and other authors (73, 92) that microbodies are present in both types of cells. If these enzymes are present in both cell types, they may be present as isoenzymes and have different characteristics. Therefore studies were conducted to determine if catalase, hydroxypyruvate reductase or glycolate oxidase activities in extracts of the mesophyll cells as obtained by homogenization in a Waring blendor, were distinguishable from the activities in extracts of the bundle sheath cells as obtained by the grinding in the roller mill.

CATALASE

The catalase activity in corn appeared to be present in both bundle sheath and mesophyll cells. Starch gel electrophoresis was used to measure the electrophoretic

pattern of catalase from whole leaf extract, mesophyll cell extract and bundle sheath extract. Each sample contained one anionic band of catalase activity and the enzyme had the same mobility in all three samples. Likewise, only one band was observed in mixtures of the three samples. There have been reports of catalase isoenzymes in plants (141, 144). Scandalios (141) has shown that in corn endosperm tissue some catalase isoenzymes are formed because the parent plants had different molecular forms of catalase. If parent X has catalase composed of AAAA and parent Y has a catalase of BBBB, then the F_1 will have five isoenzymes produced by the combinations of the two types of subunits A and B. In one report by Scandalios (140) two catalase isoenzymes were present in the leaf tissue of an inbred line of corn. In the hybrid corn leaf tissues used in my studies, isoenzymes were not observed.

HYDROXYPYRUVATE REDUCTASE

The kinetic characteristics of the hydroxypyruvate reductase activities in the two types of cells from corn leaves were studied. The Km for hydroxypyruvate in the mesophyll cells and in the bundle sheath cells was 73 μM and 81 μM respectively. These values are not considered

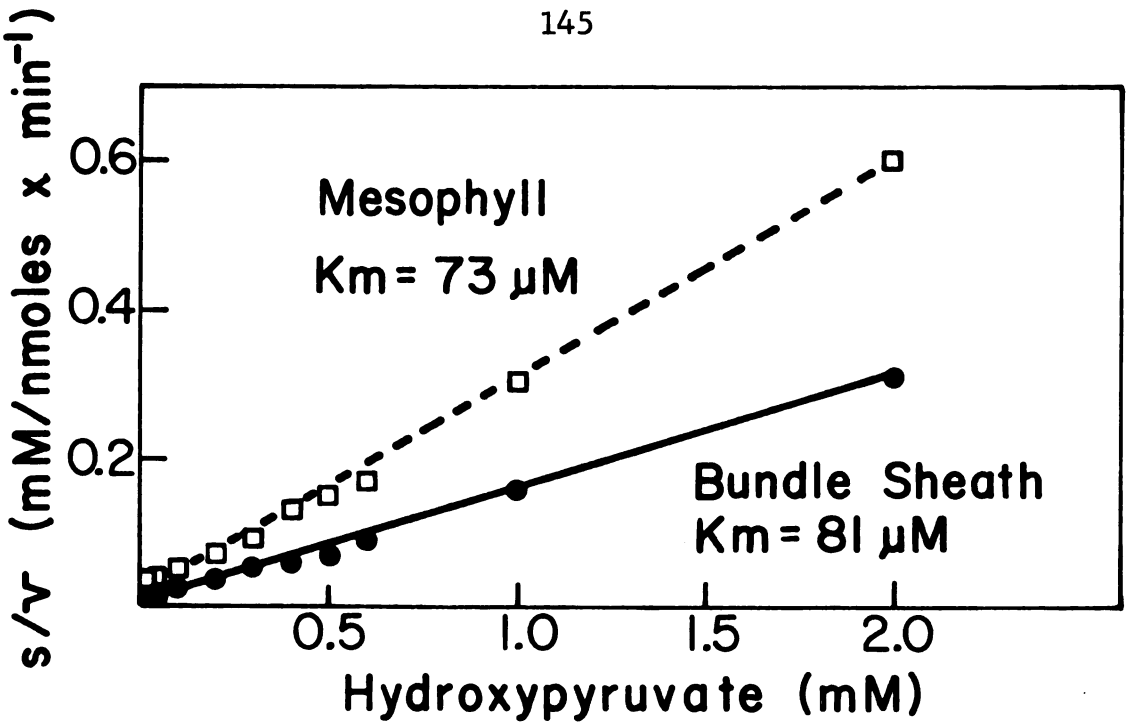
to be significantly different (Figure 16). Hydroxypyruvate reductase from spinach leaves also is known to reduce glyoxylate to glycolate (89, 159). The glyoxylate reductase activities in the two types of cells from corn leaves also exhibited similar kinetics. The Km for glyoxylate was 1.0 mM and 0.4 mM for the mesophyll cell and bundle sheath cell respectively (Figure 16). Spinach leaf hydroxypyruvate reductase has a Km for hydroxypyruvate of 50 μM which is similar to that observed for the enzyme from corn leaves (89, 159). However, the glyoxylate reductase from corn leaves had a 25-50 fold lower Km for glyoxylate than that reported for the enzyme from spinach leaves (89, 159). This difference appears significant.

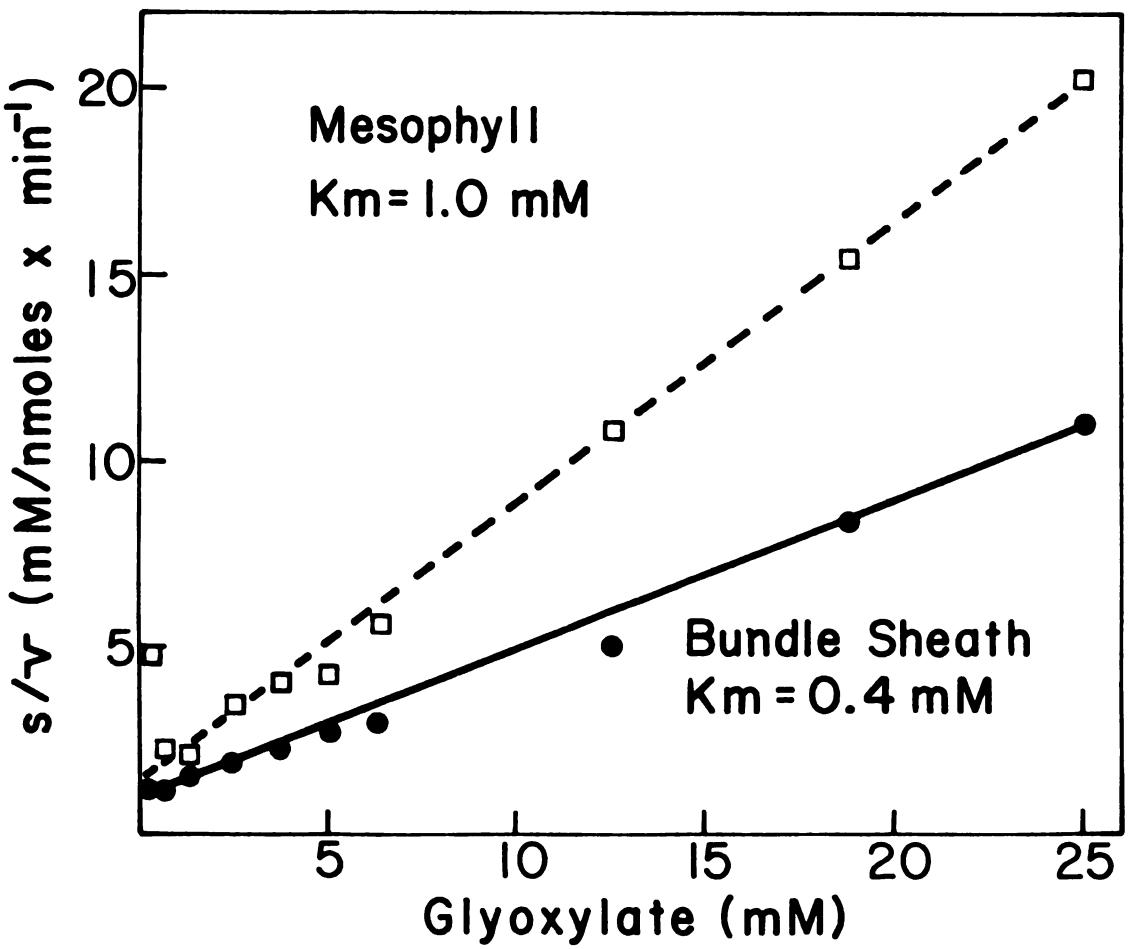
Starch gel electrophoresis was used to search for isoenzymes of hydroxypyruvate reductase. The bundle sheath cells contained a small cationic band of enzyme activity and a heavy anionic band. The mesophyll cell extract had only a small anionic band at the same R_f as from the bundle sheath cells. The mesophyll may have also contained the cationic form but the activity was too low to detect. Since nearly the same activity from each extract was applied to the gel, the reason for the weaker staining of the mesophyll extract is not known.

Figure 16

Reaction Kinetics for Hydroxypyruvate Reductase and Glyoxylate Reductase from Mesophyll and Bundle Sheath Cells of Maize

Mesophyll extracts were prepared by homogenization of corn leaf tissue in a Waring blendor. The residue from the homogenization was ground further in a roller mill to obtain the bundle sheath cell extracts. Both reductases were assayed by measuring the oxidation of NADH.


s/v (mM/nmoles x min⁻¹)
0.6
0.4
0.2
Mesophyll
Km = 73 µM
Bundle Sheath
Km = 81 µM
0.5
1.0
1.5
2.0
Hydroxypyruvate (mM)


s/v (mM/nmoles x min⁻¹)
20
15
10
5
Mesophyll
Km = 1.0 mM
Bundle Sheath
Km = 0.4 mM
5
10
15
20
25
Glyoxylate (mM)

GLYCOLATE OXIDASE

Extracts of corn leaf tissue were electrophoresed and stained for glycolate oxidase activity. Both starch gels and polyacrylamide gels were used, but no activity was ever observed. Glycolate oxidase from spinach leaves moved into both types of gels and stained very readily. In the staining reaction for glycolate oxidase, phenezine methylosulfate (PMS) supposedly links the transfer of electrons from the reduced FMN of glycolate oxidase to nitroblue tetrazolium (NBT), which in the reduced state is blue and less soluble. The necessity for PMS in the staining solution was not determined. Since the corn glycolate oxidase could not be detected after electrophoresis, the staining reaction was studied in the spectrophotometer to determine if glycolate oxidase activity could be observed with PMS and NBT. The spinach enzyme gave a measurable rate of NBT reduction, but the corn enzyme did not (Table 13). Something in the corn enzyme preparation may have been binding or destroying the PMS, but this seems unlikely since the rate of spinach glycolate oxidase was unchanged in the presence of corn extract. If the PMS concentration was increased 2.5 fold, a very slight reaction was observed under anaerobic conditions. The spinach glycolate oxidase activity was considerably higher under

Table 13. Glycolate Oxidase Activity in Corn and Spinach Leaf Extracts

The leaf extracts were assayed for glycolate oxidase by the standard DCIP linked assay as previously published (Appendix A). The PMS-NBT assay mixture consisted of 46 μmoles and 0.25 μmoles of FMN. All assays contained 200 μg of PMS except where noted. The reaction was started with the addition of 25 μmoles of glycolate. Total assay volume was 2.5 ml and all assays were anaerobic except for the last one.

Plant Extract	DCPIP Assay	PMS-NBT Assay
	Relative Rate	
Spinach	100	100
Corn	109	0
Spinach + Corn	-	100
Corn (500 μg PMS)	-	11
Spinach (aerobic)	-	19

anaerobic conditions as compared to aerobic conditions and so it is very unlikely that the corn enzyme could be detected under aerobic conditions. The necessity of PMS in the glycolate oxidase stain was not determined. Frigerio and Harbury (59) reported spinach glycolate oxidase did not link with one electron acceptors and so the spinach enzyme was very likely reacting directly with NBT as other flavoproteins are known to do (44). This failure of glycolate oxidase to reduce NBT is not limited to C_4-plants such as corn. Glycolate oxidase from wheat, a C_3-plant, also could not be detected on electrophoretic gels (E. B. Nelson, personal communication). The algae enzyme (discussed below) appeared to link to PMS (116), but it was not stained by the PMS-NBT system (E. B. Nelson, personal communication).

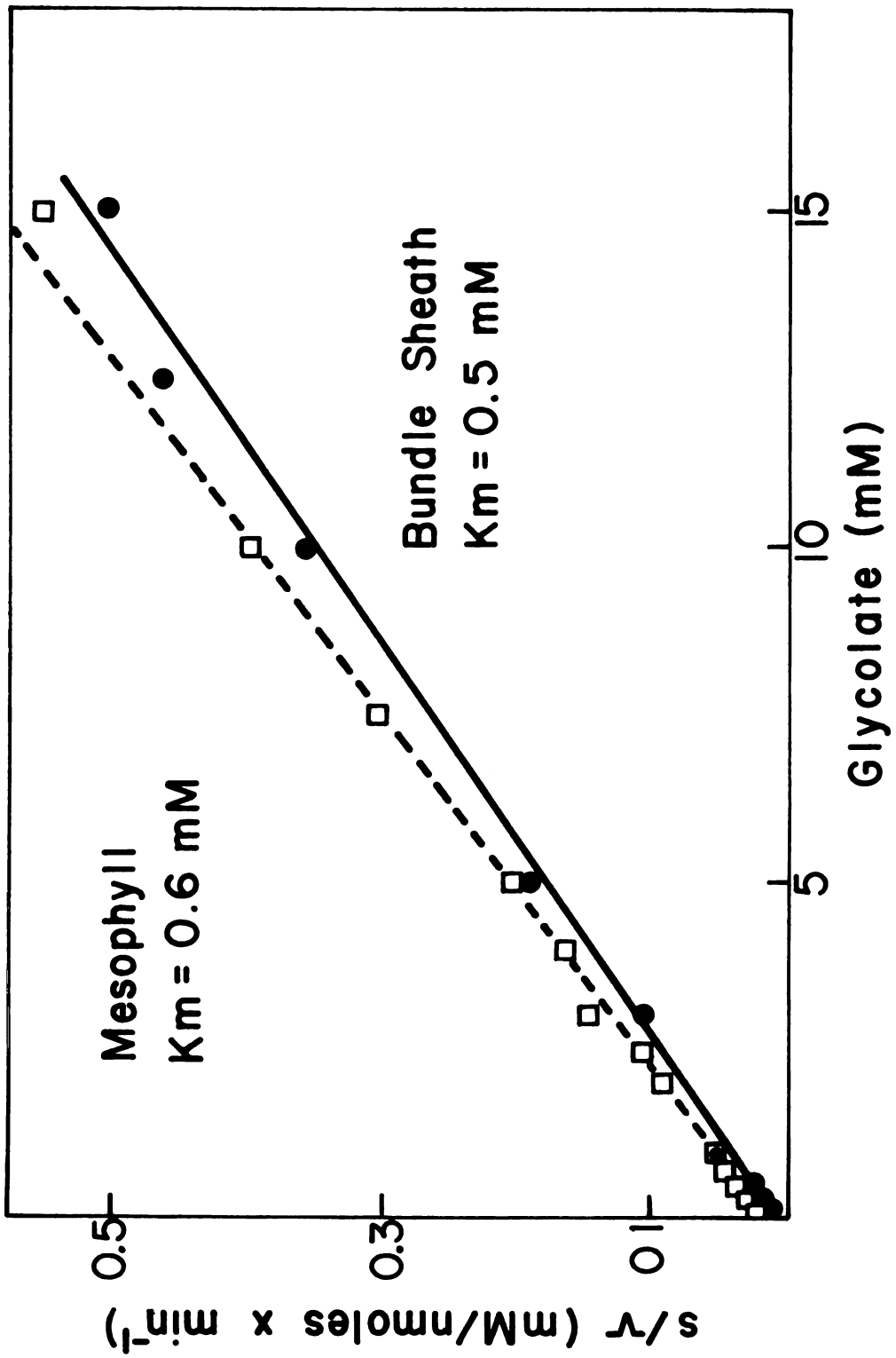
Extracts of corn leaves, after differential grinding to obtain the mesophyll cells and bundle sheath cells, were assayed by the DCIP assay to determine the kinetics of glycolate oxidase. The Km for glycolate was nearly the same in the two types of cell homogenates (Figure 17). The Km value of 0.5 mM for glycolate oxidase from corn is similar to that reported for the enzyme from spinach (154).

Nelson and Tolbert (116) found that the glycolate

Figure 17

Reaction Kinetics of Glycolate Oxidase From
Mesophyll and Bundle Sheath Cells of Maize

Mesophyll extracts were prepared by homogenization of corn leaf tissue in a Waring blendor. The residue from the homogenization was ground further in a roller mill to obtain the bundle sheath cell extract. The glycolate oxidase was assayed by measuring the reduction of DCIP.


Mesophyll
Km = 0.6 mM
Bundle Sheath
Km = 0.5 mM
0.5
0.3
0.1
5
10
15
Glycolate (mM)
s/v (mM/nmoles x min⁻¹)

oxidase from algae did not link to O_2 and was therefore called glycolate dehydrogenase. The algae enzyme could not be stained by the PMS-NBT system, it was strongly inhibited by CN^- and it would oxidize D-lactate but not L-lactate. In contrast the spinach glycolate oxidase was not significantly affected by CN^- and it oxidized L-lactate but not D-lactate (116). The characteristics of the corn glycolate oxidase were determined to see if it was related to the algae enzyme since as already mentioned, neither the algae enzyme nor the corn enzyme could be stained with the PMS-NBT system. The corn enzyme oxidized L-lactate but not D-lactate (Table 14). Likewise glycolate oxidase from sugarcane had the characteristics of the spinach enzyme. Cyanide did not appreciably affect the activity of the corn enzyme (data not shown).

The above results on catalase, hydroxypyruvate reductase and glycolate oxidase suggest that the mesophyll cells and the bundle sheath cells in C_4-plants do not contain distinct isoenzymes of these enzymes. The only significant difference found in the enzymes between C_3- and C_4-plants was in a low Km (glyoxylate) for the glyoxylate reductase activity.

Table 14. Glycolate Oxidase Activity From Various Sources

Spinach and sugarcane samples were whole leaf homogenates while the two corn extracts were prepared by the differential grinding procedure. The algae extract was prepared by E. B. Nelson.

Substrate	Extract				
	Spinach	Sugarcane	Corn		Algae
			Bundle Sheath	Mesophyll	
	nmoles x min^{-1} x ml^{-1}				
Glycolate	213	25	27	12	18.3
D-Lactate	0	0	0	0	18.8
L-Lactate	163	25	21	8	0

DISCUSSION

By differential grinding of corn leaf tissue it has been shown that the enzymes of the glycolate pathway were preferentially located in the bundle sheath cells but that some activity was also very likely in the mesophyll cells. Even though the two types of cells appear to have specialized functions, no isoenzymes or kinetic differences for glycolate oxidase, hydroxypyruvate reductase or catalase were found between the two cell types. The corn did have an NADH-glyoxylate reductase activity with a lower Km than is found in spinach leaves. Whether this activity is catalyzed by the NADH-hydroxypyruvate reductase, as it is in spinach, or whether a separate enzyme is present in corn is not known. An NADPH-glyoxylate reductase with a low Km for glyoxylate is present in spinach chloroplsts but this enzyme is not very reactive with NADH (159, 180).

Spinach glycolate oxidase reduced NBT in a PMS-NBT system. Since the necessity of PMS was not established, the glycolate oxidase reduction of NBT may have been direct rather than linked through the PMS. The corn, wheat and algae enzymes would not stain on electrophoretic gels with a PMS-NBT staining solution. Dixon (44) in his report on the characteristics of flavoproteins mentions only one

enzyme which exhibits different acceptor specificities depending upon the source of the enzyme. Xanthine oxidase from chicken liver and pigeon liver will not react with O_2 whereas the mammalian enzyme does react with O_2. Glycolate oxidase appears to be another example where acceptor specificity depends upon the source of the enzyme. The glycolate oxidase from all plants and algae studied so far will reduce DCIP. The algae enzyme will also reduce PMS but not O_2 nor several other acceptors (116). The corn and wheat enzymes will reduce O_2 (155) but appear not to reduce a PMS-NBT system.

Further discussion about the distribution of the glycolate pathway enzymes in the bundle sheath and mesophyll cells in C_3- and C_4-plants has been published (129, Appendix A).

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APPENDIX

APPENDIX A

D. W. Rehfeld, D. D. Randall and N. E. Tolbert. 1970. Enzymes of the Glycolate Pathway in Plants Without CO_2-Photorespiration. Can. J. Bot. 48:1219-1226.

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Enzymes of the glycolate pathway in plants without CO_2-photorespiration[1]

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Dedicated to the memory of the late Dr. G. P. Krotkov and the late Dr. C. D. Nelson

REHFELD, D. W., D. D. RANDALL, and N. E. TOLBERT. 1970. Enzymes of the glycolate pathway in plants without CO_2-photorespiration. Can. J. Bot. **48**: 1219–1226.

Extracts, mainly from mesophyll cells, were obtained by grinding cells in a Waring Blendor; then extracts of parenchyma sheath cells were obtained by exhaustive grinding of the blender residue in a roller mill or mortar with sand. The specific activities of P-glycolate phosphatase, glycolate oxidase, catalase and reduced nicotinamide adenine dinucleotide- (NADH-) hydroxypyruvate reductase were fourfold higher in extracts of the parenchyma sheath cells than in the mesophyll cells from corn, sugarcane, and *Atriplex rosea*. P-Glycerate phosphatase was mainly located in the mesophyll cells. The total activity of glycolate oxidase in plants without CO_2-photorespiration averaged about one-third that found in other plants on a wet-weight basis. Glycolate oxidase activity in *Atriplex rosea*, without CO_2-photorespiration, was about the same as in *Atriplex patula*, with CO_2-photorespiration. It is concluded that enzymes for glycolate metabolism are present in all leaves in substantial amounts and are located in both cell types, although a higher specific activity is in the parenchyma sheath cells. Thus it is proposed that photorespiration occurs in all plants, but that CO_2 evolution from glycolate metabolism is not manifested in plants which have high levels of activity for the C_4-dicarboxylic acid cycle of CO_2 fixation.

Introduction

From the extensive studies initiated by Krotkov, Nelson, and co-workers (7, 8, 23), plants have been classified according to whether they do or do not exhibit photorespiration. These measurements were based upon a light-dependent CO_2 evolution either as a gush when the lights are turned off (7, 8, 23), as $^{14}CO_2$ evolution from newly formed photosynthetic products (9, 26), or upon a CO_2 compensation point. Photorespiration, as measured by CO_2 release, may not be manifested in plants with high levels of phosphoenolpyruvate (PEP) carboxylase, because they are efficiently refixing the CO_2 released. Photorespiration appears to occur in these types of plants when assessed by O_2 exchange (6, 11), by metabolism of glycolate-^{14}C and glycine-^{14}C (13, 17, 25), by addition of hydroxysulfonates to show glycolate accumulation during photosynthesis (24) and, as shown in this paper, by the presence of the enzymes involved in glycolate metabolism or photorespiration. Consequently the term "without photorespiration" for corn and similar plants is misleading, and in this paper it has been modified to "without CO_2-photorespiration" to designate the measurement involved.

Though there are many differences between plants with and without CO_2-photorespiration, for this paper the pertinent properties common to plants with CO_2-photorespiration are (*a*) CO_2-fixation by the reductive pentose phosphate pathway, (*b*) the absence of well-developed parenchyma sheath cells (2, 4), and (*c*) high levels of enzymes for glycolate metabolism (16, 22). Plants without CO_2-photorespiration are characterized by (*a*) initial CO_2-fixation by the C_4-dicarboxylic acid cycle, (*b*) presence of well-developed parenchyma sheath cells or green veins, and (*c*) low levels of glycolate oxidase and ribulosediphosphate (RuDP) carboxylase.

The metabolism of glycolate appears to be directly related to photorespiration (9, 15, 24, 25). In photorespiration, light is necessary for photosynthetic glycolate biosynthesis in the chloroplast, O_2 uptake occurs during glycolate oxidation to glyoxylate by glycolate oxidase in the peroxisomes (21, 22), and CO_2 release occurs in the conversion of two glycines to one serine (13). Peroxisomes were isolated from some plants with CO_2-photorespiration, but low recoveries of the particles were reported for plants without CO_2-photorespiration. A similar relationship was observed when we were comparing plants upon the basis of glycolate oxidase (16, 22).

The magnitude of photorespiration might also be related to the amount of glycolate biosynthesis. The only enzyme, so far related to this

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process, is a specific P-glycolate phosphatase, and, indeed, lower levels of it were found in some plants without CO_2-photorespiration (18). Alternatively, plants without CO_2-photorespiration usually have high levels of a 3-P-glycerate phosphatase, which has similar but not identical properties to the P-glycolate phosphatase (D. D. Randall and N. E. Tolbert, unpublished).

Slack and Hatch (20) showed that plants without CO_2-photorespiration have high levels of PEP carboxylase and low levels of RuDP carboxylase, which is located in the parenchyma sheath cells (19). However, Björkman and Gauhl (3) found higher levels of RuDP carboxylase in plants without CO_2-photorespiration, because the tissue was ground sufficiently to rupture most of the parenchyma sheath cells. Since high levels of glycolate oxidase are present in plants with the reductive pentose phosphate pathway, enzymes involved in glycolate metabolism also may be located in parenchyma sheath cells of plants without CO_2-photorespiration and may have been underestimated because of inadequate grinding. Whereas the Waring Blendor breaks mainly mesophyll cells, grinding by a roller mill or mortar and pestle with sand is necessary to rupture the parenchyma sheath cells. Enzyme assays on extracts from sequential grinding may be used, though not quantitatively, to differentiate between the activities in the two cell types. We have measured three peroxisomal enzymes, glycolate oxidase, catalase, and NADH-hydroxypyruvate reductase, involved in glycolate metabolism. Assays on the two phosphatases, one hydrolyzing P-glycolate and one hydrolyzing P-glycerate, were run to possibly locate the site of glycolate biosynthesis. These activities are compared with the distribution of RuDP carboxylase and PEP carboxylase between the two types of cells.

Materials and Methods

Plants and Extraction

Plants with CO_2-photorespiration were spinach, *Spinach oleracea* L., varieties unknown; wheat, *Triticum vulgare* L., variety Genesee; and *Atriplex patula*. Plants without CO_2-photorespiration were corn, *Zea mays* L., variety Michigan 500 unless otherwise specified; sugarcane, *Saccharum*, variety CL 41-223; and *Atriplex rosea*. Seeds of variegated corn mutants were furnished by Dr. Robert Lambert, Maize Genetic Collection, University of Illinois, Urbana. Dr. Elmer C. Rossman, Crop and Soil Science, Michigan State University, provided the field corn. Spinach and corn were field grown and the other plants were raised in a greenhouse. Mature leaves were used except for wheat leaves which were harvested when about 12 cm high. All leaves were extracted immediately after harvest except spinach, which was stored for as long as 1 week at 4°. Leaves were washed with water and blotted dry, and the midrib removed from spinach, corn, and sugarcane leaves. The remaining steps were performed in a cold room at 4° or in ice baths.

Extracts for all plants were obtained by homogenizing 20 g of leaf tissue in 100 ml of grinding medium with a Waring Blendor at high speed for 30 s, except for spinach, which was blended for only 10 s. The grinding medium consisted of 0.02 *M* glycylglycine, pH 7.5. Each homogenate was squeezed through eight layers of cheesecloth. The residue remaining in the cheesecloth was then run repeatedly through a serrated roller mill for further crushing until the residue, squeezed on the roller, was nearly colorless. About 100 ml of grinding medium was used to wash and remove the extract from the rollers of the mill. This mixture was also squeezed through eight layers of cheesecloth. Each extract was adjusted to pH 7.5 and volumes recorded. The procedure for extraction of the *Atriplex* leaves differed by using 5 g of leaf tissue in 100 ml of grinding medium. After the blender homogenate was squeezed through cheesecloth, the residue was ground exhaustively with a mortar and pestle with sand and extracted with buffer. Neither whole leaves nor the Waring Blendor residue of *A. rosea* could be extracted with the roller mill because of the gelatinous nature of these crushed leaves.

The roller mill was developed for isolating mitochondria (12) by John D. Jones (present address: Food Research Institute, Central Experimental Farms, Ottawa, Ontario) to whom we are grateful for a copy of the design. Tissue was crushed between two diagonally serrated, finely knurled, stainless steel rollers that are appressed.

Enzyme Assays

Glycolate oxidase (EC. 1.1.3.1) was assayed anaerobically at pH 8.7 with the dye, 2,6-dichlorophenolindophenol (DCPIP), as electron acceptor (22). All values are corrected to saturating levels of DCPIP (N. E. Tolbert, submitted to Methods of Enzymology), which involves a multiplication factor of 3.95. Because the O.D. of the dye concentration which saturates the enzyme is too high for the spectrophotometer, V_m must be obtained by extrapolation from a standard plot of enzyme activity versus usable dye concentrations.

NAD-hydroxypyruvate reductase (EC. 1.1.1.29) was assayed by following the oxidation of NADH at 340 mμ (22). Glyoxylate was used as a substrate because it is more stable and less expensive than hydroxypyruvate. This enzyme from spinach reduces hydroxypyruvate 4 to 5 times faster than glyoxylate and the K_m with hydroxypyruvate is about 100-fold less than with glyoxylate. However, values reported in this paper are with glyoxylate and are not corrected to activity with hydroxypyruvate. Triton X-100 was routinely added to both the glycolate oxidase and the reductase assays since it was used in assays on peroxisomes to overcome latency caused by the particle. This use of Triton X-100 may not have

been necessary since the present grinding procedures without sucrose should not have preserved many of the peroxisomes.

Catalase (EC. 1.11.1.6) was measured spectrophotometrically by following the decrease in H_2O_2 concentration (21). Triton X-100 was not added in this assay since there is no latency in this assay with peroxisomes.

P-Glycolate phosphate (EC. 3.1.3.18) and P-glycerate phosphatase were assayed for 10 min at 30° with 5 micromoles of substrate in 20 m*M* sodium cacodylate, pH 6.3. For the P-glycolate phosphatase assay, the reaction mixture contained 1 m*M* $MgCl_2$. Reactions were stopped upon the addition of 10% trichloroacetic acid, the precipitate removed by centrifugation, and the released inorganic phosphate measured (5).

Protein was determined by the Lowry procedure (14) and chlorophyll by Arnon's procedure (1). Activities are expressed as nanomoles of substrate changed per minute. Specific activities are based on grams fresh weight, milligrams protein, or milligrams chlorophyll as indicated.

Results

Release of Enzymes from Corn and Sugarcane Leaves

Two plants without CO_2-photorespiration, corn and sugarcane, have been compared with two plants with CO_2-photorespiration, spinach and wheat. Spinach represents a leaf quickly homogenized in a Waring Blendor and wheat, like corn and sugarcane, a leaf that is difficult to grind completely with the blender. Wheat plants with CO_2-photorespiration do not have well-developed parenchyma sheath cells. For spinach and wheat the Waring Blendor solubilized most of each enzyme, which is in mesophyll cells (Table I). The extraction of 3-P-glycerate phosphatase from wheat leaves was not complete by the blender for reasons unknown.

After the leaves of corn and sugarcane were ground for 30 s by the Waring Blendor, about one-third of the total glycolate oxidase was solubilized, about half of the P-glycolate phosphatase and NADH-hydroxypyruvate reductase, and even more than half of the catalase was released (Table I). Microscopic examination of the residue revealed many intact parenchyma sheath cells attached to vein cells but few intact mesophyll cells. The actual percentage of parenchyma sheath cells broken by the blender is unknown but the value must be significant. Most of the remaining parenchyma sheath cells were crushed by vigorous grinding in the roller mill and the rest of these enzymes released. The

TABLE I

Enzyme activities in leaf extracts after differential grinding*

	With CO_2-photorespiration				Without CO_2-photorespiration			
	Spinach		Wheat		Corn		Sugarcane	
	Blender	Mill	Blender	Mill	Blender	Mill	Blender	Mill
Glycolate oxidase								
% total	98	2	100	0	31	69	31	69
S.A., protein	142	47	47	—	8	43	8	20
S.A., chlorophyll	4 657	2192	1335	—	158	2070	142	869
NADH-hydroxypyruvate reductase								
% total	98	2	97	3	39	61	48	51
S.A., protein	140	70	49	15	10	38	29	48
S.A., chlorophyll	4 586	3111	1362	1 500	218	1801	739	2176
Catalase								
% total	97	3	97	3	53	47	64	36
S.A. $\times 10^{-3}$, protein	180	110	180	60	20	40	20	20
S.A. $\times 10^{-3}$, chlorophyll	6 060	4750	4880	6 210	440	2090	460	700
P-Glycolate phosphatase								
% total	99	1	92	8	47	53	45	55
S.A., protein	435	202	336	195	47	148	73	139
S.A., chlorophyll	14 350	9100	9300	19 350	1055	7290	1865	6230
P-Glycerate phosphatase								
% total	96	4	74	26	72	28	57	44
S.A., protein	210	186	39	99	22	22	122	150
S.A., chlorophyll	6 900	8300	1070	9 750	486	1015	3130	6850

*Specific activity (S.A.) is expressed as nmoles min^{-1} mg^{-1}.

percentage distribution of these enzymes between the two homogenates from the sequential grinding procedure is indicative of their cellular location, but it is not quantitative. In the differential grinding procedure, as a first approximation, the enzymes released by the Waring Blendor will be referred to as in mesophyll cells and those released by a subsequent roller mill or mortar and sand extraction will be referred to as in parenchyma sheath cells. After prolonged grinding of up to 2 min on the blender many of the parenchyma sheath cells still remained unbroken as observed microscopically, and the total solubilization of glycolate oxidase or P-glycolate phosphatase was far from complete (Fig. 1). The phosphatase for 3-P-glycerate is, however, almost completely solubilized by the Waring Blendor. From these results it appears that the enzymes for glycolate metabolism are located in both cell types, while the 3-P-glycerate phosphatase is located almost exclusively in the mesophyll cells. From similar arguments it has been proposed that PEP carboxylase and the C_4-dicarboxylic acid cycle are located in the mesophyll cells, while RuDP carboxylase and the reductive pentose phosphate pathway for CO_2 fixation are mainly localized in the parenchyma sheath cells (3).

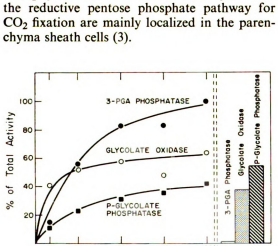

FIG. 1. Rate of enzyme release by Waring Blendor. Different leaf samples of field-grown corn, variety A509 × MS1334, were ground in the Waring Blendor for indicated length of time. After homogenization for 120 s, the residue from that sample was further crushed by the roller mill and the percentage of the total enzymatic activity released is indicated on the right side. 3-P-Glycerate (3-PGA) phosphatase, ●; glycolate oxidase, ○ and open column; P-glycolate phosphatase, ■ and closed column.

Breakage of the surviving parenchyma sheath cells by the roller mill released glycolate oxidase, catalase, NADH-hydroxypyruvate reductase, and P-glycolate phosphatase of a much higher specific activity on a protein or chlorophyll basis than obtained by the Waring Blendor rupture of the mesophyll cells. These higher specific activities in the roller mill fractions suggest a higher concentration of the enzymes for glycolate metabolism in parenchyma sheath cells.

The percentage distribution of catalase in the two homogenates did not follow that for glycolate oxidase (Table I). From sugarcane leaves more catalase was in the Waring Blendor fraction than in the roller mill fraction and for corn about an equal distribution was found. On the basis of specific activities the roller mill fraction was still the highest. Also the percentage distribution of NADH-hydroxypyruvate reductase and P-glycolate phosphatase was more nearly equal between two cell fractions than was found for glycolate oxidase.

Investigation with Corn Mutants

Ten variegated corn mutants were grown in the field, mature leaves from plants before tasseling were subjected to the differential grinding procedure, and the two homogenates examined for activity of glycolate oxidase and the two phosphatases (Table II). The distribution between the two cell types after differential grinding and the total level of enzyme activity for glycolate metabolism varied little from that found with normal green plants. Microscopic examination of the leaves did not show a preponderance of green cells of one type. Thus the use of mutant corn varieties failed to delineate in which cell types these enzymes were located. There is the possibility that glycolate metabolism would occur in chlorophyll-less mutant cells adjacent to green cells, and thus the distribution would be the same as found in normal corn. As mentioned above, the distribution of the phosphatases in the normal plants seemed to be distinctly different between the two cell types. In all of the mutants the distribution of these two phosphatases between the two homogenates also did not vary radically from that found in normal corn plants. It was not possible to find a positive correlation for the minor variations shown in Table II with the type of striation seen visually in the leaf.

Level of Glycolate Oxidase in Plants with and without CO_2-Photorespiration

The specific activities of glycolate oxidase varied several-fold depending upon the type, variety, age, and nutrition of the plant and also upon the grinding procedures. Higher values are obtained with older leaves and after more vigorous grinding. The specific activity values obtained for this oxidase are summarized in Table III. Current values are the sum of the activities of the Waring Blendor extract plus the subsequent roller mill extraction, or they were obtained by grinding whole leaves directly in the roller mill. Both procedures gave similar results. The specific activities from spinach are maximum, and nearly two-fold higher than average values previously reported. This variation can be attributed to a great deal of variation experienced in the plant material. The higher values for wheat previously reported were due to the use of older tissue. In the present experiments young seedlings about 12 cm high were used.

The level of glycolate oxidase, on the basis of fresh weight, in leaves of plants without CO_2-photorespiration generally ranged from 20 to 50% and averaged one-third of that found in plants with CO_2-photorespiration. On the basis of protein specific activity, plants without CO_2-photorespiration had only 10 to 20% of the activity of spinach or wheat leaves. If one compares the two *Atriplex* species, *A. rosea* without CO_2-photorespiration contained nearly as much glycolate oxidase as *A. patula*. However, a pattern of lower activity of glycolate oxidase in

TABLE II

Variation in total (nmoles min^{-1} g^{-1} wet wt.) and blender-extracted* (% of total) enzyme activities in corn mutants

	Glycolate oxidase		P-Glycolate phosphatase		P-Glycerate phosphatase	
Mutants†	Total	Extracted by blender, %	Total	Extracted by blender, %	Total	Extracted by blender, %
Lineate	802	38	2960	45	1020	60
Striate-1	604	24	3070	32	1200	58
Striate-2 (waxy)	585	29	2995	33	1178	57
Striate-2	750	25	3140	31	1405	52
Fine stripe-1	367	29	2940	29	1005	63
Japonica-1	719	35	3520	33	925	60
Iojap	332	22	3010	26	1028	53

*After 30 s of Waring Blendor treatment.
†The corn mutants were from the University of Illinois, Maize Genetic Collection. The collection numbers were as follows: lineate, 67-1915-1⊕; striate-1, 67-771-8/771-2; striate-2 (waxy), 65-346-2⊕ and 66-1795-4/-6; striate-2, 63-3432-6⊕ and 63-3463-7⊕; fine stripe-1, 66-757-7⊕; Japonica-1, 63-3783-7⊕; Iojap, 65-322-2/321-2 and 65-322-7/321-6.

TABLE III

Total glycolate oxidase activity in plant extracts,* nmoles min^{-1}

	Currently obtained		Previously reported†	
	g^{-1} fresh wt.	mg^{-1} protein	g^{-1} fresh wt.	mg^{-1} protein
With CO_2-photorespiration				
Spinach	4159	134	2030	83
Wheat	1825	63	3219	115
Atriplex patula	802	12	402‡	—
Without CO_2-photorespiration				
Corn (old)	932	16	—	—
Corn (young)	237	12	118	4
Sugarcane	561	12	190	12
Atriplex rosea	640	—	300‡	—
Amaranthus	—	—	1051	28

*All values are corrected to V_m with saturating amount of DCPIP.
†Values from Tolbert *et al.* (21) recalculated as mentioned in first footnote.
‡O. Björkman, personal communication.

TABLE IV

Enzymatic activities from *Atriplex* varieties of extracts prepared by differential grinding

Sequence of extraction procedures	%						nmoles min^{-1} mg^{-1} protein	
	Glycolate oxidase		P-Glycolate phosphatase		3-P-Glycerate phosphatase		RuDP carboxylase,	PEP carboxylase,
	A. rosea	*A. patula*	*A. rosea*	*A. patula*	*A. rosea*	*A. patula*	*A. rosea**	*A. rosea**
First: 30 s Waring Blendor	41	96	31	85	69	96	116	580
Followed by: mortar with sand	59	4	69	15	31	4	310	2

*Values from Björkman and Gauhl (3), who gave specific activity after an unspecified time of Waring Blendor homogenization and after grinding of the cells in a mortar with glass beads.

plants without CO_2-photorespiration is generally evident. Nevertheless, the activity of glycolate oxidase is very substantial in all plants, and the absence of CO_2-photorespiration cannot be attributed to very low levels of this enzyme. For corn leaves, the activity of glycolate oxidase after thorough grinding was eight times greater on a weight basis than reported earlier and the specific activity on a protein basis was four times greater. A portion of this difference can be attributed to the previous use of young corn seedlings rather than more mature leaves. However, the use of the roller mill even on young corn leaves extracted twice as much glycolate oxidase as previously reported. In sugarcane leaves the roller mill extraction triples the level of measurable glycolate oxidase activity on a weight basis although on a protein basis no change was observed.

Differential Extraction of Enzymes from Atriplex

The solubilization of the enzymes in *A. patula*, the variety with CO_2-photorespiration, followed the pattern found for spinach and wheat leaves. Most of the activity was released after a 30-s homogenization in the Waring Blendor (Table IV). In contrast, *A. rosea* is without CO_2-respiration and has well-developed parenchyma sheath cells. Less than half of the glycolate oxidase and P-glycolate phosphatase activity in *A. rosea* was released by use of the Waring Blendor. Grinding by mortar with sand was necessary to break the parenchyma sheath cells and this procedure released a large portion of these enzymes with a high specific activity. These results were similar to those found by Björkman and Gauhl (3) for RuDP carboxylase. It is concluded that these enzymes in *A. rosea*, as in corn and sugarcane leaves, are located mainly in the parenchyma sheath cells. However, a portion of the enzymes associated with glycolate metabolism are located in both types of cells, although a lower percentage and a lower specific activity is to be found in the mesophyll cells.

Discussion

The differential grinding procedure of a Waring Blendor to break mesophyll cells and a roller mill or mortar with sand to break parenchyma sheath cells is not quantitative and the results can only be indicative of enzyme distribution between these two cell types. Results are expressed as percentage distribution as well as specific activities on a protein and a chlorophyll basis. Absolute quantitation of the enzyme activities cannot be achieved with any of these units. The observed percentage distribution of enzyme activity is related to the number of each cell type broken under the given treatment. Variation in extractable chlorophyll and protein in the cell types would change the enzyme specific activity, whereas the total amount of enzyme activity in each cell type could be nearly the same. Previous citation to location of the carboxylases in a particular cell type was based upon protein specific activity (3), but the distribution may not be nearly as complete in one cell type as indicated.

By sequential grinding procedures (3) or isolation of different types of chloroplasts (19) the concept has developed that the mesophyll cells contain the C_4-dicarboxylic acid cycle and that the parenchyma sheath cells contain most of the

reductive pentose phosphate cycle for CO_2-fixation. Since glycolate biosynthesis and metabolism had always been associated with the reductive pentose phosphate cycle in plants with photorespiration, it was expected that the enzymes for the glycolate pathway would be in the parenchyma sheath cells. Indeed, in corn and sugarcane leaves at least half or more of the activity of these enzymes was in the fraction of cells ground by the roller mill or mortar with sand. However, a substantial part (about one-third to one-half) of the activities were first released upon grinding the mesophyll cells by Waring Blendor. It is tentatively concluded that the glycolate pathway of metabolism associated with photosynthesis is in both cell types. Visually there are several times (three to five) more mesophyll cells than parenchyma sheath cells in corn leaves, yet about two-thirds of the glycolate oxidase activity was found in the parenchyma sheath cells. Thus the level of glycolate oxidase per parenchyma sheath cell could be as much as 10-fold greater than in a mesophyll cell. This assumption is consistent with the higher specific activities of these enzymes in the extracts after grinding exhaustively with the roller mill.

The distribution of each enzyme activity associated with glycolate metabolism was not the same between the two cell types in corn or sugarcane leaves (Table I). Three of these enzymes, glycolate oxidase, NADH-hydroxypyruvate reductase, and catalase were located together in peroxisomes from spinach leaves (21). However, from corn or sugarcane leaves 31% of the glycolate oxidase was released by grinding the tissues for 30 s with the Waring Blendor, whereas 49 or 39% of the reductase and 53 or 63% of the catalase was released. The results suggest the possibility of peroxisomes of different enzymatic composition or soluble forms of the enzymes in the two cell types.

One objective of this paper was to examine the level of activity of enzymes for glycolate metabolism in plants without CO_2-photorespiration. These values varied widely, but on an average there was about a third as much enzymatic activity in corn leaves on a total fresh weight basis as in spinach or wheat leaves. Nearly the same level of glycolate oxidase was found in the two *Atriplex* varieties. The absence of CO_2-photorespiration in plants can not be attributed to very low levels of enzymatic activities associated with glycolate metabolism, but is more likely due to very efficient refixation of the CO_2. The reduced level of enzyme activity for glycolate metabolism may well reflect a lower level of overall photorespiration in these plants.

Complete grinding of corn and sugarcane leaves is an extremely difficult undertaking. Careful and prolonged use of the roller mill for complete cell breakage has been used as another procedure besides exhaustive grinding by mortar with sand. The isolation and investigation of peroxisomes from plants without CO_2-photorespiration has not yet been accomplished. The drastic procedures necessary to break parenchyma sheath cells are believed to break also the peroxisomes, which are very fragile. We are thus left with the dilemma of how to break parenchyma sheath cells from this type of plant without also breaking the peroxisomes.

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