Aminotransferases in Peroxisomes from Spinach Leaves*

(Received for publication, January 28, 1972)

DWAYNE W. REHFIELD† and N. E. TOLBERT§

From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

SUMMARY

Organelles in spinach leaves were isolated in sucrose gradients by isopycnic centrifugation. The peroxisomal fraction was the only cellular site for two different and irreversible aminotransferases which utilized glyoxylate as the amino acceptor in the formation of glycine. These were a serine:glyoxylate aminotransferase with a specific activity of 1.54 μmoles × min⁻¹ × mg⁻¹ peroxisomal protein and a glutamate:glyoxylate aminotransferase with a specific activity of 2.4. Both enzymes also catalyzed an alanine:glyoxylate reaction, and the serine:glyoxylate aminotransferase catalyzed a serine:pyruvate reaction. These activities of the peroxisomal serine:glyoxylate aminotransferase coincided in fractions isolated on ion exchange or isoelectric focusing columns. For serine:glyoxylate aminotransferase reaction the $K_m$ (glyoxylate) was 0.15 mM, the $K_m$ (pyruvate) was 2.82 mM, and the $K_m$ (serine) was 2.72 mM. The pH optimum was about 7. This enzyme was inhibited by n-serine, and phosphate buffer at 70 μM inhibited it 80%.

Aspartate:α-ketoglutarate aminotransferase activity was located in peroxisomes, mitochondria, and chloroplasts. By ion exchange chromatography and polyacrylamide gel electrophoresis these were separated into three isoenzymes. Isoenzyme 1 was by far the most active, and it was the only form in mitochondria and chloroplasts. The combined specific activity of the peroxisomal aspartate aminotransferases was a low 0.15 μmole × min⁻¹ × mg⁻¹ protein. Isoenzyme 1 represented about half of this activity in the peroxisomes, and the peroxisomes contained in addition small amounts of Isoenzymes 2 and 3. The metabolic sequence for glycolate metabolism in leaves was modified to include the three different peroxisomal aminotransferases for glutamate:glyoxylate, serine:glyoxylate, and aspartate:α-ketoglutarate.

Among the enzymes for glycine and serine synthesis by the glycolate pathway in leaf peroxisomes (1) are several aminotransferases. Kisaki and Tolbert (2) showed that a glutamate-glyoxylate aminotransferase was located exclusively in peroxisomes of the spinach leaf. Yamazaki and Tolbert (3) found in isolated spinach leaf peroxisomes two additional aminotransferase activities, one for serine:pyruvate and the other for aspartate:α-ketoglutarate. The aspartate aminotransferase activity in the peroxisomes was separated into three isoenzymes by polyacrylamide disc gel electrophoresis (3, 4). Soluble and particulate forms of aspartate aminotransferase have been reported in leaves (3-6), and this aminotransferase is present in glyoxysomes and mitochondria of castor bean endosperm (7) and sunflower cotyledons (8). Murerji and Ting (9) found aspartate aminotransferase in the chloroplasts and mitochondria of cactus. Serine:glyoxylate aminotransferase has been partially purified from wheat and oat leaves (10, 11), and Sallach and coworkers (12, 13) have studied an alanine:hydroxybutyrate aminotransferase in green leaves. Because an aminotransferase may catalyze several reactions, it was deemed necessary to separate and examine all of these activities in isolated leaf peroxisomes simultaneously in order to evaluate how many separate enzymes were present in the organelle.

MATERIALS AND METHODS

Plants—Spinacia oleracea L. was purchased in local markets or the variety Longstanding Bloomsdale was grown in growth chambers. No apparent differences in the aminotransferase activities of isolated peroxisomes were attributable to the origin of the spinach leaves. Leaf tissue without midveins was ground either with a mortar and pestle, or for 10 s in a blender with an equal weight of a grinding medium composed of 30% (w/w) sucrose and 20 mM glycylglycine at pH 7.5. The homogenates were squeezed through cheesecloth and centrifuged at 650 × g for 5 min to remove cellular debris. This centrifugation also removed most of the whole chloroplasts but only part of the broken plastide. The homogenate was then placed directly on top of sucrose gradients and fractionated by isopycnic centrifugation (14).

Organelle Separation—Gradients were prepared with aliquots of the following percentages of sucrose (w/w) solutions in 20 mM glycylglycine at pH 7.5: 25%, 30%, 35%, 40%, 41%, 42%, 43%, 44%, 49%, 50%, 51%, 52%, 52.5%, and 56%. Small gradients in tubes for the Beckman SW 25.2 rotor were composed of 2 ml of each solution. For large gradients in the International B-29 zonal rotor, 50 ml of each sucrose solution was used. Gradients in the B-30 zonal rotor were made with a 50-ml pad of 56% sucrose and 10-ml fractions of 25%, 30%, and 35% and 20-ml fractions of the other concentrations of sucrose.

For small gradients the homogenate from 20 g of spinach leaf

* This work was supported by National Science Foundation Grant GB-3204X and a traineeship for D. W. Rehfeld from National Institutes of General Medical Sciences, GM 1091. It is published as Journal Article No. 5796 of the Michigan Agricultural Experiment Station.

† Present address, Department of Chemistry, University of Arizona, Tucson, Arizona 85721.

‡ To whom correspondence should be addressed.
tissue was layered onto the top of a sucrose gradient, which was then centrifuged for 21/2 hours at 25,000 rpm. Fractions of 1 ml were collected from the bottom of the tube. For zonal gradients homogenate from 250 g of leaves was added next to the core of a B-30 rotor or from 500 g of leaves for the B-29 rotor. Centrifugation was for 2 hours at 30,000 rpm. The zonal rotors were unloaded from the edge and 10- or 20-ml fractions were collected. Particular fractions in the gradient were located by marker enzymes. Catalase and hydroxypropyruvate reductase were indicative of peroxisomes, cytochrome c oxidase of mitochondria, and chlorophyll of broken chloroplasts.

Intact chloroplasts were isolated separately from leaf homogenates by an initial differential centrifugation at 650 X g for 5 min. The particles were washed by resuspending in grinding medium and centrifuging in a 50-ml tube at 270 X g for 10 min through a layer of 25 ml of 30% sucrose.

Assays—A unit of enzyme activity catalyzed the formation of 1 pmole of product per min, and assays were run with a recording Gilford spectrophotometer at 25°. L-amino acids were used unless stated otherwise. Catalase was measured by following the absorbance decrease at 240 nm of 12.5 mM H_2O_2 (15). The oxidation of cytochrome c was followed at 550 nm as described previously (16). Malate dehydrogenase (17) and hydroxypyruvate reductase (18) were assayed by the oxidation of NADH. Chlorophyll was measured by Arnon's procedure (10) and protein by the procedure of Lowry et al. (20) with crystalline bovine serum albumin as the standard.

Aspartate:α-ketoglutarate aminotransferase was assayed by linking the formation of oxalacetate to malate dehydrogenase. The assay mixture contained 70 μmoles of Hepes at pH 7, 30 μmoles of α-ketoglutarate, 0.1 μmole of pyridoxal-5-phosphate, 0.09 μmole of NADH, 2 units of malate dehydrogenase (Sigma Chemical Co.), 20 μmoles of aspartate, and enzyme in a total volume of 1 ml. Reactions were started with aspartate.

Serine:glyoxylate aminotransferase was measured spectrophotometrically at 340 nm by linking the reduction of the product, hydroxypropyruvate, to the oxidation of NADH, as catalyzed by addition of excess hydroxypropyruvate reductase (10). The assay mixture contained 70 μmoles of Hepes at pH 7, 0.17 μmole of pyridoxal-5-phosphate, 1 μmole of glyoxylate, 0.05 unit of glyoxylate (hydroxypropyruvate) reductase (Sigma Chemical Co.), and enzyme in a total volume of 1 ml. The endogenous rate due to glyoxylate reduction by the added reductase was slow, because the K_m (glyoxylate) is 20 to 50 mM (18, 21). The aminotransferase reaction was started with 20 μmoles of L-serine. Since the K_m (hydroxypropyruvate) is 0.05 to 0.2 mM, the increased rate of NADH oxidation was attributed to the formation of hydroxypropyruvate from serine in the transamination reaction. A serine:pyruvate aminotransferase activity was assayed by the same protocol except that 3 μmoles of pyruvate were used instead of glyoxylate, and in this case the rate in the absence of an amino donor was negligible. An alanine:glyoxylate aminotransferase was assayed similarly by substituting 0.03 unit of lactate dehydrogenase (Sigma Chemical Co.) for the hydroxypropyruvate reductase and 20 μmoles of L-alanine for serine.

Glutamate:glyoxylate aminotransferase was assayed by a 

1 The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; TEAE-, triethylaminoethyl-; Bioine, N,N'-bis(2-hydroxyethyl)glycine.

action mixture contained 70 μmoles of phosphate or cacodylate buffer at pH 7.0, 0.1 μmole of pyridoxal-5-phosphate, 32 μmoles of L-glutamate, 5 μmoles of [1,2-3H]glyoxylate, and enzyme in a total volume of 1 ml. Assays were run for 30 min and terminated by heating in a boiling water bath for 3 min. Excess [1,2-3H]glyoxylate was removed by a Dowex 1-acetate column, which was washed with water to elute all of the [3H]glycine product. Radioactivity in the effluent was measured by scintillation counting. All samples were corrected for nonenzymatic transamination.

Electrophoresis—Polyacrylamide disc gel electrophoresis was run with 7% gels at pH 8.3 and electrode solutions at pH 8.3 (22). No sample or spacer gel was used, but rather the sample in sucrose solution was layered on top of the gel. In starch gel electrophoresis, the 12% gel and the electrode solution contained 5 mM Tris and 35 mM glycine at pH 8.3 (23). Samples of 100 μl were placed in the sample wells and subjected to electrophoresis at 500 volts for 10 to 16 hours.

The electrophoretic gels were stained for aspartate:α-ketoglutarate aminotransferase activity by using Fast Violet B salt (6-benzamido-4-methoxy-m-toluidine diazonium chloride) which reacts with oxaloacetate (24, 25). One ml of staining mixture contained 80 μmoles of TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer at pH 7, 20 μmoles of aspartate, 5 μmoles of α-ketoglutarate, 0.1 μmole of pyridoxal-5-phosphate, and 1 mg of Fast Violet B, which was added just before use. The disc gels were immersed in staining solution in test tubes. The starch gels were sliced in half, excess moisture removed, and then a piece of Whatman No. 1 chromatography paper soaked with staining solution was placed over the gel.

Isoelectric Focusing—This was conducted in a small column by a modification of the procedure described by Godson (20). The column consisted of a 5-ml pipette with the tip cut off and plugged with a small amount of polyacrylamide gel at the bottom. One milliliter of cathode solution containing sucrose was layered above the polyacrylamide plug. Four milliliters of a linear sucrose gradient containing the ampholite carriers (LKB) and up to 5 mg of protein were placed in the column. Anode solution was added to fill the column. The column was then placed in a 250-ml graduated cylinder filled with cathode solution which was stirred to help dissipate the heat. Platinum electrodes were immersed in the electrode solutions. The columns were run in a 4° cold room. To drain the column, a glass capillary tube was forced through the polyacrylamide plug and 0.1-ml fractions were collected.

Ion Exchange Chromatography—Peroxisomes in the sucrose gradient fractions were ruptured by dilution with 5 mM Tris-HCl buffer at pH 8.3 and placed on a TEAE-cellulose column which had been previously equilibrated with the same buffer. The column was washed with this buffer which removed glycolate oxidase. The remaining peroxisomal proteins were eluted with a linear gradient of 0 to 0.3 M KCl.

RESULTS

Three different aminotransferase proteins were found in peroxisomes isolated from spinach leaves and have been designated as glutamate:glyoxylate, serine:glyoxylate, and aspartate:α-ketoglutarate. Other observed aminotransferase activities can be attributed to one or more of these enzymes. The isoenzymes of aspartate aminotransferase are designated 1, 2, and 3 on the basis of relative mobility toward the anode during electrophoresis.
Aspartate aminotransferase activity in organelle fractions

The whole chloroplasts were isolated by differential centrifugation and the other organelles by sucrose gradient isopycnic centrifugation. The estimates of percentage of contamination were based upon the amount of specific marker enzymes in each of the organelle fractions. An example of this calculation is:

\[
\frac{(\text{catalase in chloroplasts}) \times \text{aminotransferase in peroxisomes}}{(\text{catalase in peroxisomes}) \times \text{aminotransferase in chloroplasts}} \times 100
\]

This gives the percentage of the total aminotransferase in chloroplasts attributable to peroxisomes.

### Table I

<table>
<thead>
<tr>
<th>Organelle fraction</th>
<th>Specific activity</th>
<th>Peroxosomal activity in fractions</th>
<th>Mitochondrial activity in fractions</th>
<th>Whole chloroplast activity in fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu\text{moles} \times \text{min}^{-1} \times \text{mg}^{-1} \text{ protein})</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Peroxosomes</td>
<td>144</td>
<td>85</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>96</td>
<td>14</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>Whole chloroplasts</td>
<td>50</td>
<td>6</td>
<td>6</td>
<td>88</td>
</tr>
</tbody>
</table>

Subcellular Location of Aminotransferases in Spinach Leaves—A typical distribution for the marker enzymes used to locate organelles on a sucrose gradient after isopycnic centrifugation is shown in Fig. 1. Similar distributions were obtained with both swinging bucket and zonal rotors, the latter being used to obtain sufficient peroxisomes for protein fractionation. Chlorophyll marked the location of broken chloroplasts. There were two peaks of cytochrome c oxidase; the major mitochondrial peak was at a density of 1.20 g x cc^{-1}, while activity was also in fractions of lower sucrose density characteristic of microsomes (27). Catalase at a density of 1.25 g x cc^{-1} located the peroxisomes which were well separated from the mitochondria and chloroplasts.

The distribution of serine:glyoxylate aminotransferase on the sucrose gradient was the same as that for catalase (Fig. 1). There was no peak of activity with other particulate fractions, and thus the serine:glyoxylate aminotransferase appears to be located exclusively in the peroxisomes. Similar data were previously obtained for the peroxisomal location of glutamate:glyoxylate aminotransferase (2, 8). Most of the alanine:glyoxylate aminotransferase activity was also located in the peroxisomal band, but there was some activity in fractions at lower densities that did not correspond with either the peak of mitochondria or broken chloroplasts. It is concluded that the serine, glutamate, and alanine aminotransferase reactions with glyoxylate are primarily peroxisomal, and this is consistent with the formation of glyoxylate only in the peroxisomes as a result of glycolate oxidation.

Aspartate aminotransferase activity was found on the sucrose gradient in the peroxisomal, mitochondrial, and supernatant fractions (Fig. 1). Although the broken chloroplast fraction from the sucrose gradients contained little aspartate aminotransferase activity, washed whole chloroplasts isolated by differential centrifugation contained considerable amounts of this activity (Table I). The percentage of the total aspartate aminotransferase activity in each of the organelle fractions attributable to contamination by the other organelles was calculated upon the basis of the percentage of activity of marker enzymes in each of the organelle fractions. Of the total aspartate aminotransferase activity in the peroxisomes, only 15% could be attributed to mitochondrial and chloroplastic contamination. Likewise over half of the activity in the mitochondrial fraction was not due to contamination by other organelles. In intact washed chloroplasts isolated by differential centrifugation only 12% of the aspartate aminotransferase activity could be attributed to mitochondrial and peroxisomal contamination. It is concluded that aspartate:α-ketoglutarate aminotransferase activity is located in all three subcellular organelles. On the other hand only peroxisomes contain aminotransferases for the formation of glycine from glyoxylate. The specific activity for glutamate:glyoxylate aminotransferase was 2.40 μmoles X min^{-1} X mg^{-1} peroxisomal protein, for serine:glyoxylate 1.54, for alanine:glyoxylate 0.87, and for aspartate:α-ketoglutarate 0.15. These data do not indicate to what extent each of these enzymes may also be located in the cytosol in vivo. The soluble fraction from
the sucrose gradient always contained these activities, which could arise either from the cytosol or from release of enzymes from broken particles.

Some Characteristics of Peroxisomal Glyoxylate Aminotransferases—Both serine:glyoxylate and alanine:glyoxylate aminotransferase activities in isolated spinach leaf peroxisomes were optimal at pH 7 under the assay conditions described (Table II). Rates of serine:glyoxylate aminotransferase activity were nearly the same at pH 7 in Hepes, cacodylate, or bicine buffer. Activity was nearly maximal in Bicine at pH 6, but declined at pH 8 in cacodylate buffer. Phosphate buffer at 70 μM and pH 7 inhibited the serine:glyoxylate aminotransferase reaction about 80%; at 10 mM phosphate the inhibition was 34%. Ammonium sulfate (12 mM) was also inhibitory. Because of the high concentrations the phosphate inhibition is probably not of physiological importance. The use of phosphate buffer, however, is to be avoided. King and Waygood (11), using phosphate buffer, had reported a pH optimum of 8.2 for serine:glyoxylate aminotransferase from wheat leaves.

Alanine:glyoxylate aminotransferase had nearly the same activity in Hepes, cacodylate, phosphate, and Bicine buffers at pH 7. The rate was about 50% less at pH 6 in cacodylate or at pH 8 in Bicine. The alanine:glyoxylate activity was not affected by phosphate buffer.

The glutamate:glyoxylate aminotransferase had the same activity in cacodylate and phosphate buffers at pH 7 (data not shown). A pH optimum of 7 for this enzyme had been previously reported for spinach peroxisomes (2) and for the enzyme from both rat and human liver (28, 29).

Of the aminotransferases in spinach leaf peroxisomes, only serine:glyoxylate aminotransferase was inhibited by phosphate. This result suggests that the serine:glyoxylate reaction may be catalyzed by a different protein than the glutamate:glyoxylate and alanine:glyoxylate reactions.

D-Serine inhibited by 85% the serine:glyoxylate aminotransferase reaction, whereas the alanine:glyoxylate aminotransferase reaction was inhibited only 35% (Table II). D-Alanine had little effect on either reaction. D-Aspartate also did not affect the aspartate aminotransferase activity of spinach leaf peroxisomes (data not shown). The D-serine inhibition of serine:glyoxylate aminotransferase did not appear to be purely competitive or noncompetitive; plots of 1/velocity versus 1/serine concentration were nonlinear in the presence of varying amounts of D-serine (Fig. 2). Although this kinetics is characteristic of an allosteric inhibitor, the enzyme may not have an allosteric site, since the interaction of D-serine with the various forms of the enzyme which exist during the aminotransferase reaction may result in complex kinetics.

Ion Exchange Chromatography of Peroxisomal Aminotransferases—Peroxisomes were broken by dilution, placed on a TEAE-cellulose column, and eluted with a linear gradient of 0 to 0.3 M KCl (Fig. 3). A peak of serine:glyoxylate aminotransferase eluted with about 0.2 M KCl but represented only about 50% recovery of activity. The peaks using either glyoxylate or pyruvate as the amino acceptor coincided.

Malate dehydrogenase and hydroxypyruvate reductase, which are major leaf peroxisomal enzymes, were partially separated from the other enzyme activities. However, the peroxisomal catalase peak coincided with the serine:glyoxylate aminotransferase activity (Fig. 3). The serine:glyoxylate aminotransferase elution profile did not correspond to any of the three aspartate aminotransferase peaks of activity. Glutamate:glyoxylate and alanine:glyoxylate aminotransferase activities were not recovered from the column even with 1.0 M KCl, suggesting that these latter activities were not catalyzed by the serine:glyoxylate aminotransferase. Brock et al. (10) have also reported that upon partial purification of serine:glyoxylate aminotransferase from oat leaves, the glutamate:glyoxylate and alanine:glyoxylate activities were lost.

Isoelectric Focusing of Peroxisomal Enzymes—The distribution of aminotransferases in a pH 3 to 10 gradient (Fig. 4) indicated that activities for serine:glyoxylate and for glutamate:glyoxylate

Table II

<table>
<thead>
<tr>
<th>Buffer and additions</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serine:glyoxylate aminotransferase</td>
</tr>
<tr>
<td>Heps (pH 7)</td>
<td>100</td>
</tr>
<tr>
<td>Cacodylate (pH 7)</td>
<td>50</td>
</tr>
<tr>
<td>Cacodylate (pH 7)</td>
<td>88</td>
</tr>
<tr>
<td>Phosphate (pH 6)</td>
<td>10</td>
</tr>
<tr>
<td>Phosphate (pH 7)</td>
<td>20</td>
</tr>
<tr>
<td>Bicine (pH 7)</td>
<td>85</td>
</tr>
<tr>
<td>Bicine (pH 8)</td>
<td>85</td>
</tr>
<tr>
<td>Heps + d-serine (pH 7)</td>
<td>15</td>
</tr>
<tr>
<td>Heps + d-serine (pH 7)</td>
<td>84</td>
</tr>
</tbody>
</table>

FIG. 2. n-Serine inhibition of serine:glyoxylate aminotransferase. Spinach peroxisomes isolated on a sucrose gradient were used and the glyoxylate concentration was at 1 mM. From 0 to 20 mM n-serine was added to the assay before the enzyme, whose activity was then measured with L-serine. Incubation of the enzyme with n-serine for 2 min gave the same results as with no incubation period.
FIG. 3. Separation of peroxisomal enzymes by ion exchange chromatography. Peroxisomes, isolated on a sucrose gradient were broken by osmotic shock, placed on a TEAE-cellulose column, and eluted with a 0 to 0.3 M KCl gradient. ●―●, aspartate aminotransferase; ○―○, serine:glyoxylate aminotransferase; △―△, catalase; □―□, malate dehydrogenase; ▲―▲, hydroxybutyrate reductase. The serine:glyoxylate aminotransferase was assayed by the serine:pyruvate aminotransferase protocol; rates for serine:glyoxylate aminotransferase activity would be approximately 10 times higher, but proportional in each fraction to the serine:pyruvate activity.

FIG. 4. Isoelectric focusing of peroxisomal aminotransferases. Peroxisomes, isolated on a sucrose gradient, were broken by osmotic shock, centrifuged to remove the traces of chloroplasts, and placed on a pH 3 to 10 isoelectric focusing column that was run at 500 volts for 9 hours. For aspartate aminotransferase, relative values of the peak fractions are in parentheses.

Fraction Number

Fraction Number

FIG. 4. Isoelectric focusing of peroxisomal aminotransferases. Peroxisomes, isolated on a sucrose gradient, were broken by osmotic shock, centrifuged to remove the traces of chloroplasts, and placed on a pH 3 to 10 isoelectric focusing column that was run at 500 volts for 9 hours. For aspartate aminotransferase, relative values of the peak fractions are in parentheses.
TABLE III
Comparison of serine:glyoxylate and serine:pyruvate aminotransferase activities in isolated spinach peroxisomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serine:glyoxylate aminotransferase</th>
<th>Serine:pyruvate aminotransferase</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>50° for 0 min.</td>
<td>945</td>
<td>90</td>
<td>10.6</td>
</tr>
<tr>
<td>50° for 5 min.</td>
<td>439</td>
<td>46</td>
<td>9.5</td>
</tr>
<tr>
<td>50° for 15 min.</td>
<td>310</td>
<td>31</td>
<td>10.0</td>
</tr>
<tr>
<td>50° for 25 min.</td>
<td>284</td>
<td>28</td>
<td>10.1</td>
</tr>
<tr>
<td>50° for 35 min.</td>
<td>232</td>
<td>26</td>
<td>9.0</td>
</tr>
<tr>
<td>Hepes buffer</td>
<td>419</td>
<td>53</td>
<td>8.0</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>97</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Serine (20 mM)</td>
<td>232</td>
<td>22</td>
<td>10.0</td>
</tr>
<tr>
<td>D-Serine (20 mM)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D-+ L-Serine (40 mM)</td>
<td>64</td>
<td>17</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* The three different types of treatments were with different enzyme preparations.

reaction, but inhibitors may have different effects on two reactions catalyzed by one enzyme. The serine:glyoxylate activity was not significantly inhibited by ATP, ADP, AMP, 3-P-L-serine, 3-P-glycerate, and p-glycerate.

Homogenates of Atriplex and of sorghum leaves contain an aminotransferase activity that was designated as serine:glyoxylate and that also catalyzed at a slower rate the serine:pyruvate aminotransferase reaction (30). An earlier report (3) that spinach leaf peroxisomes contained a serine:pyruvate aminotransferase should be changed to mean a serine:glyoxylate aminotransferase. Sallach and coworkers (12, 13) have studied an alanine:hydroxypruvate aminotransferase in green leaves, which appears equivalent to the designation serine:pyruvate aminotransferase.

Irreversibility of Glyoxylate Aminotransferases—Earlier reports using crude homogenates and reaction times of an hour had implicated an aminotransferase reaction using glycine as the amino donor (31-33). Other reports using partially purified enzymes had been unable to detect glycine:α-keto acid aminotransferase activity (2, 10, 29, 34), as if the amino acid:glyoxylate aminotransferase reactions were nearly irreversible. In the present studies, no reversible reactions were detected for glutamate:glyoxylate, serine:glyoxylate, or alanine:glyoxylate aminotransferase activities from isolated peroxisomes. However a very small and probably physiologically insignificant exchange reaction between glyoxylate and glycine was observed by a radioactive assay.

Aspartate:α-Ketoglutarate Aminotransferases in Spinach Leaves—Since this activity was found in the supernatant, whole chloroplasts, mitochondria, and peroxisomes (Fig. 1 and Table I), the isoenzymic nature of the enzyme in its different cellular locations was studied by polyacrylamide gel electrophoresis (Fig. 5), starch gel electrophoresis (Fig. 6), TEAE-cellulose chromatography (Fig. 7), and isoelectric focusing (pH 3 to 10) (Fig. 3). A major single enzyme band [Isoenzyme 1] with the same electrophoretic mobility was found in both isolated chloroplasts and mitochondria, and this isoenzyme was also the dominant one in the crude cell homogenate (Figs. 5 and 6). From whole chloroplasts obtained by an initial low speed centrifugation of the homogenate, a band at the anode front also

---

FIG. 5. Separation of aspartate aminotransferase isoenzymes on polyacrylamide gels. The peroxisomes and mitochondria were isolated on sucrose gradients. Whole chloroplasts were in the pellet obtained by centrifuging the spinach homogenate at 659 X g. Washed whole chloroplasts were the pellet obtained by centrifuging the resuspended chloroplast fraction through a layer of 30% sucrose.

FIG. 6. Starch gel electrophoresis of aspartate aminotransferase isoenzymes. The gel and electrode tanks contained 5 mM Tris-glycine buffer at pH 8.3, and the electrophoresis was at 400 volts for 10 hours.

FIG. 7. Separation of peroxisomal aspartate aminotransferase isoenzymes by TEAE-cellulose chromatography. An aliquot from each peak was then assayed by polyacrylamide gel electrophoresis as in Fig. 5.
stained with dye in the absence of added aspartate in the assay. If the chloroplasts were washed by resuspension and centrifugation through a 30% sucrose layer, this nonenzymatic band was not detected. The nature of the reacting material in the chloroplasts is unknown.

From the isolated peroxisomes three isoenzymes of aspartate aminotransferase were obtained (Figs. 3, 5, 6, 7). The fastest moving Isoenzyme 1 had the same electrophoretic mobility as the chloroplast or mitochondrial form. The slower moving Isoenzymes 2 and 3 on polyacrylamide gels were found only in the peroxisomal fraction from the sucrose gradients (Fig. 5). Electrophoresis of mixtures of the particles showed that the fast moving band in all three organelles was the same. The two distinct peroxisomal isoenzymes were detectable in the original homogenate by polyacrylamide gel, if sufficient homogenate was used, but the fast moving Isoenzyme was dominate in the homogenate (Fig. 5). With starch gel electrophoresis (Fig. 6) the two slow moving peroxisomal isoenzymes of aspartate aminotransferase did not move far from the origin and did not separate.

The three peroxisomal aspartate aminotransferases were well separated on a TEAE-cellulose column with each peak representing a different isoenzyme mobility on polyacrylamide electrophoresis (Fig. 7). Isoenzyme 3 eluted from TEAE at the lowest KCI concentration (Peak A) and had the lowest electrophoretic mobility on polyacrylamide; Isoenzyme 1 eluting at highest KCl concentration (Peak C) had the fastest electrophoretic mobility. In addition Isoenzyme 3 from the TEAE-cellulose column, upon polyacrylamide electrophoresis appeared to partially resolve into two or three closely spaced bands. Removal of KCl by dialysis from Peak A of the TEAE-cellulose fractions did not change the electrophoretic pattern. The aspartate aminotransferase Isoenzyme 1 from peroxisomes in Peak C from the TEAE-cellulose column was inhibited by phosphate buffer more than Isoenzymes 2 and 3.

It has been mentioned in a previous section that none of the three isoenzymes of aspartate aminotransferase activity from TEAE-cellulose columns coincided with other aminotransferase activities in the peroxisomes. In a fraction where aspartate aminotransferase and serine:glyoxylate aminotransferase overlapped, rates with combined substrates equalled the sum of the individual aminotransferase activities. Also none of the three peroxisomal aspartate aminotransferases isoenzymes, when separated by a pH 3 to 10 isoelectric focusing column (Fig. 4) corresponded to any of the glyoxylate aminotransferases. Recovery of the aspartate aminotransferase from the column was nearly 100%, and boiled controls of each fractions had no activity. Thus the three peroxisomal aspartate aminotransferase isoenzymes cannot be attributed to the nonspecificity of the glyoxylate aminotransferase.

Aspartate aminotransferase Isoenzyme 1 from peroxisomes seemed identical with the form of that aminotransferase in the mitochondria and chloroplasts. Based upon the intensity of the stain in polyacrylamide gels, this isoenzyme comprised 40 to 50% of the small amount of total aspartate aminotransferase activity in the peroxisomes. It is possible that Isoenzyme 1 in the peroxisomal fraction from the sucrose gradient may be due to contamination by other organelles. However, Isoenzymes 2 and 3 of slower electrophoretic mobility were exclusively found in the peroxisomes.

**DISCUSSION**

The metabolic sequence visualized in leaf peroxisomes (Fig. 8) is based upon the investigations of the enzymatic composition of the isolated particle and the known sequence for glycolate and glycerate metabolism as established by 14C tracer methodology (35). Glycolate is formed in the chloroplasts during photosynthesis and is oxidized in the peroxisomes to glyoxylate (Reaction 1) and H2O2, which is destroyed by catalase (Reaction 2). Therefore it is consistent to find that two different aminotransferases, glutamate:glyoxylate (Reaction 3) and serine:glyoxylate (Reaction 4), for glyoxylate conversion to glycine are also exclusively located in the leaf peroxisome. Serine:glyoxylate aminotransferase has been localized mainly in the bundle sheath cells of plants with β-carboxylation (30), which is also consistent with the known presence of more peroxisomes in these cells than in mesophyll cells (36, 37). The physiological irreversibility of the glyoxylate aminotransferase reactions is similar to that for other irreversible metabolic steps during glycolate biosynthesis and metabolism, which combined represent a metabolic process manifested by photorepiration.

In the glycolate pathway 2 moles of glyoxylate are converted by transamination to 2 moles of glycine, which in turn are converted to 1 mole of serine. If all of the glycine were converted to serine and all of the serine underwent a further transamination to hydroxypyruvate in the peroxisome, there would be only half enough serine for the conversion of glycolate to glycine (Fig. 8). Since the serine:glyoxylate aminotransferase has a lower K_m (glyoxylate) (0.15 mM) than the glutamate:glyoxylate aminotransferase (4.4 mM) (2), the serine:glyoxylate reaction should preferentially be utilized in the peroxisomes for glycine formation. However a second peroxisomal aminotransferase, glutamate:glyoxylate is necessary for conversion of all of the glyoxylate to glycine. The serine:glyoxylate aminotransferase reaction would involve only an internal transfer of amino nitrogen within the peroxisomes, independent of the rest of the cell. The glutamate:glyoxylate aminotransferase reaction requires other cellular sources of glutamate or alanine. The glutamate:glyoxylate aminotransferase with a specific activity of 2.4 μmoles min⁻¹ mg⁻¹ peroxisomal protein and serine:glyoxylate of 1.54 specific activity are sufficiently active for the calculated metabolic fluxes (4). An alanine:glyoxylate transamination reaction also appeared to be catalyzed by these two enzymes, and a serine:pyruvate reaction, which has previously been described, can be catalyzed by the serine:glyoxylate aminotransferase. This latter reaction was of lower specific activity and is not considered to participate in our current scheme for the glycolate pathway in peroxisomes.

The peroxisomal glycerate to serine interconversion (Reactions 6 and 7) is an anaerobic reversible metabolic pathway. In the conversion of serine to hydroxypropionate during glycolate metabolism in the light, most of the reaction is probably catalyzed by the serine:glyoxylate aminotransferase (Reaction 4). However during dark metabolism and synthesis of serine from glyceraldehyde and hydroxyproprionate in peroxisomes, a transamination is necessary which cannot be carried out by the serine:glyoxylate aminotransferase because of the irreversibility of that reaction and because glycolate and glyoxylate are only formed in the light. Consequently to form serine from hydroxypropionate another aminotransferase (Reaction 6) is required in peroxisomes. This latter reaction may be catalyzed by one of the already known
Fig. 8. Scheme for involvement of aminotransferases in peroxisomal metabolism. Enzymes indicated by numbers are: 1, glycolate oxidase; 2, catalase; 3, glutamate:glyoxylate aminotransferase; 4, serine:glyoxylate aminotransferase; 5, serine hydroxymethyltransferase; 6, an unidentified aminotransferase; 7, NADH:hydroxypyruvate reductase; 8, NAD:malate dehydrogenase; 9, aspartate:α-ketoglutarate aminotransferase.

Aminotransferases of the particle or by as yet undetected enzyme. Peroxisomes contain an active glutamate:hydroxypyruvate activity catalyzed by the glutamate:glyoxylate aminotransferase (2), and in Fig. 8 we have shown this reaction as a possibility for serine formation from hydroxypyruvate. We have not excluded the possibility that one of the aspartate aminotransferases may also function in serine formation.

The peroxisomal malate dehydrogenase (Reaction 8) and aspartate:α-ketoglutarate aminotransferases (Reaction 9) are postulated to function in a malate-aspartate shuttle (38-40) of reducing equivalents (as malate) into the organelle. In Fig. 8, only one aspartate aminotransferase in the peroxisome is envisaged, yet two and possibly three isoenzymes were found in the organelle. The phenomenon of multiple forms of aspartate aminotransferase in peroxisomes was not observed in chloroplasts or mitochondria where only one common isoenzyme was present. However, the total peroxisomal aspartate aminotransferase activity of all three isoenzymes was only about one-tenth of the activity of the glutamate:glyoxylate and serine:glyoxylate aminotransferases.

The presence of enzyme activity in different subcellular organelles or sites has often been attributed to isoenzymic forms of that enzyme. This was true for NAD-malic dehydrogenase in spinach leaves which has different isoenzymes in the peroxisomes, mitochondria, and cytosol (17, 41). In the present investigation a major isoenzyme 1 of aspartate aminotransferases was found in the cytosol, chloroplasts, mitochondria, and peroxisomes. It was only in the peroxisomes that additional isoenzymes 2 and 3 were found. The presence of aspartate aminotransferase Isoenzyme 1 in the various subcellular sites is noteworthy, and suggests that this isoenzyme for all of the organelles may be synthesized at one location.

Acknowledgments—We thank Robert Donaldson, George Lorimer, Sandra Wardell, and Angelika Schnarrenberger for help in isolating the peroxisomes.

REFERENCES
Aminotransferases in Peroxisomes from Spinach Leaves
Dwayne W. Rehfeld and N. E. Tolbert


Access the most updated version of this article at [http://www.jbc.org/content/247/15/4803](http://www.jbc.org/content/247/15/4803)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/247/15/4803.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/247/15/4803.full.html#ref-list-1](http://www.jbc.org/content/247/15/4803.full.html#ref-list-1)