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Evidence for the Presence in Tobacco Leaves of Multiple Enzymes for the Oxidation of Glycolate and Glyoxylate

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ABSTRACT

The enzymic oxidation of glycolate to glyoxylate and glyoxylate to oxalate by preparations purified from tobacco (*Nicotiana tabacum* var Havana Seed) leaves was studied. The K_m values for glycolate and glyoxylate were 0.26 and 1.0 millimolar, respectively. The ratio of glycolate to glyoxylate oxidation was 3 to 4 in crude extracts but decreased to 1.2 to 1.5 on purification by $(NH_4)_2SO_4$ fractionation and chromatography on agarose A-15 and hydroxylapatite. This level of glyoxylate oxidation activity was higher than that previously found for glycolate oxidase (EC 1.1.3.1). The ratio of the two activities was changed by reaction with the substrate analog 2-hydroxy-3-butynoate (HBA) which at all concentrations inhibited glyoxylate oxidation to a greater extent than glycolate oxidation. The ratio of the two activities could also be altered by changing the O_2 concentration. Glycolate oxidation increased 3.6-fold when the O_2 atmosphere was increased from 21 to 100%, whereas glyoxylate oxidation increased only 1.6-fold under the same conditions. These changes in ratio during purification, on inhibition by HBA, and under varying O_2 concentrations imply that tobacco leaves contain at least two enzymes capable of oxidizing glycolate and glyoxylate.

Leaf discs of tobacco, in the presence of added glyoxylate, show decreased photorespiration and glycolate synthesis and increased net photosynthesis (13). In order to understand better how glyoxylate regulates photorespiration, a study of the fate of labeled glyoxylate fed to leaf discs in the light was undertaken. A large amount of the added glyoxylate was transformed into oxalic acid and the enzymic conversion of glyoxylate to oxalate by extracts of tobacco leaves also proceeded readily.

Glycolate oxidase (EC 1.1.3.1) has previously been shown to catalyze the conversion of glyoxylate to oxalate and of L-lactate to pyruvate, as well as the oxidation of glycolate to glyoxylate (4, 11, 17). Richardson and Tolbert (14) concluded that both glycolate and glyoxylate were oxidized by the same enzyme. The ratio of the two activities may vary with pH but the activity with glycolate as substrate has always been reported to be at least several-fold higher than with glyoxylate. In the tobacco leaf extracts used here, the ratio of glycolate to glyoxylate oxidation changed during purification of the activities. The characterization of the activities was undertaken to determine whether tobacco contains more than one form of glycyolate oxidase or perhaps an enzyme specific for one of the substrates. It was found that the ratio of the activities also changed with O_2 concentration and on inhibition by HBA[1]. HBA, an acetylenic substrate analog (1), has been shown to be an inhibitor of plant glycolate oxidase (9, 10), but its effect on glyoxylate oxidation has not been reported. The presence of multiple enzymes for the oxidation of glycolate in plants has been described (2, 6, 8), but their catalytic activities have not been investigated in detail.

[1] Abbreviations: HBA, 2-hydroxy-3-butynoate; FMN, flavin mononucleotide.

MATERIALS AND METHODS

Materials. HBA was the generous gift of Dr. Philip Jewess, Shell Research, Sittingbourne, Kent, U.K.; [1-^{14}C]glyoxylate was purchased from Amersham Corp.

Assay of Glycolate and Glyoxylate Oxidase Activity by HPLC. The conversion of glycolate to glyoxylate and glyoxylate to oxalate was measured in reaction mixtures (usually 0.2–0.5 ml total volume) consisting of 10 mM K-phosphate (pH 7.5), 20 mM glycolate or glyoxylate, and enzyme equilibrated in 50 mM K-phosphate (pH 7.5). Samples of the reaction mixture (0.1 ml) were pipetted into tubes containing 0.02 ml 0.4 N H_2SO_4 at zero time and at 5–10 min intervals. The precipitated protein was removed from the acid-treated sample with a microfilter apparatus (Schleicher and Schuell, Inc.) containing a 0.20 μm membrane. The reactants and products of the assay mixture were separated by HPLC on a Perkin-Elmer Series 3B Liquid Chromatograph (Fig. 1). Peak areas, determined with a Hewlett-Packard 3390A Integrator, were converted to nM concentrations for the products of the enzymic reaction. The rate of the reaction was linear for at least 10 min. The definition of a unit of activity, used here, is the amount of enzyme catalyzing the formation of 1.0 μmol of the appropriate product (glyoxylate or oxalate) per min. Enzyme assays usually contained 10 to 15 munits of activity.

A second method of assay, based on O_2 uptake in Warburg vessels (17), was used to confirm the validity of the HPLC method. A third type of assay, used primarily during purification of the enzyme, was based on the spectrophotometric measurement of the reduction of 2,6-dichlorophenolindophenol (16). This method could not be used for the crude enzyme extract because of interfering reactions. The conversion factor of a decrease in absorbance of 0.1/min being equivalent to 1.5 μl of O_2 uptake in 10 min (16) was used to compare the various methods of assay.

Preparation of Enzyme. Mature leaves were cut from greenhouse-grown tobacco (*Nicotiana tabacum* var Havana Seed), washed with a dilute soap solution, rinsed with tap water, and the cut ends placed in a beaker of water for 15 to 30 min. The leaves were blotted dry, the midribs removed, and the lamina weighed into 30-g portions. The leaves were cut into strips (~2.6 cm wide) and frozen in a large mortar with liquid N_2. The frozen pieces were ground by hand into a fine powder; additional liquid N_2 was added, if needed, to prevent thawing. The powder was transferred to a beaker containing 200 ml extraction buffer (0.05 M K-phosphate [pH. 7.5], 20% ethylene glycol, and 2.5 mM DTT) and 20 ml settled volume Dowex 1 (×8)-Cl^- (20–50 mesh) anion exchange resin. A second 30-g batch of leaves was ground and added to the

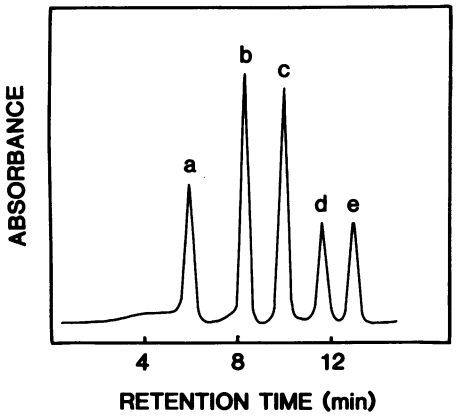

FIG. 1. Separation of organic acids by HPLC. A mixture of oxalic (a), glyoxylic (b), glyceric (c), glycolic (d), and formic (e) acids containing 1.8, 55, 90, 100, and 200 nmol, respectively, was separated by HPLC on an Aminex HP-87H column, 300 × 7.8 nm, with 0.03 N H_2SO_4 as solvent; flow rate, 0.6 ml/min; temperature 25°C; detection, UV at 210 nm.

first. After the suspension was stirred for an additional 5 min, it was filtered through four layers of cheese cloth and the filtrate centrifuged at 40,000*g* for 10 min. Two more 30-g portions of leaf strips were treated by the same procedure and the supernatants from both centrifugations combined for $(NH_4)_2SO_4$ precipitation.

To each 100 ml of supernatant, 26 g of $(NH_4)_2SO_4$ were added with stirring. The suspension was centrifuged at 40,000*g* for 10 min. The precipitate was suspended in ~10 ml of 50 mM K-phosphate (pH 7.5) and centrifuged at 40,000*g* for 10 min. The clear supernatant was designated the 1AS fraction. To the supernatant obtained from the first addition of $(NH_4)_2SO_4$, 9 g of $(NH_4)_2SO_4$ were added for each 100 ml of the original volume. The precipitate was collected by centrifuging at 40,000*g* for 10 min, dissolved in 0.05 M K-phosphate (pH 7.5), and centrifuged at 40,000*g* for 10 min. This supernatant was designated the 2AS fraction.

An agarose A-15 m column (4.0 × 28 cm) was equilibrated with 50 mM K-phosphate (pH 7.5). Enzyme, either 1AS or 2AS fraction, was added to the top, washed in, and then eluted with the same buffer at a rate of 1 ml/min. Enzyme activity was recovered between 90 and 140 ml of the elution volume. The volume of the eluate containing enzyme was measured and 35 g of $(NH_4)_2SO_4$/100 ml of eluate were added. The precipitate was collected by centrifugation at 40,000*g*, dissolved in 50 mM K-phosphate (pH 7.5), dialyzed for at least 2 h against 1 L of the same buffer to remove traces of $(NH_4)_2SO_4$, and then stored at −20°C. The highest purification was achieved if the operations were carried out as quickly as possible through the agarose chromatography step. Dilute enzyme from the agarose A-15 m column could be stored frozen for at least 1 week without appreciable loss of activity.

The agarose A-15 fraction was chromatographed on hydroxylapatite (Bio-Rad) which had been equilibrated with 50 mM K-phosphate (pH 7.5). The enzyme was added to the top of the column (2.0 × 10 cm) and washed in with 100 ml of the same buffer. The enzyme was eluted by a linear phosphate gradient (200 ml each 50 mM and 0.5 M K-phosphate, pH 7.5). Enzyme eluted at ~0.15 M K-phosphate and was concentrated by adding an equal volume of saturated $(NH_4)_2SO_4$ to the fractions containing enzyme. The precipitate was collected by centrifugation and then dissolved in 50 mM K-phosphate (pH 7.5).

Identification of Oxalate as Product Formed Enzymically from Glyoxylate. [1-^{14}C]Glyoxylate (10 mmol, 0.5 μCi) was incubated with 50 munits of enyme and 50 mmol K-phosphate (pH 7.5) in a total volume of 0.5 ml. After 1 h, the reaction was stopped by the addition of 0.05 ml 1 N H_2SO_4. After removal of precipitated protein by centrifugation, $CaCl_2$ solution was added to the supernatant. The precipitate which developed was collected by centrifugation and dissolved in 0.03 N H_2SO_4. The precipitate contained only oxalate (HPLC assay).

The ^{14}C-labeled product, isolated as described above, was further examined by reaction with oxalate decarboxylase. The samples were added to the main compartment of a Warburg flask after adjustment of the pH to 3.0. Oxalic acid decarboxylase (Sigma), 1 unit/flask, was placed in the side arm, and 0.05 ml 4 M ethanolamine, absorbed on a paper wick, was placed in the center well. After temperature equilibration (10 min, 30°C) in the Warburg bath, the system was closed, the enzyme tipped in, and the flasks shaken for 2 h. The radioactivity in the center well was determined by scintillation counting using 0.10 ml Protosol (New England Nuclear) in 10 ml of scintillation fluid. An aliquot of the reaction mixture in the main flask was assayed for formic acid by HPLC.

Removal of FMN by Acid Precipitation. The procedure described by Choong *et al.* (3) to prepare the apoenzyme of lactate oxidase was used. Enzyme (A-15 fraction, 1.0 ml) was treated with 4.0 ml of a saturated $(NH_4)_2SO_4$ solution (pH adjusted to 2.0 with dilute H_2SO_4). The precipitate was collected by centrifugation (10,000*g* for 10 min) and dissolved in 1.0 ml 0.10 M Na-acetate (pH 5.7) containing 1 mM EDTA. The enzyme was reprecipitated by the further addition of $(NH_4)_2SO_4$, and collected by centrifugation. It was redissolved in 1.0 ml 0.1 M K-phosphate (pH 7.5), centrifuged to remove the insoluble material, and assayed by the HPLC method.

RESULTS

Enzyme Assay by HPLC and Comparison with Other Methods. A method for measuring glycolate or glyoxylate oxidation was developed based on the detection and quantitative determination by HPLC of the products of the reactions, glyoxylate or oxalate, respectively (Fig. 1). There are several advantages of the HPLC method. (a) Assays could be performed in very small volumes, thus conserving enzyme. (b) The sensitivity of the technique made it possible to perform assays on samples taken at very short intervals. (c) The stoichiometry of the reaction could be determined and side reactions which might occur are readily detected by product analysis. For example, for every μmol of glyoxylate which disappeared from the reaction mixture, it was found that 1 μmol of oxalate appeared. In addition, it was clear that there was no decomposition of glyoxylate by H_2O_2 produced in the reaction since no formic acid could be detected. Presumably, sufficient catalase was present in the partially purified preparations to prevent the accumulation of H_2O_2.

The above method of assay was compared with two other assays for several preparations of enzyme. All three gave equivalent results, *e.g.* the ratios of glycolate/glyoxylate oxidation for an agarose fraction determined by HPLC, by O_2 uptake, and dye reduction were 1.5, 1.8, and 1.7, respectively, under standard conditions.

Change in Ratio of Glycolate and Glyoxylate Activities during Purification. Enzyme preparations were assayed with both glycolate and glyoxylate as substrates during all stages of purification. The ratios of activity are summarized in Table I. The difference in ratios between the 1AS and 2AS fractions was observed for at least 10 or more preparations. The activity found in the 2AS fraction (~50% of the total) was unstable and, thus, aside from chromatography on an agarose column, was not purified or characterized further. When the 1AS fraction was chromatographed on agarose and hydroxylapatite, the ratio changed only slightly. The overall degree of purification for the 1AS fraction was approximately 65-fold, based on activity measured with glycolate.

Effect of FMN on Oxidation of Glycolate and Glyoxylate. There was no increase in either glycolate or glyoxylate oxidation by the

Table I. *Change in Ratio of Glycolate and Glyoxylate Oxidation during Purification*

Enzyme was purified from tobacco leaf extracts and assayed by HPLC for rates of glycolate and glyoxylate oxidation as described in "Materials and Methods."

Fraction	Specific Activity[a]	Glycolate/ Glyoxylate Oxidation
	munits/mg protein	*ratio*
Extract	1.7[b]	4.0[c]
1AS (0–37% $[NH_4]_2SO_4$)	4.0	1.6
A-15 agarose	22.0	1.4
Hydroxylapatite	110.0	1.3
2AS (37–50% $[NH_4]_2SO_4$)	2.8[d]	2.5
A-15 agarose	6.0[d]	2.8

[a] Value given is for glycolate oxidation.
[b] Approximate value, range from 1.0 to 2.3.
[c] Approximate value, range from 3 to 4.
[d] Estimated value, preparation rapidly lost activity.

1AS, 2AS, or A-15 (1AS) fractions upon addition of FMN (data not shown). In contrast, the enzyme from spinach requires FMN for full activation (17). When the A-15 (1AS) enzyme preparation from tobacco was precipitated with $(NH_4)_2SO_4$ under acid conditions (see "Materials and Methods"), no activity could be detected unless FMN was added. The ratio of glycolate to glyoxylate oxidation was essentially unchanged, but the restored activity was only 15% of the original. With other flavin enzymes such as lactate oxidase (3) and glycolate oxidase from spinach (6), as much as 85 or 95% of the activity could be restored. These results indicate that, for the tobacco enzyme, either the removal of FMN from the holoenzyme results in structural change or that the apoenzyme is labile.

Characterization of Glycolate and Glyoxylate Oxidation: K_m, pH Optimum. The kinetic parameters were determined for enzyme from the agarose A-15 (1AS) fraction by the HPLC assay. For both activities, there was a broad pH optimum, with glycolate between 7.5 and 8.0 and with glyoxylate between 7.3 and 7.8. Tolbert *et al.* (15) reported a pH optimum between 7.8 and 8.6 for glycolate, and pH 7.7 for glyoxylate for enzyme from tobacco leaves. These determinations and all other data reported in this paper were done in phosphate buffer which may have a stimulating or protective effect, since enzyme prepared in the absence of phosphate had much lower activity. Other ions, such as borate or Tris, were inhibitory to varying degrees. The K_m for glycolate, at pH 7.5, was 0.26 mM in good agreement with some previous K_m values, *e.g.* 0.38 mM for enzyme from spinach (17), 0.25 mM for enzyme from peas (12). For glyoxylate, however, the K_m of 1.0 mM was lower than other reported values, *e.g.* 5.4 mM (14) for enzyme from spinach and 6.6 mM (12) for enzyme from peas.

Effect of O_2 Concentration on Oxidation of Glycolate and Glyoxylate. The rates of glycolate and glyoxylate oxidation under atmospheres of varying O_2 concentration are shown in Figure 2. The O_2-dependence curve for the conversion of glycolate to glyoxylate is similar to those published previously. For example, Frigerio and Harbury (6) found a 4.5-fold increase in activity of spinach glycolate oxidase on increasing the O_2 concentration from 21 to 100%. The increase observed here was 3.6-fold. The O_2-dependence curve is quite different for the conversion of glyoxylate to oxalate. Saturation with O_2 occurs at a much lower concentration, and the increase in activity from 21 to 100% O_2 was only 1.6-fold. This difference in the effect of O_2 on glyoxylate oxidation compared to glycolate oxidation has not been previously observed. Two points should be made about Figure 2. (a) While the atmosphere above the reaction mixtures was 100% N_2, undoubtedly some dissolved O_2 was present since oxalate formation under these conditions was verified by the procedure described in "Materials and Methods." (b) The flattening of the curve at high O_2 concentrations is not due to the inhibition of the reaction by the oxalate produced since the point at 100% O_2 is determined from several samples taken at various times and thus with very different concentrations of oxalate present.

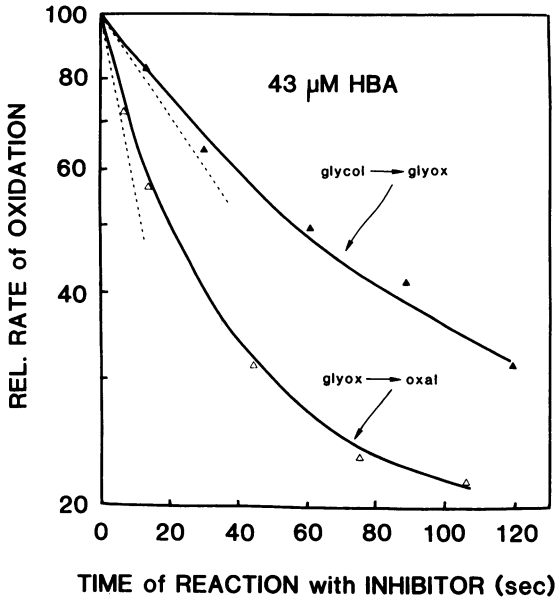

FIG. 2. The rates of glycolate (○) and glyoxylate (△) oxidation as a function of O_2 in the atmosphere. Enzyme and 30 mmol K-phosphate buffer (pH 7.5) were added to the main compartment (total volume, 0.6 ml) of Warburg flasks and either glycolate or glyoxylate (0.1 ml, 10 mmol) was added to one of the side arms. The second side arm was fitted with a rubber septum through which samples were withdrawn for assay. Each pair of vessels, one with glycolate and one with glyoxylate, were shaken and gassed for 10 min at 30°C with one of the following gasses: N_2, 5% O_2 in N_2 air, or 100% O_2. The stopcocks were then closed and the substrate tipped in. Samples (0.1 ml) were withdrawn with a hypodermic syringe at 1, 5, 10, 15, and 20 min and immediately pipetted into test tubes containing 0.01 ml 0.4 M H_2SO_4. The samples were assayed by HPLC according to the procedures described in "Materials and Methods." The rate of glyoxylate or oxalate production was determined during the period of constant rate. A separate experiment under N_2 with [1-^{14}C]glyoxylate (0.2 μCi) in addition to the unlabeled glyoxylate was carried out. The radioactive product, oxalate, was isolated and its identity confirmed as described in "Materials and Methods."

Inactivation of Glycolate and Glyoxylate Oxidation by HBA. The effect of this compound on the oxidation of glycolate and glyoxylate is shown as a semilog plot in Figure 3. The inactivation is clearly complex and does not follow pseudo first-order kinetics. A similar type of inactivation curve for glycolate oxidase from peas was observed by Jewess *et al.* (10). They, however, studied the inhibitory effect of the compound only on glycolate oxidation. It is obvious from Figure 3 that glyoxylate oxidation is more sensitive to the inhibitor than is glycolate oxidation. The values for $t_{1/2}$ calculated from curves such as those in Figure 3 are summarized in Table II. In all cases, $t_{1/2}$ with glyoxylate as substrate was significantly lower than with glycolate as substrate. The second-order rate constants calculated from $t_{1/2}$ inactivation of glycolate and glyoxylate oxidation by HBA are also given. Jewess *et al.* (10) calculated a value of $3 \pm 0.5 \times 10^2$ M^{-1} s^{-1} for glycolate oxidation.

A second experiment was designed to determine if preincubation with substrate could prevent inhibition by HBA (Table III). The control reactions, with no inhibitor, have a ratio of glycolate/

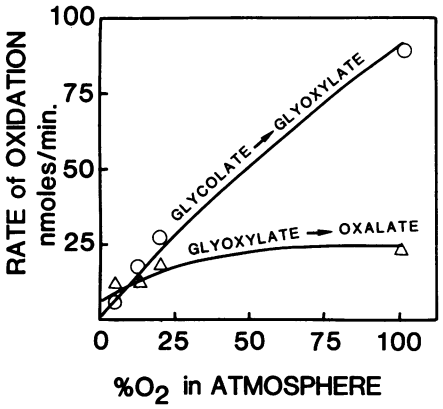

FIG. 3. Rate of inactivation of glycolate and glyoxylate oxidation by HBA. Enzyme was incubated with HBA (43 μM) in K-phosphate (pH 7.5). The reaction was started by the addition of inhibitor. Samples (0.10 ml) were withdrawn at indicated time intervals and pipetted into tubes containing either 10 mmol glycolate or glyoxylate. Samples (0.1 ml) were withdrawn from this reaction mixture at zero time, 5, 10, 15 min. Treatment of samples and determination of product formed is described in "Materials and Methods." The rate of oxidation of glycolate (▲) and glyoxylate (△) for each time interval of inactivation was then calculated and shown in the above figure.

Table II. *Rate of Inactivation of Glycolate and Glyoxylate Oxidation by HBA*

Inactivation curves were determined as described in Figure 3 for the concentrations of HBA indicated in the Table. Values for $t_{1/2}$ were calculated from tangents drawn to the curve at the initial rate of inactivation.

HBA Concn.	Inactivation Rate ($t_{1/2}$)		k	
	Glycolate	Glyoxylate	Glycolate	Glyoxylate
μM	s		$M^{-1} s^{-1}$	
23	40	16	753	1,880
43	31	13	519	1,239
85	17	7	479	1,237

Table III. *Protection by Substrate of Glycolate and Glyoxylate Oxidation against Inactivation by HBA*

Enzyme was incubated 5 min without substrate, with glycolate (20 mM), or with glyoxylate (20 mM) before HBA (final concentration, 1.4×10^{-5} M) was added. After 5 min incubation, the mixtures were passed through columns of Sephadex G-25 (7 mm × 7.5 cm) equilibrated with 50 mM K-phosphate (pH 7.5). Protein recovered was subdivided. One portion was assayed in the standard HPLC assay with glycolate and another with glyoxylate as substrate. Samples were taken at 0, 5, 10, 15, 20 min, and the rates of glycolate and glyoxylate oxidation were determined.

Preincubation Conditions/ Additions	Rate of Oxidation		Glycolate/ Glyoxylate Activity
	Glycolate	Glyoxylate	
	nmol/1.0 min		*ratio*
No substrate, no HBA	86	48	1.8
Glycolate, no HBA	103	55	1.9
Glyoxylate, no HBA	98	46	2.1
No substrate, + HBA	34	2.7	12.4
Glycolate, + HBA	91	30	3.0
Glyoxylate, + HBA	91	24	3.7

glyoxylate activity of 1.8 to 2.1. When the enzyme was incubated with inhibitor alone, the ratio increased to 12.4. This is consistent with the observation made in the previous experiment that glyoxylate oxidation is more sensitive to the inhibitor than glycolate oxidation. When the enzyme was incubated with either substrate before the addition of inhibitor, both activities were protected, but not equally since the ratio rose from 1.8 to 3.0 with glycolate as protectant and to 3.8 with glyoxylate. While glycolate activity was protected almost completely, glyoxylate oxidation activity was not.

DISCUSSION

There are three lines of evidence which support the hypothesis that there are at least two enzymes with distinct ratios of activity for the oxidation of glycolate and glyoxylate present in tobacco leaf preparations, one of which has a ratio of activities of 1.0 and the other which is predominantly a glycolate oxidase.

These lines of evidence were obtained from (a) the change in the ratio of activities observed during purification, (b) the inhibition by HBA, and (c) the effect of O_2. Each will be discussed in turn.

First, changes in the ratio of activities on purification would indicate the isolation of one form of the enzyme. However, modifiers of one or both activities might be removed during purification or change in pH, and salt concentration might alter activity and make such ratio changes misleading. But when such changes are observed consistently and reproducibly, as they are in this study, the existence of multiple forms of enzymes provides the simplest explanation of the results.

The second line of evidence is based on the contrasting inactivation rates of glycolate and glyoxylate oxidation by HBA. Flavin-containing enzymes, such as L-lactate oxidase and glycolate oxidase, have been shown to be rapidly and irreversibly inactivated by acetylenic substrate analogs such as HBA (1, 5, 7, 10). Cromartie and Walsh (5) used the equal inactivation of the α-hydroxy acid oxidase and amino acid oxidase activities of a preparation as evidence that both activities were catalyzed by the same enzyme. In contrast to the behavior of other flavin-containing enzymes (5, 7), Jewess *et al.* found that the time-dependent inactivation of glycolate oxidase of pea leaves could not be represented by a linear function (10). They suggested that the curvature could be attributed to the presence of isozymes or higher mol wt forms of the enzyme such as those found by Frigerio and Harbury (6). However, they did not measure the rate of inactivation with any substrates other than glycolate. The same type of inactivation curves were found for the enzyme activities in the tobacco leaf preparations. In addition, the results presented in Figure 3 and Table II indicate that glyoxylate oxidation is more sensitive to inhibition by HBA than is glycolate oxidation. If, as postulated (1, 7), inactivation by HBA is caused by reaction with FMN and results in a modified cofactor, then it is hard to reconcile these observed changes in ratio with the previously accepted view that one enzyme is responsible for both activities. Thus, the kinetics of the reaction and the different degrees of inhibition support the hypothesis that more than one enzyme capable of oxidizing glycolate and glyoxylate is present.

The argument for the presence of an enzyme that is primarily a glycolate oxidase would come from data such as presented in Table III. In this experiment and in a number of others with HBA (data not shown), enzyme with virtually no glyoxylate oxidase could be prepared (ratios 7–12). In all of these studies, it was never possible to prepare enzyme that had a ratio of activities less than 1.0.

It is obvious from Figure 2 that glyoxylate oxidation can (a) proceed under very much lower concentrations of O_2 than glycolate oxidation, and (b) does not increase greatly as the O_2 concentration is raised. Again, the observations are compatible with the hypothesis that at least two enzymes are present, one of which has a glycolate/glyoxylate oxidation ratio of 1 or less and has the lower K_m for O_2. For this enzyme, the rate-limiting step would not be the O_2-requiring step. The other enzyme with a ratio $\gg 1$

exhibits the behavior with respect to O_2 reported previously for glycolate oxidase (6).

The physical separation of two activities has not yet been achieved. The electrophoresis of the enzyme on polyacrylamide gels was unsatisfactory and, even under a number of altered conditions of pH and buffer composition, the migration of the protein was minimal (data not shown). Activity with both glycolate and glyoxylate could be detected on the gel but the electrophoretic pattern streaked. Grodzinski and Colman (8) partially separated two bands of protein showing activity with glycolate but could detect no activity with glyoxylate as substrate.

The reaction sequence for flavin-containing enzymes is very complex, and the detailed reaction mechanism of plant glycolate oxidase has not been studied. Conclusions drawn in this paper on the oxidation of glycolate and glyoxylate, and by others, are based on the assumption that glycolate oxidase resembles closely such other flavin-enzymes as lactate oxidase. Such an assumption may have to be modified, but until such time, the most logical hypothesis to account for the observations presented here is that there are at least two enzymes capable of oxidizing glycolate and glyoxylate present in tobacco leaves.

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LITERATURE CITED

1. Abeles RH, AL Maycock 1976 Suicide enzyme inactivators. Accts Chem Res 9: 313–318
2. Baker AL, NE Tolbert 1967 Purification and some properties of an alternate form of glycolate oxidase. Biochim Biophys Acta 131: 179–187
3. Choong YS, MG Sheperd, PA Sullivan 1975 Preparation of the lactate oxidase apoenzyme and studies on the binding of flavin mononucleotide to the apoenzyme. Biochem J 145: 37–45
4. Clagett CO, NE Tolbert, RH Burris 1949 Oxidation of α-hydroxy acids by enzymes from plants. J Biol Chem 178: 977–987
5. Cromartie TH, CT Walsh 1975 Rat kidney L-α-hydroxy acid oxidase: isolation of enzyme with one flavine coenzyme per two subunits. Biochemistry 14: 2588–2596
6. Frigerio NA, Ha Harbury 1958 Preparation and some properties of crystalline glycolic acid oxidase of spinach. J Biol Chem 231: 135–157
7. Ghisla S, H Ogata, V Massey, A Schonbrunn, RH Abeles, CT Walsh 1976 Kinetic studies on the inactivation of L-lactate oxidase by [the acetylenic suicide substrate] 2-hydroxy-3-butynoate. Biochemistry 15: 1791–1797
8. Grodzinski B, B Colman 1972 Disc electrophoresis of glycollate oxidizing enzymes. Phytochemistry 11: 1281–1285
9. Jenkins CLD, LS Rogers, MW Kerr 1982 Glycollate oxidase inhibition and its effect on photosynthesis and pigment formation in *Hordeum vulgare*. Phytochemistry 21: 1849–1858
10. Jewess PJ, MW Kerr, DP Whitaker 1975 Inhibition of glycollate oxidase from pea leaves. FEBS Lett 53: 292–296
11. Kenten RH, PJG Mann 1952 Hydrogen peroxide formation in oxidations catalyzed by plant α-hydroxyacid oxidase. Biochem J 52: 130–134
12. Kerr MW, D Groves 1975 Purification and properties of glycollate oxidase from *Pisum sativum* leaves. Phytochemistry 14: 359–362
13. Oliver DJ, I Zelitch 1977 Increasing photosynthesis by inhibiting photorespiration with glyoxylate. Science 196: 1450–1451
14. Richardson KE, NE Tolbert 1961 Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase. J Biol Chem 236: 1280–1284
15. Tolbert NE, CO Clagett, RH Burris 1949 Products of the oxidation of glycolic acid and L-lactic acid by enzymes from tobacco leaves. J Biol Chem 181: 905–914
16. Zelitch I 1955 Glycolic acid oxidase and glyoxylic acid reductase. Methods Enzymol 1: 528–535
17. Zelitch I, S Ochoa 1953 Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. J Biol Chem 201: 707–718