and by estimation of the manganese oxidation product with hydrazine. The oxidation product accumulates in both pyrophosphate and orthophosphate media.

3. With low Mn^{++} concentrations the oxidation was demonstrated by the increased oxygen uptake of the system in presence of oxalic acid. Under these conditions the manganese is involved in a cycle of oxidation and reduction.

4. The oxidation of Mn^{++} by peroxidase systems can be used as a test for hydrogen peroxide. By this test it has been shown that plant α-hydroxyacid oxidase produces hydrogen peroxide while catalysing the oxidation of L-lactic and glycollic acids.

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Hydrogen Peroxide Formation in Oxidations Catalysed by Plant α-Hydroxyacid Oxidase

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Kolesnikov (1948*a*) found that extracts of barley leaves oxidized glycollic acid, and Clagett, Tolbert & Burris (1949) showed the presence in leaves of many plants of an α-hydroxyacid oxidase catalysing the oxidation of lactic, glycollic and glyoxylic acids. More recent work (Tolbert & Burris, 1950) indicates that different enzymes may be responsible for the oxidation of these acids. While considerations of the total oxygen uptake suggested that hydrogen peroxide is formed during the oxidations, Tolbert & Burris (1950) were unable to demonstrate its presence, and like Kolesnikov (1948*b*) suggested that an organic peroxide might be formed. Kenten & Mann (1952) demonstrated the oxidation of manganese by flavoprotein enzyme systems coupled with peroxidase systems. It was found that α-hydroxyacid oxidase could replace the known flavoprotein enzymes in producing systems capable of oxidizing manganese, and it was concluded that this was due to the formation of hydrogen peroxide by α-hydroxyacid oxidase systems. In the present work this hydrogen peroxide formation is demonstrated by more conventional methods and its influence on the course of the oxidations is investigated.

MATERIALS AND METHODS

α-Hydroxyacid oxidase. This was prepared from leaves of tobacco (*Nicotiana tabacum* L.). The leaves were minced in a cooled domestic meat mincer and squeezed through madapollam. The temperature of the sap was kept below 5° during the subsequent procedure except during centrifuging. Sufficient 2 M-K_2HPO_4 was added to the sap to give a final concentration of 0·1 M. The procedure of Clagett *et al.* (1949) was then followed up to and including the first $(NH_4)_2SO_4$ fractionation. The active precipitate from this fractionation was dissolved in water (25 ml./100 ml. original sap) and either dialysed against 0·01 M-phosphate buffer at pH 7 overnight in the refrigerator or treated with $Ca_3(PO_4)_2$ gel to reduce the catalase content. This treatment was based on the observation of Agner (1938) that catalase is adsorbed by the gel at pH 5·5. The gel was made by the method of Singer & Kearney (1950) and had a dry weight of 20 mg./ml. The enzyme solution was treated successively with small amounts of gel at pH 5·5. After each addition the suspension was left 30 min. at 0° and then centrifuged. The supernatant was then tested for α-hydroxyacid oxidase and catalase. The amounts of gel required varied with the different enzyme preparations. In one case 20 ml. enzyme solution were treated with 4 ml. of gel followed by two treatments with 1 ml. The catalase activity was then 2%, and the α-hydroxyacid oxidase activity 50%, of that before gel treatment. In

some cases the α-hydroxyacid oxidase was subsequently adsorbed on the gel at pH 8 and eluted with 0·2M-pyrophosphate buffer at pH 7·5 as described by Kenten & Mann (1952). The preparations were stored frozen.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparations was estimated by the method of Sumner & Somers (1943). The decomposition of 0·01N-H_2O_2 in 0·0067M-orthophosphate at pH 7 at 0° by a suitable amount of catalase was followed by iodine titration on samples taken at 0, 3, 6, 9 and 12 min. From the equation $k=(1/t)\log_{10}\{a/(a-x)\}$, where a is the initial H_2O_2 concentration and $a-x$ is the concentration at time t, k values for the time intervals were calculated and the k value at zero time obtained by extrapolation. Using this value for k the catalase-activity $=k$/g. enzyme of the preparations used varied from 5000 to 6000. For each experiment a sample of the preparation was suspended in 0·2M-phosphate buffer (10 mg./ml. buffer) at the required pH and the insoluble material removed by centrifuging. A considerable purification was effected by this procedure.

Carbonyl compounds. These were estimated by the bisulphite method of Clift & Cook (1932).

Formic acid. This was estimated by the method of Pirie (1946).

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixtures was 3 ml. O_2 uptakes were measured directly with KOH in the centre cups. CO_2 outputs were measured by Warburg's direct method (Dixon, 1943).

EXPERIMENTAL AND RESULTS

Glycollic acid

Clagett *et al.* (1949) found that their preparations of α-hydroxyacid oxidase decomposed H_2O_2 rapidly and that added catalase did not affect the O_2 uptake with glycollic acid as substrate. According to Tolbert, Clagett & Burris (1949) the oxidation of glycollic acid proceeds in two stages through glyoxylic acid to formic acid. In the present experiments using small amounts of enzyme to keep the catalase activity low and working at pH 8·5, which is close to the optimum for the oxidation of glycollic to glyoxylic acid, the enzyme-catalysed reaction studied is mainly the oxidation of glycollic acid to glyoxylic acid. Under these conditions it is possible to demonstrate an effect of added catalase using the dialysed ammonium sulphate preparation of the oxidase (Fig. 1). The composition of the reaction mixtures is given in Table 1. In presence of added catalase the O_2 uptake is rapid until the total uptake approaches 1 atom O/mol. glycollic acid. This rapid stage is mainly due to the oxidation of glycollic acid to glyoxylic acid and is followed by a slow uptake due to the oxidation of the glyoxylic acid. In absence of added catalase the rate of O_2 uptake is

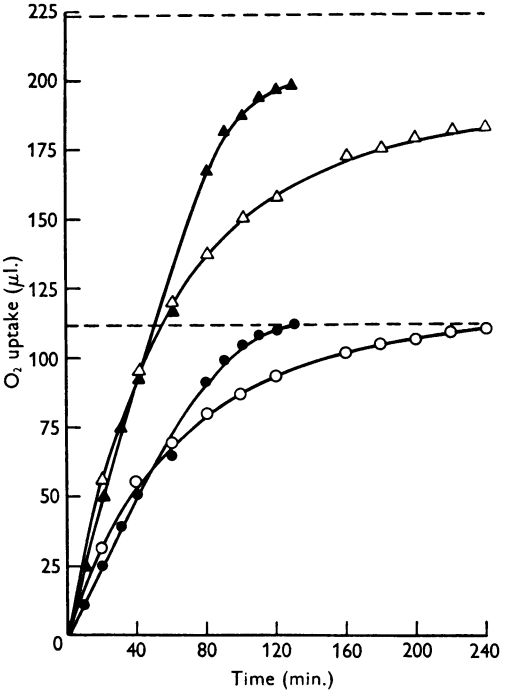

Fig. 1. The effect of catalase on the oxidation of lactate and glycollate by α-hydroxyacid oxidase. △—△, lactate, no added catalase; ○—○, lactate + catalase; ▲—▲, glycollate, no added catalase; ●—●, glycollate + catalase. The dotted lines show the theoretical uptakes.

Table 1. O_2 *uptake and formation of* CO_2 *and other reaction products during the oxidation of lactate glycollate and glyoxylate by α-hydroxyacid oxidase*

(With DL-lactate as substrate the gel-purified preparation of α-hydroxyacid oxidase was used in 0·033M-pyrophosphate buffer at pH 7·5. With glycollate and glyoxylate the dialysed ammonium sulphate precipitate was used in 0·033M-orthophosphate buffers at pH 8·5 and 7·7 respectively. Catalase (where present) 0·1 ml.)

Substrate	Catalase	O_2 uptake (μmoles)	CO_2 output (μmoles)	Keto acid (μmoles)	Formic acid (μmoles)
DL-Lactate (10 μmoles)	−	8·2	6·8	2·0	.
	+	5·1	0·6	8·5	.
Glycollate (10 μmoles)	−	8·9	7·5	1·3	.
	+	5·0	0·7	8·7	.
Glyoxylate (20 μmoles)	−	9·3	7·8	.	6·8
	+	8·0	0·7	.	1·4

double that in its presence and proceeds rapidly until the total O_2 uptake approaches 1 mol. O_2/mol. glycollic acid. In the experiment with glycollic acid shown in Fig. 1 the reactions were stopped when the O_2 uptake in presence of catalase was 1 atom O/mol. glycollic acid. The results suggest that the reaction proceeds according to the equation

$$CH_2OH.COOH + O_2 \rightarrow CHO.COOH + H_2O_2. \quad (1)$$

In presence of catalase the H_2O_2 is decomposed according to the equation

$$2H_2O_2 \rightarrow 2H_2O + O_2. \quad (2)$$

Analysis of the reaction mixtures (Table 1) showed that in presence of catalase 87 % of the glycollic acid was now present as carbonyl compounds, presumably glyoxylic acid, while in absence of added catalase only 13 % was accounted for in this form. H_2O_2 did not accumulate (in absence of added catalase) since no evolution of O_2 was observed when catalase was added from the side arms at the end of the experiment. The H_2O_2 must therefore be removed in a secondary reaction. The low value for carbonyl compounds coupled with the high output of CO_2 in absence of added catalase suggests that this secondary reaction may be between the H_2O_2 and the glyoxylic acid. Hatcher & Holden (1925) showed that glyoxylic acid reacts rapidly with H_2O_2 according to the equation

$$CHO.COOH + H_2O_2 \rightarrow CO_2 + H_2O + H.COOH. \quad (3)$$

Solutions of the sodium salt reacted in this way more rapidly than those of the free acid. In the present work it has been found that the reaction proceeds rapidly in phosphate buffers at pH's 7·7 and 8·5. The results therefore suggest that the oxidation of glycollate catalysed by α-hydroxyacid oxidase proceeds according to Eqns. (1) and (2) in presence of added catalase and according to Eqns. (1) and (3) in its absence.

Glyoxylic acid

The oxidation of glyoxylic acid is catalysed by the α-hydroxyacid oxidase preparations at a much slower rate than that of glycollic acid. Despite the fact that larger amounts of the enzyme are therefore required for this oxidation, it is possible to show an effect of added catalase on the rate of O_2 uptake using the dialysed ammonium sulphate preparations (Fig. 2). The composition of the reaction mixtures is given in Table 1. The effect of catalase is not as marked as with glycollic acid as substrate. In the early stages of the reaction the uptake in absence of added catalase is about 50 % higher than in its presence, but the percentage difference decreases with time, and in both cases the O_2 uptake eventually approaches 1 atom O/mol. glyoxylic acid. The effect of added catalase was not more marked with enzyme preparations purified by treatment with calcium phosphate gel to reduce the catalase activity. Hydrogen peroxide does not accumulate since no evolution of O_2 was observed when catalase was added from the side arm at the end of the experiments. In absence of added catalase the CO_2 output was 84 % of that required if the H_2O_2 produced reacted with glyoxylic acid according to Eqn. (3), and formic acid estimations showed the

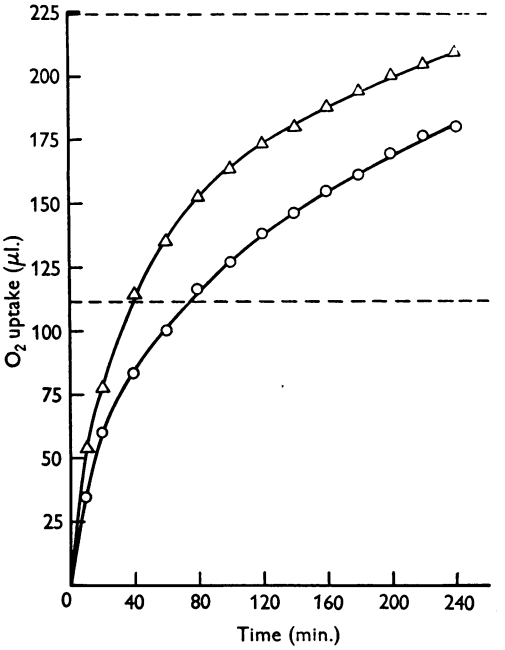

Fig. 2. The effect of catalase on the oxidation of glyoxylate by α-hydroxyacid oxidase. △—△, no added catalase; ○—○, catalase added. The dotted lines show the theoretical uptakes.

presence of 87 % of the formic acid expected based on the CO_2 output (Table 1). In presence of added catalase the CO_2 output was only 9 % and the formic acid found only 20 % of that in absence of added catalase. The results show that the oxidation of glyoxylic acid catalysed by α-hydroxyacid oxidase is accompanied by the formation of H_2O_2. In absence of catalase this H_2O_2 reacts with the substrate to give formic acid and CO_2. This secondary reaction accounts for the increased rate of O_2 uptake in the early stages of the oxidation. It does not produce a doubling of the rate of uptake since it also lowers the concentration of glyoxylic acid available for the enzyme-catalysed reaction. It is probable that the enzyme reaction is between glyoxylic acid monohydrate and O_2 according to the equation

$$\begin{matrix} COOH + O_2 \\ | \\ CH(OH)_2 \end{matrix} \rightarrow \begin{matrix} COOH + H_2O_2 \\ | \\ COOH \end{matrix}. \quad (4)$$

In the presence of catalase the net reaction should be the sum of Eqns. (4) and (2) and in absence of catalase the sum of Eqns. (4) and (3). In both cases, therefore, the total O_2 uptake should be 1 atom O/mol. glyoxylic acid. In the present experiments the O_2 uptake normally reaches 90–100% of this value in the absence of added catalase but only 70–80% in its presence (Fig. 2). This would be expected if oxalic acid is in fact the product of the enzyme-catalysed reactions, since Tolbert *et al.* (1949) have shown that oxalic acid competitively inhibits the oxidation of glyoxylic acid by the enzyme.

Evidence that oxalic acid is the product of the enzyme-catalysed reaction was obtained by its isolation from a large-scale reaction mixture in presence of added catalase.

A reaction mixture containing 15 ml. 0·1 M-glyoxylate, 5 ml. catalase, 50 ml. of a dialysed preparation of α-hydroxyacid oxidase and 75 ml. 0·2 M-orthophosphate buffer at pH 7·7 in a total volume of 225 ml. was shaken in air at room temperature for 5 hr. The disappearance of glyoxylate was followed by bisulphite titrations on 1 ml. samples at hourly intervals. After 5 hr. 42% of the glyoxylate had disappeared according to the bisulphite titrations. The reaction mixture was then heated at 100° for 5 min., acidified with 2 N-acetic acid to pH 4 and centrifuged. The supernatant was adjusted to pH 5·5 with 8 N-NH_4OH and 20 ml. 20% (w/v) $CaCl_2.6H_2O$ were added. The mixture was allowed to stand overnight and was then centrifuged. The precipitate was dried at 100°, powdered, and the oxalic acid extracted by the method of Pucher, Vickery & Wakeman (1934). The powder was suspended in 5 ml. 4 N-H_2SO_4, mixed with Gooch asbestos, and extracted with ether in a soxhlet for 24 hr. The ether extract was allowed to stand over anhydrous Na_2SO_4 overnight, filtered and evaporated to dryness. The residue was taken up in 10 ml. water and filtered. The filtrate was concentrated on a hot plate and then taken to dryness *in vacuo* over H_2SO_4 yielding at first colourless needles which on prolonged drying gave an opaque whitish product; yield 48·6 mg. of m.p. 179° (uncorr.) with decomposition. The melting point of anhydrous oxalic acid is 187° (corr.). The product was dissolved in ether, filtered, the filtrate was allowed to stand over anhydrous Na_2SO_4 overnight, filtered and evaporated to dryness. The residue redissolved in water and taken to dryness at 37° gave 46 mg. of colourless crystals, m.p. 100° (uncorr.). Mixed melting point with an authentic sample of hydrated oxalic acid (m.p. 101° uncorr.) was 100° (uncorr.). The yield based on glyoxylate disappearance was 58%.

L-*Lactic acid*

Tolbert *et al.* (1949) have shown that α-hydroxyacid oxidase catalyses the oxidation of L-lactic acid to pyruvic acid. D-Lactic acid is not attacked (Clagett *et al.* 1949). The effect of catalase on the oxidation of L-lactic acid catalysed by α-hydroxyacid oxidase is best shown with enzyme preparations freed from all but traces of catalase activity by treatment with calcium phosphate gel. With such preparations the addition of catalase almost halves the rate of O_2 uptake with lactate. The total O_2 uptake is 1 atom O/mol. L-lactic acid in presence of catalase but 1·6–1·8 atoms O/mol. L-lactic acid in absence of added catalase. Typical results are shown in Fig. 1. The composition of the reaction mixtures is given in Table 1. The results suggest that the reaction proceeds according to the equation

$$CH_3.CHOH.COOH + O_2 \rightarrow CH_3.CO.COOH + H_2O_2. \quad (5)$$

In the absence of catalase no H_2O_2 accumulates since on adding catalase when the O_2 uptake was complete no significant output was obtained. It has been shown by Negelein & Brömel (1939) with D-amino-acid oxidase using alanine as substrate that the H_2O_2 formed oxidizes the pyruvic acid, which is the other product of the enzyme-catalysed reaction, to acetic acid according to the equation

$$CH_3.CO.COOH + H_2O_2 \rightarrow CH_3.COOH + H_2O + CO_2. \quad (6)$$

Since pyruvic acid and H_2O_2 are the products of the action of α-hydroxyacid oxidase on L-lactic acid, the absence of accumulated H_2O_2 is presumably due to its reaction with pyruvic acid. This is supported by the values given in Table 1 for CO_2 output during the reaction and carbonyl compounds finally present in the reaction mixtures. In the absence of added catalase there was a high CO_2 output coupled with a low content of carbonyl compounds, and in the presence of added catalase a low CO_2 output coupled with a high content of carbonyl compounds. It is clear, therefore, that in the absence of catalase the net result of the oxidation of L-lactic acid by α-hydroxyacid oxidase is given by Eqns. (5) and (6), and in the presence of catalase by Eqns. (5) and (2).

DISCUSSION

The results of the present work show that the oxidation of L-lactic acid, glycollic acid, and glyoxylic acid catalysed by α-hydroxyacid oxidase takes place with the formation of H_2O_2. The final products of the oxidation depend on the amount of catalase present. Thus, according to Tolbert *et al.* (1949), pyruvic acid is the product of the oxidation of L-lactic acid. The present results suggest that this is only the case if sufficient catalase is present to stop the oxidation of the pyruvic acid to acetic acid by the H_2O_2 produced in the enzyme-catalysed reaction.

The oxidation of glycollic acid was shown by Tolbert *et al.* (1949) to proceed in two stages to formic acid and CO_2 with glyoxylic acid as the intermediate. According to the present results, formic acid is not the immediate product of the enzyme-catalysed reaction, but is formed by a secondary reaction between H_2O_2 and glyoxylic acid. In the complete absence of catalase the oxidation of glycollic acid should proceed entirely to

formic acid and CO_2, and that of glyoxylic acid to equimolecular amounts of formic acid and oxalic acid with evolution of CO_2 if, as seems likely, oxalic acid is the direct product of the enzyme-catalysed oxidation of glyoxylic acid. In the presence of sufficient catalase to stop the secondary reaction between glyoxylic acid and H_2O_2, no formic acid should be formed. As previously suggested, the most likely product under these circumstances would be oxalic acid. Nord & Vitucci (1947) have shown that certain fungi convert glycollic to oxalic acid with the postulated intermediate formation of glyoxylic acid.

In the present work the amounts of added catalase were such as to give a catalase activity of the same order as that of 3 ml. of tobacco-leaf sap. In view of the results obtained it appears that when untreated sap is used to catalyse the oxidation of lactate and glycollate its catalase activity would be sufficient to prevent the non-enzymic oxidation of pyruvate and glyoxylate by H_2O_2. Results with α-hydroxyacid oxidase preparations made from the sap would depend on the catalase activity of such preparations.

The influence of catalase on the oxidations catalysed by α-hydroxyacid oxidase provide an explanation of at least some of the variable O_2 uptakes and CO_2 outputs obtained by Clagett *et al.* (1949), Tolbert *et al.* (1949) and Tolbert & Burris (1950).

SUMMARY

1. Hydrogen peroxide is produced during the oxidation of lactate, glycollate and glyoxylate catalysed by plant α-hydroxyacid oxidase. This has been shown by the effect of catalase on the oxygen uptake.

2. The oxidation products depend on the amount of catalase present. In the absence of catalase, the enzyme-catalysed reactions are followed by non-enzymic reactions between hydrogen peroxide and pyruvate or glyoxylate, with the formation of acetate or formate respectively.

3. It is suggested that oxalate is the product of the enzyme-catalysed oxidation of glyoxylate.

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The Biochemistry of the Nitrifying Organisms

1. THE AMMONIA-OXIDIZING SYSTEMS OF *NITROSOMONAS*

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Micro-organisms of the genus *Nitrosomonas* are autotrophs, typically found in soil, which oxidize ammonia to nitrite. Since the energy yielded by this oxidation is apparently the only primary energy source available to the organisms, no cell growth or proliferation can take place without ammonia oxidation. On the other hand, Nelson (1931) showed that ammonia oxidation could take place in the absence of carbon dioxide, i.e. under conditions that excluded the possibility of cell growth or proliferation, the energy released being (presumably) dissipated as heat instead of being used, in part at least, for carbon dioxide assimilation. It therefore follows that while any compound that inhibits ammonia oxidation by the organisms must inhibit their proliferation, the fact that a com-