Oxidation of Formate by Peroxisomes and Mitochondria from Spinach Leaves

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1. Spinach (*Spinacia oleracea* L.) leaf extracts catalyse the oxidation of formate to CO₂.
2. Two enzymic systems are responsible for this oxidation, the peroxidatic action of catalase (EC 1.11.1.6) and NAD-dependent formate dehydrogenase (EC 1.2.1.2).
3. Formate dehydrogenase is mainly, if not exclusively, located in the mitochondria. This enzyme has a pH optimum of 6–6.5 and a K_m for formate of 1.7 mM in the presence of 1 mM-NAD⁺.
4. Peroxidatic action of catalase is presumed to take place in peroxisomes, since these seem to be the subcellular site of catalase. Formate oxidation at pH 5 by chloroplast and mitochondrial fractions is due to their ability to generate H₂O₂ and the presence of contaminating catalase.
5. During photorespiration, peroxidatic oxidation of formate by catalase can occur over a wide range of pH values, but the rate of this reaction is probably controlled by the concentration of formate present, to an extent dependent on the pH.

Leaves from various plants readily catalyse the oxidation of formate to CO₂. In the light, this CO₂ can be fixed photosynthetically and so [¹⁴C]formate supplied to illuminated leaves labels intermediates of the Calvin cycle and its associated metabolic pathways (Tolbert, 1955; Doman & Romanova, 1962; Kent, 1972). In the dark, most of the CO₂ is released (Cossins & Sinha, 1965; Leek, 1972).

The enzymic mechanisms by which leaves convert formate into CO₂ have not been fully characterized. Although high activities of formate dehydrogenase (formate-NAD oxidoreductase, EC 1.2.1.2) are found in many seeds, the activities decrease rapidly on germination, so that the amount of this enzyme in the primary leaves is very small (Davison, 1949; Peacock, 1970). Mazelis (1960) demonstrated a particulate formate-oxidizing system in leaves of cabbage and other plants; the rate of formate oxidation by this system was increased by the addition of NAD⁺. Davies (1956), using a spectrophotometric assay, demonstrated formate dehydrogenase activity in crude mitochondrial fractions from pea leaves. However, it is now known that such preparations are heavily contaminated with other organelles (Tolbert *et al.*, 1969) and it cannot be concluded that the enzyme is located in mitochondria.

Leek *et al.* (1972) identified two systems for the oxidation of formate to CO₂ in particulate preparations from leaves of spinach beet (*Beta vulgaris* L.). Formate oxidation activity at pH 6

was located in peroxisome preparations and was due to the peroxidatic action of catalase, but the source of H₂O₂ in these preparations was not identified. A formate dehydrogenase activity was also detected in these preparations, but detailed studies on its subcellular location were not carried out. Such studies have proved difficult because of the fragility of spinach-beet organelles on density gradients (Tolbert *et al.*, 1969) and so the studies have been extended to leaves of spinach (*Spinacia oleracea* L.), whose organelles are readily separated on sucrose gradients (Tolbert *et al.*, 1968; Rocha & Ting, 1970).

Experimental

Materials

Chemicals. Normal laboratory reagents were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K. Coenzymes and metabolites were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. [¹⁴C]Formate was obtained as its sodium salt from The Radiochemical Centre, Amersham, Bucks., U.K. Butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was obtained from CIBA-Geigy Ltd., Duxford, Cambridge, U.K.

Plants. Field-grown spinach was purchased locally. Only undamaged adult leaves were used.

Methods

Homogenization and differential centrifugation. A method modified from that of Tolbert *et al.* (1968) was used. Washed leaves (100g), from which the

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major veins had been removed, were homogenized for three 2s periods at top speed in an MSE Ato-Mix homogenizer with 200ml of 0.5M-sucrose containing 20mM-glycylglycine buffer adjusted to pH7.5 with KOH. The homogenate was filtered through four layers of muslin and then centrifuged at 500g for 25min in a Servall Superspeed centrifuge type RC2. The supernatant was further centrifuged at 6000g for 20min. Precipitates were gently resuspended in homogenizing medium. All these operations were carried out at 0–4°C.

Density-gradient centrifugation. A step gradient was constructed from the following solutions of sucrose in 20mM-glycylglycine-KOH buffer, pH7.5; 5ml of 2.0M-sucrose, under 10ml of 1.8M-sucrose, under 8ml of 1.5M-sucrose, under 7ml of 1.3M-sucrose. A 6000g precipitate, obtained as described above, was resuspended in homogenizing medium and 2ml of this suspension applied to the top of the gradient, which was then centrifuged at 21000 rev./min for 4h in the 4×43ml swing-out head of the MSE Superspeed 65 ultracentrifuge. Fractions (2–5ml) were removed from the bottom of the gradient after the tube had been pierced with a needle. All these operations were carried out at 2°C.

Spectrophotometric enzyme assays. Catalase (H_2O_2 – H_2O_2 oxidoreductase, EC 1.11.1.6) was assayed by the decrease in absorbance at 240nm as H_2O_2 was decomposed (Luck, 1963). Glycollate oxidase (glycollate– O_2 oxidoreductase, EC 1.1.3.1) was assayed by the formation of glyoxylate phenylhydrazones (Feierabend & Beevers, 1972). Cytochrome *c* oxidase (ferrocytochrome *c*– O_2 oxidoreductase, EC 1.9.3.1) was assayed by the decrease in absorbance at 550nm caused by oxidation of reduced cytochrome *c* (Tolbert *et al.*, 1969). For each of these assays, it was established that the rate of the reaction was proportional to the amount of extract added in the range normally used.

Radiochemical enzyme assays. Decarboxylation of formate was assayed by the release of ^{14}C from [^{14}C]formate. The general technique was described by Leek *et al.* (1972). Formate oxidation at pH5 by spinach extracts was assayed by incubating 0.1mM-formate, containing 2×10^5 c.p.m. of [^{14}C]formate, 0.2ml of extract and 66mM- K_2HPO_4 buffer adjusted to pH5 with citric acid in a total volume of 3ml at 25°C for 2h. Formate oxidation at pH7 was measured by incubating either 0.1mM-formate containing 2×10^5 c.p.m. of label or 10mM-formate containing 10^6 c.p.m. of label with 0.2ml of extract, 1mM- NAD^+ , and 66mM- K_2HPO_4 buffer adjusted to pH7 with citric acid, in a total volume of 3ml at 25°C for 2h. The concentrations stated refer to the final concentration in the reaction mixtures.

The rate of CO_2 release was in all cases linear with time for at least 2h and the amount of CO_2 released

in 2h was proportional to the volume of extract added up to at least twice that normally used. Addition of 0.01% of Triton X-100 did not increase the rate of formate oxidation by any fraction under these assay conditions. Heating the extracts at 100°C for 5min before assay abolished 80% or more of the CO_2 release.

Determinations. Chlorophyll was determined spectrophotometrically by the method of Arnon (1949). Protein was precipitated from the solution to be assayed by 5% (w/v) trichloroacetic acid, dissolved in 0.1ml of 3% (w/v) NaOH and assayed by the Lowry method as modified by Leggett-Bailey (1962). Dry bovine serum albumin was used as a standard.

Results

Crude spinach-leaf homogenates and particulate fractions obtained from them by differential centrifugation readily oxidized formate to CO_2 . NAD^+ had no effect on the rate of this reaction at pH5 and below, but it stimulated markedly at pH6 and above. These results suggest that two systems of formate oxidation are present in these extracts, as was observed with spinach-beet extracts by Leek *et al.* (1972), i.e. an NAD^+ -dependent system, which may be assayed at pH7, and a system independent of NAD^+ , which may be assayed at pH5. Fig. 1 shows the

Fig. 1. Subcellular distribution of enzymes in spinach leaves; differential centrifugation

The height of each bar gives the percentage of the activity recovered in each fraction, and the numbers in parentheses give the overall percentage recovery of the activity of the crude homogenate. The assays of formate oxidation were carried out as described in the Experimental section, with 0.1mM-formate. ■, 500g precipitate; ▨, 6000g precipitate; □, 6000g supernatant.

Fig. 2. Subcellular distribution of enzymes in spinach leaves; density-gradient centrifugation

Assays were as described in the Experimental section. The height of each bar gives the percentage of the activity recovered in a given fraction and the numbers in parentheses below give the percentage recovery of the activity applied to the gradient. (a) Glycollate oxidase (96); (b) cytochrome *c* oxidase (94); (c) formate oxidation at pH 5 (82); (d) chlorophyll (100); (e) catalase (93); (f) formate oxidation at pH 7 assayed at 0.1 mM-formate (79); (g) formate oxidation at pH 7 assayed at 10 mM-formate (78); (h) protein (104). Some 74% of the chlorophyll was found in fraction 7.

distribution of formate oxidation activity at pH 5 and pH 7 among the fractions obtained by differential centrifugation of a spinach-leaf homogenate, together with the distributions of various marker enzymes. Activity at both pH values is mostly found in the sedimentable fractions. This distribution resembles that of cytochrome *c* oxidase rather than that of glycollate oxidase, a peroxisome marker enzyme (Tolbert *et al.*, 1968). Fig. 2 shows the results obtained when organelles from a 6000g precipitate were separated on a sucrose density gradient. The peroxisomes (glycollate oxidase) were well separated from the mitochondria (cytochrome *c* oxidase) and chloroplasts. A large part of formate oxidation activity at pH 5 was associated with the chloroplast fraction, but there was more activity in the mitochondrial fraction than could be accounted for by the chlorophyll present. Little activity was found in the peroxisomes.

In contrast, formate oxidation at pH 7 seemed to be associated with both peroxisomes and mitochondria. Detailed studies were carried out on the properties of formate oxidation at pH 5 and at pH 7, with fractions obtained from sucrose gradients. These fractions are referred to below as 'peroxisomal', (fraction 2), 'chloroplast' (fraction 7) or 'mitochondrial' (fraction 5) on the basis of their major components (Fig. 2), but it must be remembered that they are cross-contaminated to some extent.

Formate oxidation at pH 5

The studies of Leek *et al.* (1972) suggested that formate oxidation at low pH values by subcellular fractions from spinach-beet leaves was due to peroxidatic action of catalase. Formate oxidation at pH 5, by a resuspended 6000g precipitate from spinach leaves or by the chloroplast, peroxisome

Table 1. *Effect of various reagents on formate oxidation at pH 5 by organelles from spinach leaves*

Assays were carried out as described in the Experimental section. Where indicated, light was excluded from the reaction mixtures by wrapping the flasks in a double layer of aluminium foil. —, Results not shown.

Reagent added	CO ₂ released (nmol/h per ml)			
	6000g fraction	Mitochondrial fraction	Chloroplast fraction	Peroxisome fraction
None	41.8	2.1	3.1	1.6
1800 units of bovine catalase	55.2	1.9	14.7	1.6
4 mM-H ₂ O ₂	66.5	10.0	3.1	6.7
16.7 mM-3-Amino-1,2,4-triazole	2.5	0.1	0	0.2
1 mM- β -Mercaptoethanol	10.9	0.1	0	0.8
1 mM-Azide	2.7	0.1	0	0
1 mM-NAD ⁺	41.8	2.0	3.1	1.6
2 mM-EDTA	—	—	2.1	—
Exclude light	29.2	2.0	2.4	1.6

Table 2. *Formate oxidation by spinach-leaf organelles by fraction-mixing experiments*

Samples (0.2ml) of each fraction were assayed either separately or together at pH 5 for 2h by the method described in the Experimental section.

Fraction	CO ₂ released (nmol/2h)	
	Predicted by simple addition	Determined
Peroxisomes	—	8.4
Mitochondria	—	8.7
Chloroplasts	—	10.5
Mitochondria + peroxisomes	17.1	16.8
Mitochondria + chloroplasts	19.2	29.2
Peroxisomes + chloroplasts	18.9	37.8

or mitochondrial fractions derived from this, was severely inhibited by catalase inhibitors such as 3-amino-1,2,4-triazole (Margoliash & Novogrodsky, 1958), azide or β -mercaptoethanol (Table 1). Therefore peroxidatic action of catalase seems to account for formate oxidation at low pH values in spinach fractions. Since peroxisome fractions contain a high activity of catalase, their low rate of formate oxidation must be due to lack of H₂O₂, and addition of H₂O₂ markedly increased this rate (Table 1). H₂O₂ also increased the rate of formate oxidation by the mitochondrial fraction but not by the chloroplast fraction. In contrast, excess of catalase stimulated formate oxidation at pH 5 by the chloroplast fraction, but not by the mitochondrial or peroxisome fractions. Table 2 shows the results obtained when organelle fractions were assayed separately or together for formate oxidation at pH 5. Assays of equal volumes of peroxisomal and mitochondrial fractions in the same reaction mixture gave the sum of the two rates obtained when the fractions were assayed separately. However, the combined rates for (mitochondria +

chloroplasts) or (chloroplasts + peroxisomes) were respectively about 50 or 100% greater than those predicted by simple addition.

Oxidation of formate to CO₂ by peroxidatic action of catalase requires both H₂O₂ and catalase activity. There is little catalase in chloroplast fractions (Fig. 2), and the addition of more catalase increased the rate of formate oxidation, indicating that catalase activity was rate-limiting. It therefore appears that chloroplasts can generate H₂O₂. This H₂O₂ production is stimulated by light, since excluding light from the reaction mixture decreased the rate of formate oxidation by chloroplast fractions, and thus it may be compared with the light-stimulated H₂O₂ production in chloroplasts reported by other workers (Mehler, 1951*a,b*; Kiseki & Tolbert, 1969; Whitehouse *et al.*, 1971; Zelitch, 1972). This reaction was inhibited slightly by 2mM-EDTA. It seems that mitochondrial fractions can also generate H₂O₂, but at rates insufficient to saturate the catalase present in this fraction. This explains the increased rates of formate oxidation observed when chloroplast and peroxisomal or mitochondrial fractions are assayed together; the excess of H₂O₂ produced by the chloroplast fraction can interact with the excess of catalase in the other fractions. The reactions by which spinach mitochondrial fractions generate H₂O₂ have not been established, but mitochondria from rat liver are known to generate H₂O₂ both *in vitro* and in perfused organs (Boveris *et al.*, 1972; Oshino *et al.*, 1973).

In the 6000g precipitate, addition of either catalase or H₂O₂ produces only a small effect, indicating that the supply of H₂O₂ is almost in balance with the catalase activity (Table 1). Excluding light from the reaction mixture decreased the rate of formate oxidation by this fraction. Since light is not required for formate oxidation by the peroxisomal or mitochondrial fractions, this must be due to a decrease in the supply of H₂O₂ by the chloroplasts.

Formate oxidation at pH7

Mitochondrial fractions. Whereas 0.1mM-formate was sufficient to saturate the formate-oxidation activity of mitochondrial or peroxisomal fractions with substrate at pH5, 10mM-formate and 1mM-NAD⁺ were required at pH7 to obtain maximal rates. Assaying fractions at pH7 in the presence of 0.1mM-formate altered the subcellular distribution of this activity; more was found in the peroxisomes and less in the mitochondria than in assays at 10mM-formate (Fig. 2). Studies of the effects of catalase inhibitors showed that this was because some peroxidatic action of catalase occurred in both mitochondrial and peroxisome fractions at pH7, but to a greater extent in the latter. This activity is saturated at 0.1mM-formate, whereas the NAD⁺-dependent activity is not (see below), and so this peroxidatic action makes relatively much less contribution to formate oxidation in assays at high substrate concentration.

Fig. 3 shows the effect of pH on formate oxidation by mitochondrial fractions in the presence of 10mM-formate and 1mM-NAD⁺. The pH optimum is

Fig. 3. Effect of pH on formate oxidation by spinach mitochondrial fractions

Assays were carried out in 66mM-K₂HPO₄ buffer adjusted to the appropriate pH with citric acid as described in the Experimental section. All reaction mixtures contained 10mM-formate and 1mM-NAD⁺.

6–6.5. However, preliminary experiments showed that a small part (10–15%) of the activity at these pH values was inhibited by catalase inhibitors and stimulated by H₂O₂ and thus was due to the peroxidatic action of catalase. Although the pH optimum was still 6–6.5 when catalase inhibitors were included in the reaction mixtures, assays of the NAD⁺-dependent activity were usually carried out at pH7, when catalase inhibitors had no effect (Table 3), to avoid possible confusion in studies of the effects of other reagents.

Under these conditions NAD⁺ was essential for activity, and could not be replaced by NADP⁺ (Table 3). NADH, ADP-ribose and nitrate inhibited formate oxidation, H₂O₂ stimulated very slightly, but glycollate and glyoxylate had no effect. Azide completely abolished activity. Although the results in Table 3 are taken from experiments with different preparations, they were highly reproducible. NADH, ADP-ribose, azide and nitrate are inhibitors for formate dehydrogenases (Peacock, 1970; Peacock & Boulter, 1970; Leek, 1972). Inhibition by these reagents, together with the absolute and specific requirement for NAD⁺, strongly suggests that formate oxidation at pH7 by mitochondria is due to a formate dehydrogenase. To confirm this, attempts were made to detect NADH spectrophotometrically during formate oxidation. Fig. 4 shows a typical result. Initially, the rate of NADH generation was equal to the rate of CO₂ release, but at longer times the amount of NADH in the reaction mixture became progressively smaller than the amount of CO₂ released. This is probably due to the ready oxidation of NADH by mitochondria. Addition of oxaloacetate, which can reduce NADH to NAD⁺ by the malate dehydrogenase activity of spinach mitochondria (Yamazaki & Tolbert, 1969), at any time during the course of these experiments caused the absorbance at 340nm to fall at once to the initial value, confirming that NADH was responsible for the absorbance change.

Attempts were made to determine the apparent *K_m* of mitochondrial formate oxidation in the presence of 1mM-NAD⁺, a saturating concentration. The Lineweaver-Burk plot was linear except at the lowest concentration (0.1mM), when the rate of oxidation was higher than would be expected (Fig. 5). Addition of 3-amino-1,2,4-triazole to suppress peroxidatic action at low formate concentrations (see above) made the plot completely linear, with a *K_m* of 1.7 ± 0.2mM (mean ± s.d. of five determinations). Formate dehydrogenases usually have *K_m* values in the mM range (Peacock, 1970; Leek, 1972).

In a series of eight preparations, the specific activity of mitochondrial formate oxidation varied from 0.64 to 1.63 μmol/h per mg of protein, with a mean of 1.02 μmol/h per mg of protein at pH7 in assays at 10mM-formate and 1mM-NAD⁺.

Table 3. *Effect of reagents on formate oxidation at pH7 by mitochondrial and peroxisomal fractions*

The assay at 10mM-formate was used as described in the Experimental section. All reaction mixtures contained 1 mM-NAD⁺, except those marked *.

Reagent added	CO ₂ released (μmol/h per mg of protein)			
	Mitochondrial fraction		Peroxisome fraction	
	Rate without reagent	Rate with reagent	Rate without reagent	Rate with reagent
1 mM-NAD ⁺	0*	0.85	0.23*	1.00
1 mM-NADP ⁺	0*	0*	0.23*	0.23*
1 mM-NADH	0.7	0.25	0.64	0.38
1 mM-Glycollate	1.00	1.00	—	—
1 mM-Glyoxylate	1.00	1.00	—	—
2 mM-H ₂ O ₂	0.78	0.93	0.74	1.32
2 mM-KNO ₃	0.82	0.41	0.64	0.29
0.1 mM-ADP-ribose	0.80	0.65	0.64	0.49
16.7 mM-3-Amino-1,2,4-triazole	1.63	1.63	1.01	0.89
1 mM-Azide	0.80	0	0.74	0
1 mM-β-Mercaptoethanol	0.80	0.79	0.74	0.65

Fig. 4. *Formation of NADH during formate oxidation by spinach mitochondria*

The assay was carried out with 10mM-formate and 1 mM-NAD⁺ as described in the Experimental section, except that 0.4ml of the mitochondrial preparation was included in the reaction mixture. ●, CO₂ released; ▲, NADH formed.

Peroxisome fractions. Even in assays at 10mM-formate, there was more formate oxidation activity at pH7 in the peroxisome fraction than could be accounted for by the mitochondrial contamination, assessed by cytochrome *c* oxidase content (Fig. 2). Table 3 shows that this activity had very similar properties to that of the mitochondrial fraction, and its pH profile was also similar. However,

Fig. 5. *Formate oxidation by mitochondria: Lineweaver-Burk plot*

Assays were carried out as described in the Experimental section. ●, No aminotriazole added; ▲, 16.7mM-aminotriazole was included in the reaction mixtures.

Table 4. *Effects of glycollate and glyoxylate on formate oxidation by spinach peroxisome fractions*

Assays were carried out as described in the Experimental section.

Reagent added	CO ₂ released (μ mol/h per mg of protein)		
	pH5	pH7 (0.1 mM-formate)	pH7 (10 mM-formate)
None	0.05	0.11	0.53
1 mM-Glycollate	0.66	0.14	3.71
1 mM-Glyoxylate	0.17	0.14	2.12
1800 units of catalase	0.05	0.12	0.53
0.1 mM-2-Pyridyl- α -hydroxymethanesulphonate	0.05	—	0.46
1 mM-Glycollate + 0.1 mM-2-pyridyl- α -hydroxymethanesulphonate	0.06	—	0.74
1 mM-Glyoxylate + 0.1 mM-2-pyridyl- α -hydroxymethanesulphonate	0.08	—	0.90
17 mM-Aminotriazole	0.01	—	0.37
1 mM-Glycollate + 17 mM-aminotriazole	0.01	—	0.40
1 mM-Glyoxylate + 17 mM-aminotriazole	0.01	—	0.40

omission of NAD⁺ from the reaction mixtures did not completely abolish activity and there was significant inhibition by catalase inhibitors (Table 3). It is therefore probable that the apparent slight excess of formate oxidation activity in the peroxisome fraction at pH7 is due to peroxidatic action of catalase (as was concluded for the activity in this fraction in assays at pH7 with 0.1 mM-formate) and that most, if not all, of the formate dehydrogenase activity is located in the mitochondria.

Formate oxidation during photorespiration

During photorespiration, H₂O₂ is generated in peroxisomes by the action of glycollate oxidase (Tolbert, 1971). Addition of glycollate markedly increased the rate of formate oxidation by peroxisome fractions at pH5 (Table 4). Glyoxylate, which is also oxidized by glycollate oxidase with the production of H₂O₂ (Richardson & Tolbert, 1961), also stimulated formate oxidation. 2-Pyridyl- α -hydroxymethanesulphonate, a powerful inhibitor of glycollate oxidase (Zelitch, 1971), almost completely abolished the stimulation by glycollate and glyoxylate.

Whereas formate oxidation at pH7 was stimulated by glycollate or glyoxylate when the assay at 10 mM-formate was used, these compounds had little effect on the rate of oxidation of 0.1 mM-formate. In the former case, 2-pyridyl- α -hydroxymethanesulphonate and 3-amino-1,2,4-triazole almost completely abolished this stimulation, confirming that the supply of H₂O₂ to catalase is responsible for this effect of glycollate and glyoxylate.

Discussion

Spinach leaf extracts can oxidize formate to CO₂ by two reactions. The first is via a formate dehydrogenase, whose activity is located in the mitochondria.

Although it cannot be ruled out that small quantities are present in the peroxisomes, the amount present is little more than could be accounted for by mitochondrial contamination when allowance is made for the effect of catalase inhibitors. The low activities of formate dehydrogenase reported in peroxisomal fractions from spinach-beet leaves by Leek *et al.* (1972) might well have been due to mitochondrial contamination.

The second reaction is the peroxidatic action of catalase, which accounts for the whole of formate oxidation by particulate fractions from spinach and spinach-beet leaves at pH5. It requires both catalase and H₂O₂ and its activity after subcellular fractionation will follow the rate-limiting step. Most of the catalase found in the particulate fractions after differential centrifugation is located in peroxisomes, and there is no cytochemical evidence for the presence of catalase in organelles other than the peroxisomes (Tolbert, 1971). If we accept that catalase *in vivo* is wholly located within peroxisomes, then the oxidation of formate at pH5 by chloroplast and mitochondrial fractions may be seen to be an artifact caused by the generation of H₂O₂ by these fractions and the presence of contaminating catalase. The report of Zelitch (1972), that illuminated spinach chloroplasts catalyse formate oxidation, may be due to the same cause. H₂O₂ produced by chloroplasts or mitochondria *in vivo* would first have to diffuse into peroxisomes before it could bring about peroxidatic oxidation of formate.

Chance (1952) showed that the peroxidatic action of catalase uses formic acid as a substrate and also that the enzymic action is independent of pH in the range 5–8 provided that the same concentration of formic acid is supplied at each pH value by increasing the concentration of total formate as the pH increases. In the experiments reported in the present paper,

assaying at pH7 with 10mM-formate is therefore essentially analogous to assaying at pH5 with 0.1mM-formate, from the point of view of catalase. In both cases the rate of formate oxidation by the peroxisome fractions was limited by the supply of H_2O_2 (Table 4). In assays at pH7 at 0.1mM-formate, neither H_2O_2 nor catalase increased the rate of formate oxidation, suggesting that the limiting factor under these conditions may be the concentration of formic acid. Neither chloroplast nor mitochondrial fractions peroxidatically oxidized formate at pH7 in assays at 10mM-formate, and therefore H_2O_2 cannot be generated at sufficient rates by these preparations, in contrast with the situation at pH5. The reason for this difference is unknown.

During photorespiration, much H_2O_2 will be produced in peroxisomes by the oxidation of glycollate to glyoxylate. Under these conditions it seems likely that the rate of formate oxidation will be controlled by the concentration of formate present, to an extent dependent on the pH. The high K_m of the mitochondrial formate dehydrogenase for formate suggests a similar control of its activity by formate concentration.

The mechanisms of formate oxidation elucidated in this paper throw light on the results of other workers. In the studies of Mazelis (1960) and Cossins & Sinha (1965) there was a significant rate of formate oxidation in the absence of NAD^+ and so peroxidatic action of catalase was probably operative, especially as the assays of Cossins & Sinha (1965) were carried out at pH5.6. The formate dehydrogenase activity reported in crude leaf mitochondrial fractions by Davies (1956) was probably a mitochondrial enzyme. The peroxisomal preparations of Leek *et al.* (1972) appear to generate H_2O_2 much more rapidly than do spinach peroxisomes. The rate of formate oxidation by peroxisome preparations from spinach-beet leaves is increased slightly by addition of either catalase or H_2O_2 , and so the H_2O_2 supply is in balance with the catalase activity. In consequence, these preparations contain significant formate oxidation activity at low pH values, in contrast to spinach peroxisomes. Thus the subcellular distribution of formate oxidation owing to peroxidatic action of catalase differs according to the rate-limiting step. On illumination the rate of formate oxidation by leaves appears to increase, although this is difficult to measure exactly because most of the CO_2 is then fixed by photosynthesis (Tolbert, 1955; Doman & Romanova, 1962; Kent, 1972). The rapid rate of formate oxidation by peroxisomes in the presence of glycollate produced in illuminated leaves would account for this increase.

What is the origin of the formate used by these systems *in vivo*? A direct photosynthetic incorporation of CO_2 into formate has been proposed

by Kent (1972). Glyoxylate is a good precursor of formate in plant tissues (Cossins & Sinha, 1965) and so formate could be produced from this compound during photorespiration. Systems converting glyoxylate into formate have been described in plant mitochondria (Prather & Sisler, 1972), peroxisomes (Halliwell *et al.*, 1972) and chloroplasts (Zelitch, 1972). The role of these reactions and the fate of formate in illuminated leaves have been discussed by Halliwell (1973). Some of this formate would certainly be metabolized by the oxidation systems described in the present paper.

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