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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

3. GLYCOLLIC OXIDASE AND FUMARASE ACTIVITY

By W. S. PIERPOINT

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

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How far the cytochrome-linked oxidations of the mitochondrial preparations from tobacco leaves account for the oxygen uptake of intact leaves is a matter for speculation. Oxidases other than cytochrome oxidase are present in the leaves and may serve as 'terminal oxidases' in some conditions (James, 1953). Recent evidence has suggested that one of these, glycollic oxidase, is responsible for much of the uptake in detached leaves exposed to light (Zelitch, 1958). This has prompted these observations on the oxidation of glycollic acid by the leaf-mitochondrial extracts and the distribution of this activity among the other leaf fractions.

The observations on fumarase were expected to throw some light on the low rate of fumarate oxidation previously reported for these extracts (Pierpoint, 1959) and also on the integrity of the mitochondria. In animal tissues a considerable proportion of the fumarase is associated with mitochondria, but disruptive treatments liberate it in a non-sedimentable form (Kuff, 1954). The fumarase content of animal mitochondrial preparations is thus a useful indicator of mitochondrial damage (Mahler, Wittenberger & Brand, 1958). Much less is known about the location of plant fumarase, although active preparations have been made from pea-epicotyl mitochondria (Davies, 1956), and it has been detected in sedimented fractions of lupin leaves (Brummond & Burris, 1954).

EXPERIMENTAL

Oxygen uptake. Manometric conditions were the same as those described by Pierpoint (1960). Oxygen uptakes from glycollate (40 μ moles) and succinate (40 μ moles) were compared in the medium used to measure oxidative

phosphorylation (final vol. 1.9–2.0 ml.), and oxygen uptake from fumarate was measured in the simpler medium used when only gas exchanges were being followed.

In one experiment oxidation of glycollate was also followed (Table 3) in a medium similar to that used by Zelitch (1957) for the estimation of glycollic oxidase. Its composition was: sodium glycollate, 20 μ moles; riboflavin 5'-phosphate, 0.1 μ mole; 2-amino-2-hydroxymethylpropane-1:3-diol buffer (tris), pH 8.3, 0.3 m-mole; water to a final vol. of 2.1 ml. The enzyme activities measured in this manner were all within the region where activity was proportional to the amount of enzyme added.

Phosphorylation. The disappearance of orthophosphate was measured as previously described (Pierpoint, 1960). The P/O ratios quoted are only approximate, as no correction was made for phosphorylation during the 10 min. when the manometer flasks were equilibrating.

Fumarase activity. This was measured by a slight modification of the method of Racker (1950). The reaction mixture contained tris or phosphate buffer, pH 7.8 (0.15 m-mole), malate (0.15 m-mole), cysteine (2 μ moles) and enzyme preparation in a final volume of 3 ml. Increase in $E_{240\text{ m}\mu}$ was measured at room temperature (21–25°) in a Unicam SP. 500 spectrophotometer, with the reaction mixture without malate as an optical blank. The reaction was followed for 20 min., although it proceeded linearly for at least 90 min.

A unit of fumarase was defined (Racker, 1950) as that which caused an increase in extinction of 0.001/min. Under the assay conditions, activity was proportional to added enzyme for at least 30 units.

The estimation of fumarase in the reverse direction, by observation of the disappearance of fumarate (0.05 m-mole) at 300 m μ , was only 5–7% as sensitive as the above method, mainly because of the relatively high absorption of the enzyme preparations at this wavelength.

Chemical estimations. Chlorophyll and protein N were estimated as described by Pierpoint (1960).

Chemicals. Tris and phosphate buffers were prepared as described by Pierpoint (1960).

RESULTS

Oxidation of glycollate by tobacco-leaf preparations

The mitochondrial preparations oxidized glycollate more quickly than any other organic acid so far tested. The rate was usually 130 % of that of succinate under the same conditions, and only once was it less (87 %). Oxygen uptake from a mixture of glycollate and succinate was greater than that from either substance alone, but less than their sum (86 %). In contrast with this, tricarboxylic acid-cycle substrates did not augment the oxygen uptake due to succinate (Pierpoint, 1959). No phosphorylation accompanied the glycollate oxidation, and, in the presence of glycollate, phosphorylation from succinate was depressed by a quarter.

There are two other respects in which the oxidation of glycollate differed from that of succinate. It was not increased by the presence of cytochrome *c* in the incubation medium, and it was resistant to the presence of cyanide (Table 1). Bisulphite, an inhibitor of glycollic oxidase (Zelitch, 1957), inhibited it appreciably (Table 1), but also affected succinoxidase. It inhibited the phosphorylation from succinate more than the oxygen uptake, so that at a concentration of 4 μ moles, the P/O ratio was decreased by a quarter.

The presence of crystalline ox-liver catalase (27 μ g./flask) did not decrease oxygen uptake from either glycollate or succinate.

The mitochondrial preparations oxidized lactate at a rate similar to that of succinate. This oxidation was inhibited by bisulphite to approximately the same degree as that of glycollate.

Table 1. *Differences between the oxidation of glycollate and succinate by mitochondrial fractions*

The mitochondrial preparations were incubated in the medium described in Pierpoint (1960), modified as below, and with either glycollate or succinate as substrate. The values for oxygen uptake, which are expressed as μ l. of oxygen/mg. of protein N/30 min. (q_{O_2}), are corrected for the appropriate endogenous activities.

Expt. no.	Modification	Oxygen uptake in the presence of succinate (q_{O_2})	Oxygen uptake in the presence of glycollate (q_{O_2})
1	None	82	108
	Cytochrome <i>c</i> omitted	55	113
2	None	94	80
	Cyanide present (20 μ moles)	0	86
3	None	69	90
	Bisulphite present (2.4 μ moles)	50	20
	Bisulphite present (4 μ moles)	33	11

Fumarase activity of tobacco-leaf preparations

The mitochondrial preparations usually had a fumarase activity of about 1000 optical units/mg. of protein N. This is approximately 0.05 % of the activity of crystalline pig-heart fumarase measured under slightly different conditions (Massey, 1951). A few properties of the activity were observed in the course of checking the method of estimation.

The pH optimum was between 7.8 and 8.0. Activity fell away steeply on either side of this region, and was down to about half at pH 7.2 and 8.5. There was no perceptible difference between the activities measured in phosphate buffer and those in tris buffer at the same pH. This was true even after the enzyme preparation had been washed in 0.25 M-sucrose. But the traces of phosphate retained by the preparation may have been enough to mask a phosphate stimulation such as occurs with the fumarases of *Escherichia coli* (Mann & Woolf, 1930), pig heart (Massey, 1953) and yeast (Favelukes & Stoppani, 1958).

At lower substrate concentrations phosphate inhibited the fumarase. The inhibition varied with the concentration of phosphate and involved an alteration of the Michaelis constant (Fig. 1) suggesting that fumarate and phosphate competed for combination with some site on the enzyme. A similar competition occurs with yeast fumarase

Fig. 1. Competitive inhibition of fumarase by phosphate. Enzyme activity at different substrate concentrations was measured in the presence of 0.05 M-tris buffer (○), 0.05 M-phosphate (●), 0.027 M-phosphate (□) and 0.0067 M-phosphate (■). The results are graphed by the method of Lineweaver & Burk (1934).

(Favelukes & Stoppani, 1958), although here it is complicated by a phosphate-stimulation effect. The Michaelis constant obtained for the tobacco-leaf enzyme in tris buffer differed a little from one preparation to another (2.2–4.2 mM), but was not much affected by changes in the concentration of tris.

Although added cysteine had only a small effect on its activity (0–10%), the tobacco-leaf fumarase is probably SH-dependent, like the yeast enzyme (Favelukes & Stoppani, 1958). Both *p*-chloro-

mercuribenzoate and iodoacetate inhibited the enzyme, and the inhibitions were partially reversed by cysteine. The reversal of the inhibition by *p*-chloromercuribenzoate was more complete and is shown in Table 2.

Distribution of glycollic oxidase and fumarase among tobacco-leaf fractions

Although the mitochondrial preparations actively oxidized glycollate, their content of glycollic oxidase, measured by the method of Zelitch (1957), was less than one-tenth of the total in the extract (Table 3). The bulk of the activity (85%) was present in the supernatant fraction, where it was twice as concentrated, relative to protein, as in the extract. No more than a trace of it (2–3%) was sedimented on further centrifuging at 55 000 *g* for 2 hr.

In contrast, about 90% of the fumarase of the disintegrated leaves was concentrated in the mitochondrial fraction, and most of the remainder was in the chloroplast fraction (Table 4). The high recovery of enzyme activity makes it unlikely that there was any enzyme activation or inhibition, and direct tests showed that the low fumarase activity in the supernatant fraction was not due to an inhibition by the extraction medium.

Treatments that would be expected to destroy mitochondrial structure liberated the fumarase in a

Table 2. *Effect of p-chloromercuribenzoate and cysteine on fumarase activity*

Samples (0.01 ml.) of a tobacco-leaf-mitochondrial preparation were incubated at room temperature with either phosphate buffer (pH 7.8, 0.2 m-mole) alone or buffer plus *p*-chloromercuribenzoate (PCMB; 0.15 μ mole). After 20 min. malate (0.15 m-mole) was added and the extinctions (240 m μ) were measured every 3 min. Cysteine (1 μ mole) was added to all tubes after 30 min. and again after 50 min.

	Fumarase activity (extinction units)	
	Without PCMB	With PCMB
Initial activity	11.5	1
After first cysteine addition	11.5	5
After second cysteine addition	11.5	7

Table 3. *Distribution of glycollic oxidase among tobacco-leaf fractions*

Leaves (29 g.) were ground with sand (15 g.) in the usual medium (110 ml.), and the fractions sedimented at 1000 and 10 000 *g*. Each sedimented fraction was washed, and the washings of the mitochondrial fraction were added to the supernatant fraction. Chlorophyll, protein N and glycollic oxidase were measured in samples from each fraction, as well as in the initial extract. Endogenous oxygen uptake was subtracted from the uptake in the presence of glycollate in calculating the glycollic oxidase activity.

Fraction	Protein N		Chlorophyll		Glycollic oxidase	
	mg.	% of total	mg.	% of total	μ l. of oxygen uptake/30 min./mg. of protein N	% of total
Initial extract	80	100	31.6	100	170	100
Chloroplast	11.5	15	12	39	21	1.8
Mitochondrial	8.9	11	9.2	29	110	7.5
Supernatant	33	42	1.25	4	340	85

Table 4. *Distribution of fumarase among tobacco-leaf fractions*

Leaves (29 g.) were ground with sand (15 g.) in the usual medium (150 ml.), and the fractions sedimented at 1000 and 10 000 *g*. Each sedimented fraction was washed, the washings of the chloroplast fraction being added to the supernatant from which the mitochondrial fraction was sedimented, and the washings of this fraction to the final supernatant. Chlorophyll, protein N and fumarase were estimated in all fractions.

Fraction	Protein N		Chlorophyll		Fumarase		
	mg.	% of total	mg.	% of total	Units	% of total	Units/mg. of protein N
Initial extract	80	100	24.7	100	9600	100	120
Chloroplast	14	18	15.2	62	535	6	40
Mitochondrial	8	10	6.4	26	8000	85	1000
Supernatant	50	64	0.8	3.2	<300	<3	<6

less sedimentable form. In particular, three-quarters of the fumarase in a preparation that had been kept at 37° for 2 hr. and then frozen and thawed three times remained in the supernatant after centrifuging at 50 000 g for 2½ hr.

Fumarate oxidation by mitochondrial preparations

The rate of fumarate oxidation by mitochondrial preparations was about 2.8 µg.atoms of oxygen taken up/mg. of protein N/30 min. that is between a third and a half of the rate of malate or succinate oxidation. This is an improvement over the rates previously obtained (Pierpoint, 1959), possibly because more extraction medium was used, but it still suggests that the rate-limiting step in the oxidation is in the conversion of fumarate into malate.

There was, however, no evidence that the rate of fumarate oxidation was limited by the fumarase activity of the preparations. In the experiment described in Table 4, the mitochondrial fraction catalysed an oxygen uptake from fumarate of 1.8 µg.atoms/mg. of protein N/30 min. The fumarase of this preparation, however, catalysed the dehydration of 46 µmoles of malate/mg. of protein N/30 min. in the spectrophotometric estimation. This value was only decreased to 28 µmoles when the estimation was made more comparable with the manometric oxidation by changing the pH to 7.3, omitting the cysteine and decreasing the malate concentration. As the fumaric oxidase was estimated at 30° and fumarase at room temperature, this 15-fold difference between their activities is a minimal value.

DISCUSSION

Oxidation of glycolate

The oxidation of glycolate by the mitochondrial preparations differed from the oxidation of tricarboxylic acid substrates. It was not much affected by the presence of succinate and was not associated with phosphorylation reactions. The most likely mechanism for the oxidation is:

catalysed by the bisulphite-sensitive glycollic oxidase (Kenten & Mann, 1952; Zelitch, 1957). The mitochondrial preparations contained sufficient catalase activity to decompose rapidly the hydrogen peroxide formed, so that the added catalase did not lower oxygen uptake as in the experiments of Kenten & Mann (1952).

Zelitch (1958) has demonstrated a large accumulation of glycolate in illuminated tobacco leaves when they are exposed to either bisulphite or bisulphite-aldehyde addition compounds. On the basis of the specific inhibition of glycollic oxidase

by these substances (Zelitch, 1957), he suggested an important role for this enzyme in the respiration of leaves in sunlight. Bisulphite was the least specific of these inhibitors, but its inhibition of tobacco-leaf succinoxidase (Table 1) raises the possibility that, in the experiments of Zelitch, this substance at least may have prevented the oxidation of glycolate by a cytochrome system. Although no evidence for such a cytochrome-dependent oxidation was observed in the present work, the argument for a respiratory function of glycollic oxidase would be strengthened if the aldehyde bisulphite compounds were shown not to affect the cytochrome oxidations carried out by the tobacco-leaf-mitochondrial preparations.

Intracellular location of fumarase and glycollic oxidase

In rat liver about half the fumarase is located in the mitochondria, from which it is released by sonic disruption (Kuff, 1954; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The rest is distributed between smaller sedimentable particles and the supernatant fraction. The intracellular location of the extramitochondrial enzyme is in doubt, but there is some reason for believing that its presence on the small sedimentable particles is due to adsorption during cell disruption (Kuff, 1954).

Virtually all the tobacco-leaf fumarase is present in the mitochondrial fraction, where its activity per mg. of protein N is greater than in any other fraction. There was no reason to believe that any activation or inhibition of the enzyme occurred in any fraction, so it is reasonable to conclude (Alexander & Wilson, 1955) that in the intact cell it is attached to some component of the fraction other than the chlorophyll-containing fragments. It is likely that some, or even most, of it is attached to the same components that carry the enzymes of the tricarboxylic acid cycle; its release from this fraction by gentle disruptive treatments supports this view. But some of it may well be associated with other constituents of this fraction. Of importance in this context is the fate of the nucleic acid-containing particles of tobacco leaves (Pirie, 1950) in extraction media with high salt content. They may well have sedimented with the mitochondria, bearing any fumarase from the supernatant fluid with them. If this were so, sedimentable differences between the leaf and the animal fumarases may be more apparent than real.

Tolbert & Cohan (1953) suggested that about a tenth of the total glycollic oxidase of barley leaves is associated with chloroplasts. The difficulty about conclusions concerning such small proportions of a total activity is, however, well recognized (Alexander & Wilson, 1955). There is no alternative

but to regard the glycollic oxidase of the sedimentable fractions of tobacco leaves as a contaminant from the supernatant fraction. If the enzyme is associated with any microscopic structure of the tobacco cells, the linkage is labile and is completely disrupted during the disintegration of the leaves. Such a linkage would be quite unlike that holding fumarase on to the mitochondria.

SUMMARY

1. Preparations from tobacco leaves, which sedimented between 1000 and 10 000 g, oxidized glycollate and lactate. The oxidations did not appear to involve cytochromes and were probably brought about by glycollic oxidase.

2. The glycollic oxidase of these preparations was only a small (7.5%) proportion of the total in the leaf extract. The bulk of the activity was in the supernatant fraction and was not sedimented by centrifuging at 55 000 g for 2 hr.

3. In contrast, the sedimentable preparations contained nearly all (85–92%) of the fumarase in the extracts. Ageing at 30°, followed by freezing and thawing, liberated three-quarters of this activity in a form not sedimented at 50 000 g.

4. The fumarase was inhibited by *p*-chloro-mercuribenzoate and iodobenzoate and partially reactivated by cysteine. It was competitively inhibited by phosphate.

5. The fumarase activity was in excess of that

required for its expected role in the oxidation of fumarate by the preparations.

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Carbon Assimilation by *Pseudomonas oxalaticus* (OX1)

3. OXALATE UTILIZATION DURING GROWTH ON OXALATE*

By J. R. QUAYLE AND D. B. KEECH†

Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

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It has been shown (Quayle & Keech, 1959*a*) that one of the key enzymes of the ribulose 1:5-diphosphate cycle of carbon dioxide fixation (Bassham *et al.* 1954), carboxydismutase, is not synthesized when *Pseudomonas oxalaticus* (OX1) is grown on oxalate. This indicates that such a cycle cannot be of major importance in the conversion of oxalate carbon atoms into cellular material in oxalate-

grown cells, in contrast with formate-grown cells of the same organism (Quayle & Keech, 1959*b, c*). The present work, by giving information on the activities of phosphoribulokinase and phosphoriboisomerase during adaptation of oxalate-grown cells to growth on formate, confirms this conclusion. The sequence of compounds which become labelled when the organism is growing on [¹⁴C]-oxalate has also been determined, and a route for incorporation of oxalate into cellular constituents, based on these results and on reactions observed in

* Part 2: Quayle & Keech (1959*c*).

† Present address: Department of Biochemistry, Western Reserve University, Cleveland, Ohio, U.S.A.