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

2. OXIDATIVE PHOSPHORYLATION*

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A sucrose solution is suitable for the isolation of functional mitochondria from some animal tissues (Hogeboom, Schneider & Pallade, 1948), but not sufficient for use in the isolation of mitochondria from leaves. Smillie (1955), working with pea leaves, added ethylenediaminetetra-acetic acid; James & Das (1957), working with bean leaves, added phosphate and magnesium as well; Pierpoint (1959), working with tobacco leaves, increased the buffering power and added various substances, of which citrate was the most important. Even with these additions the tobacco mitochondria appeared to be somewhat functionally damaged and were heavily contaminated with chloroplast fragments.

Attempts are being made both to assess the damage done to these tobacco-leaf mitochondria and to purify them further. This paper provides information about their respiratory quotients and demonstrates that the oxidations catalysed by them are 'loosely' coupled to the phosphorylation of adenosine nucleotides.

EXPERIMENTAL

Mitochondrial preparations. Two modifications were made to the procedure already described (Pierpoint, 1959). The pH of the extraction medium (sucrose, 0.4 M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0.2 M; potassium phosphate, 0.01 M; ethylenediaminetetra-acetic acid, 5 mM; sodium citrate, 0.02 M) was decreased to 7.7, and the amount of medium used was increased so that 25–30 g. of leaves was disrupted in 100–130 ml.

Oxygen uptake. Oxygen uptake was measured in the

conventional Warburg apparatus. Details of the procedure, including the incubation temperature (30°), were the same as those described by Pierpoint (1959).

In experiments in which only gas exchanges were being followed the samples of the mitochondrial preparations were added to manometer flasks containing: substrate acid, 40 μ moles; sucrose, 0.3 m-mole; phosphate buffer, pH 7.3, 0.1 m-mole; magnesium sulphate, 10 μ moles; adenosine triphosphate (ATP), 2 μ moles; cytochrome *c*, 0.018 μ mole; diphosphopyridine dinucleotide (DPN), 0.2 μ mole; thiamine pyrophosphate, 0.2 μ mole; coenzyme A (CoA), 0.025 μ mole. The final volume was 1.5 ml. and the pH was 7.2–7.3.

When both phosphorylation and oxygen uptake were being followed, the medium in the manometer flasks contained: substrate acid, 40 μ moles; sucrose, 0.4 m-mole; phosphate buffer, pH 7.3, 12 μ moles; magnesium sulphate, 10 μ moles; manganese sulphate, 0.1 μ mole; sodium fluoride, 15 μ moles; ATP, 2 μ moles; cytochrome *c*, 0.018 μ mole; DPN, 0.2 μ mole; thiamine pyrophosphate, 0.2 μ mole; glucose, 10 μ moles; hexokinase (Sigma Chemical Co., type 11), 1 mg. CoA (0.025 μ mole) was also present in most experiments. The final volume varied a little, but was generally 2.0–2.1 ml.

Evolution of carbon dioxide. Evolution of the gas was measured manometrically by the 'direct method' (Umbreit, Burris & Stauffer, 1957).

Orthophosphate disappearance. Samples (1 ml.) were taken from the contents of manometer flasks and added to centrifuge tubes containing 1 ml. of cold trichloroacetic acid (5%, w/v). The tubes were cooled in ice-water for 10 min. and then centrifuged at 1500 *g* for the same length of time. The supernatant solution was decanted, diluted with four times its volume of sodium acetate (0.1 M) and portions were taken for phosphate estimation.

Phosphate esterification occurred in the manometer flasks during the equilibration period, before it was possible to follow oxygen uptake. In measuring P/O ratios (μ moles of P esterified/ μ g. atom of oxygen uptake) it was necessary

* Part 1: Pierpoint (1959).

to correct for this by removing some manometer flasks for estimation of P immediately after the equilibration period. Measurement of the adenosine triphosphatase activity occurring in the equilibration period was obtained in the same way.

In calculating P/O ratios, allowance was made for endogenous oxygen uptake and adenosine triphosphatase activity, both of which were assumed to continue unaffected in the presence of substrate. The correction for the enzyme activity was in most cases very small; that for endogenous oxygen uptake was larger and raised the P/O ratio for succinate oxidation by 10%. The main reason for assuming that endogenous uptake was not affected by substrate-stimulated respiration is that it was brought about by a different mechanism. It was, for example, unaffected by cyanide and other inhibitors of succinoxidase, and was unaccompanied by phosphate disappearance.

Chemical estimations. When labile organic phosphates were present, orthophosphate was measured by the method of Lowry & Lopez (1947). Otherwise, as in fractions from chromatographic experiments, the more sensitive methods of Holden & Pirie (1955) were used for orthophosphate and total phosphate.

Protein N was estimated by a micro-Kjeldahl method (Pierpoint, 1959).

Chromatography of phosphates. The contents of the manometer flasks were deproteinized with an equal volume of 5% (w/v) trichloroacetic acid, and the protein was removed by centrifuging (1500 g for 10 min.). The supernatant was extracted twice with ether, adjusted to pH 7 with ammonia solution, and, after dilution to 15 ml. with mm-ammonia solution, was added to a column (7 cm. $\times$ 1.2 cm.) of Dowex 2 (100–200 mesh; $\times$ 8 cross-linkage). The column was further washed with mm-ammonia solution and the phosphates were eluted with solutions similar to those used by Khym & Cohn (1953). Eluent 1 (ammonium chloride, 0.025M; potassium borate, 0.01M; pH 8.4) eluted orthophosphate; eluent 2 (ammonium chloride, 0.03M) eluted glucose 6-phosphate; eluent 3 (potassium chloride, 0.2M; hydrochloric acid, 0.02M) eluted adenosine nucleotides in a single peak.

Radioactive measurements. ^{32}P radioactivity in fractions (10 ml.) from the chromatographic columns was measured in an M6 Geiger counter. The counting period was 10 min. and the results were corrected for 'lost counts' and background activity. As the estimations in each experiment were done on the same day, no correction for radioactive decay was made.

Chemicals. Phosphate buffers were prepared from $\text{m-NaH}_2\text{PO}_4$ by adding sodium hydroxide soln. and diluting them to the required molarity. Tris buffers were similarly made from 2-amino-2-hydroxymethylpropane-1,3-diol and hydrochloric acid. The solutions were finally adjusted to the required pH values after dilution.

RESULTS

Respiratory quotients for the oxidation of organic acids

Respiratory quotients for the oxidation of a number of tricarboxylic acid-cycle substrates (Table 1) are similar to those reported for mitochondrial preparation from other plant sources,

such as soya-bean hypocotyls (Switzer & Smith, 1957). As with soya-bean preparations the low value for succinate was probably due to the slow rate at which fumarate was oxidized. The similarity of the other R.Q. values to those expected for complete oxidation of substrates of the tricarboxylic acid cycle is probably coincidental, for during the oxidation of these substances, other acids accumulate (Pierpoint, 1959).

With pyruvate and malonate as substrates there was neither uptake of oxygen nor evolution of carbon dioxide.

Oxidative phosphorylation

Disappearance of orthophosphate accompanying the oxidations. In the presence of a number of co-factors, orthophosphate disappeared during the mitochondrial oxidation of succinate (Fig. 1). This phosphate removal started during the 10 min. when the manometer flasks were equilibrating and proceeded almost linearly with time until nearly all the orthophosphate had gone. Orthophosphate did not disappear when the leaf preparations were briefly boiled before incubation, or when substrate was absent. Without substrate the orthophosphate concentration often increased slightly (1–5%), probably because of the action of adenosine triphosphatases.

The phosphorylation reactions that led to the disappearance of phosphate were 'loosely' coupled (Remmert & Lehninger, 1959) to the oxidative reactions. Omission of some substances from the incubation medium considerably diminished the phosphorylation, although this had little effect on oxygen uptake. In particular, ATP or ADP, hexokinase and glucose, substances that might be expected to accept the phosphate groups of unstable esters, were necessary for high phosphorylation rates (Table 2). In the absence of glucose and hexokinase the adenosine triphosphatase activity was increased about eightfold, masking much of the phosphate disappearance.

Table 1. *Respiratory quotients for the oxidation of acids by mitochondrial preparations*

The gas exchanges catalysed by mitochondrial preparations were measured over 2 hr. periods, both in the presence and absence of substrates. Allowances were made for the endogenous activity in calculating respiratory quotients for the oxidation of the substrates.

Substrate	Endogenous respiratory quotient	Respiratory quotient in the presence of substrate
Citrate	0.21	1.5
α -Oxoglutarate	0.26	1.4
Succinate	0.20	0.28
Malate	0.20	1.4

Neither DPN nor triphosphopyridine nucleotide had any effect on oxygen uptake or phosphate removal.

Occasional mitochondrial preparations would catalyse oxidation but not phosphate removal. Invariably such preparations had endogenous oxygen uptakes that were at least a quarter of the uptake in the presence of succinate. This high ratio of endogenous to succinoxidase activity, like the loss of phosphorylating powers, is probably a

symptom of mitochondrial damage, and may be a useful indicator of it.

P/O ratios for the oxidation of organic acids. Table 3 shows the P/O ratios obtained for the oxidation of tricarboxylic acid substrates by the tobacco-leaf preparations. No attempt was made to limit these oxidations to single steps of the tricarboxylic acid cycle, so that the P/O ratio values

Fig. 1. Orthophosphate disappearance and oxygen uptake from mitochondrial preparations. Samples of the mitochondrial preparations (0.6 mg. of protein N) were incubated in manometer flasks containing the medium described in the Experimental section (final vol. 1.7 ml.; CoA omitted). Oxygen uptake (○) was followed after a 10 min. equilibration period, and samples were removed at various times for phosphate estimation (●). A series of control manometers, without succinate, were used to measure endogenous oxygen uptake and appearance of phosphate due to adenosine triphosphatases. The values plotted have been corrected for both these changes.

Table 2. *Effect of components of the incubation medium on phosphorylation*

Samples of the mitochondrial preparations (0.6–0.95 mg. of protein N) were incubated with succinate as described in the Experimental section. The medium contained triphosphopyridine dinucleotide (TPN, 0.1 μmole), half the stated quantities of cytochrome *c*, thiamine pyrophosphate, DPN and orthophosphate, and it was modified as described below. Oxygen uptake was followed for 30 min., when the reactions were stopped and samples taken for orthophosphate estimations. The values given below have been corrected for endogenous oxygen uptake, and for adenosine triphosphatase activity. AMP, Adenosine monophosphate.

Expt. no.	Modification to medium	Oxygen uptake (%)	Phosphorylation (%)
1	None	100	100
	ATP omitted	95	25
	ADP replaced ATP	110	100
2	ADP replaced ATP	100	100
	AMP replaced ATP	110	50
	ATP omitted	106	30
3	None	100	100
	Glucose omitted	100	100
	Hexokinase omitted	120	54
	Glucose and hexokinase omitted	115	28
4	None	100	100
	TPN, fluoride and manganese sulphate omitted	97	90

Table 3. *P/O ratios for the oxidation of organic acids*

The ability of samples (0.5–1.0 mg. of protein N) of mitochondrial preparations to take up oxygen and orthophosphate was measured over a 30 min. period as described in the Experimental section. Hexokinase was, however, omitted from the medium in Expt. 5, and the substrate concentrations were altered in Expt. 6. The values given are corrected for endogenous oxygen uptake and adenosine triphosphatase activity.

Expt. no.	Substrate	Oxygen uptake (μg atoms)	Orthophosphate disappearance (μmoles)	P/O ratio
1	Succinate	6.1	8.0	1.3
2	Malate	6.2	7.8	1.3
	Citrate	2.6	3.8	1.5
3	Fumarate	3.8	5.0	1.3
4	Isocitrate	3.0	6.3	2.1
	Isocitrate plus TPN (0.2 μmole)	3.4	6.2	1.8
5	α-Oxoglutarate	6.9	6.5	0.9
6	Malate (14 μmoles)	4.5	9.6	2.1
	DPNH (9 μmoles)	4.0	1.3	0.3

may be the result of a number of oxidative and phosphorylative reactions.

The P/O ratio values for the oxidation of α -oxoglutarate were measured in the absence of hexokinase. They are not therefore strictly comparable with the other values in Table 3, or with higher values obtained for pea-leaf preparations (Smillie, 1956). Hexokinase was omitted as it inhibited oxygen uptake from α -oxoglutarate by 90%. Glucose and fluoride were also inhibitory but to a smaller (30%) degree. The mechanism of these inhibitions is obscure, but that due to hexokinase and glucose may reflect a requirement of ATP for the oxidation. ATP stimulates the oxidation of α -oxoglutarate although it has no effect on succinate oxidation (Pierpoint, 1959).

The P/O ratio given in Table 3 for the oxidation of reduced DPN (DPNH) is much lower than that for the DPN-dependent oxidation of malate obtained in the same experiment. Similar low values have been observed with animal preparations. They have been attributed to permeability barriers preventing the free diffusion of DPNH (Lehninger, 1951), and the inhibition of phosphorylation by the DPN formed (Lehninger, 1949). Higher P/O values (0.6–0.7) were obtained with tobacco-leaf fractions when lower concentrations of DPNH were used.

Both isocitrate and fumarate caused bigger oxygen uptakes (Table 3) than those previously recorded (Pierpoint, 1959). The improvement in the first case was due to the use of isocitrate prepared by Dr H. B. Vickery (Vickery & Wilson, 1958). It was oxidized ten times as rapidly as was a commercial DL sample of this substance. The increased oxidation of fumarate is commented on in the next paper (Pierpoint, 1960).

Bovine serum albumin stabilizes some enzymes of plant mitochondria (Price & Thimann, 1951), and improves oxidative phosphorylation in insect mitochondria (Sacktor, O'Neill & Cochran, 1958). It was therefore tested against the tobacco mitochondria. It diminished both the oxidation of succinate and the accompanying phosphorylations by a third, leaving the P/O ratio unaltered.

Effect of 2:4-dinitrophenol on phosphate disappearance.

2:4-Dinitrophenol (DNP) inhibited phosphate removal by tobacco-leaf preparations. This inhibition did not result in increased oxygen uptake, which often occurs when oxidation and phosphorylation are more tightly coupled. In fact oxygen uptake was inhibited also, although to a smaller degree (Fig. 2). Phosphate disappearance associated with α -oxoglutarate oxidation was just as sensitive to DNP as that with other substrates (Table 4).

The adenosine triphosphatases of tobacco-leaf preparations, like those of a similar preparation from mosquito (Avi-dor & Gonda, 1959), were unaffected by DNP. The inhibition of phosphorylation by this compound was not therefore a consequence of the acceleration of the breakdown of phosphate esters.

Fig. 2. Effect of DNP on mitochondrial activities. Samples of tobacco-leaf preparations (0.65 mg. of protein N) were incubated as described in the Experimental section in the presence of succinate and various amounts of DNP. Oxygen uptake (●) was followed for 30 min., when the reactions were stopped and orthophosphate disappearance (○) was estimated. Corrections were made for endogenous oxygen uptake, adenosine triphosphatase activity and phosphate disappearance during the equilibration period.

Table 4. *Effect of dinitrophenol on the oxygen and phosphorus metabolism of tobacco-leaf preparations*

Mitochondrial preparations were incubated as described in the Experimental section, either in the presence or the absence of DNP (0.14 mM). Oxygen and phosphorus uptakes were measured over a 30 min. period and corrected for endogenous oxygen uptake and adenosine triphosphatase activity.

Substrate	Inhibition of oxygen uptake (%)	Inhibition of phosphorus disappearance (%)	P:O ratio	
			DNP absent	DNP present
Succinate	20	96	0.7	0.03
Malate	20	90	1.3	0.15
Isocitrate	50	100	2.1	0
α -Oxoglutarate	14	95	0.9	0.05

Accumulation of phosphate esters during oxidation. The reactions leading to the disappearance of orthophosphate are those involved in the formation of adenosine nucleotides and glucose 6-phosphate. The accumulation of both these phosphate esters during the oxidation of succinate is shown in Fig. 3. The mitochondrial preparation used in this experiment esterified about 40% of the added phosphate, with a P/O ratio of 0.95. The amount of phosphate formed due to adenosine triphosphatases was only a small (5%) proportion of the total present. The chromatographic analyses show the formation of a little glucose 6-phosphate at the expense of ATP when the incubation was done in the absence of substrate. When succinate was present, however, there was a larger increase in the amount of glucose 6-phosphate as well as a small increase in nucleotide phosphate. Additional

Fig. 3. Formation of glucose 6-phosphate and ATP during succinate oxidation. Portions of a leaf-mitochondrial preparation (0.8 mg. of protein N) were suspended in the medium described in the Experimental section, except that the concentration of phosphate was increased to $18 \mu\text{moles}$ and that of ATP decreased to $1.2 \mu\text{moles}$. Incubation at 30° in manometer flasks was carried out for 0 hr. (●) and 1.25 hr. (x) in the presence of substrate, and 1.25 hr. in its absence (○). The contents of duplicate flasks were pooled for chromatography. Total P, orthophosphate and $E_{260 \text{ m}\mu}$ were measured on samples from the resulting fractions, but only total P is shown in the graph. The peaks of phosphorus in the top graph represent, from the left, orthophosphate, glucose 6-phosphate and nucleotide phosphates respectively.

evidence that the disappearing orthophosphate was incorporated into nucleotide phosphate was obtained from experiments in which radioactive orthophosphate was used and the glucose and hexokinase were omitted from the reaction medium. Fig. 4 summarizes the results of one such experiment. The mitochondrial preparation had the high adenosine triphosphatase characteristic of preparations tested in the absence of glucose and hexokinase. During incubation in the absence of substrate, the orthophosphate increased by a third. This breakdown of ATP is shown in the top graph of Fig. 4, as well as the decrease (20%) in the specific activity of orthophosphate that results from it. When substrate was present during the incubation there was no change in

Fig. 4. Incorporation of phosphate into nucleotides during malate oxidation. A mitochondrial preparation was suspended in sucrose (0.25M) and divided into three lots (2 mg. of protein N). One (middle graph) was added to the medium described in the Experimental section, plus $[^{32}\text{P}]$ -orthophosphate ($2 \mu\text{C}$) and malate ($40 \mu\text{moles}$). The second (bottom graph) was boiled for 5 min. and then added to the same medium. The third (top graph) was added to the medium from which malate had been omitted. All were incubated at 30° for 40 min., deproteinized and then chromatographed. Samples of the fractions collected (10 ml.) were taken for total phosphate estimations (●) and radioactivity measurements (⊗). The two peaks of phosphate represent orthophosphate, on the left, and nucleotide phosphates.

the concentration of nucleotide phosphates, and these substances became radioactive with a specific activity about a third of that of orthophosphate. Fig. 4 also shows that there is a small incorporation of radioactivity into nucleotides in the absence of substrate. Such an orthophosphate-ATP exchange reaction is well known in animal mitochondria and their fragments, and is thought to reflect some transphosphorylation reaction of the oxidative-phosphorylation process (Cooper & Lehninger, 1957). With the tobacco-leaf preparations, however, it might equally well be due to the oxidation of traces of citrate derived from the isolation procedure, or to photosynthetic phosphorylation (Arnon, Whatley & Allen, 1958) associated with the chloroplast fragments.

The asymmetric shape of some of the orthophosphate peaks in Fig. 4 was not caused by contaminating phosphates. The specific activities of fractions taken from different parts of these peaks were identical. Moreover, the same asymmetry was observed when authentic orthophosphate was eluted from these resins with 0.03M-ammonium chloride. It may result from the presence of different ionic species of orthophosphate, or from interference caused by bicarbonate contamination of the alkaline eluents (Björk & Boman, 1959).

Stability of oxidation and phosphorylation reactions. Once the mitochondrial preparations were centrifuged from the disintegrated leaves, washed and suspended in a sucrose medium, they were stable enough for convenient experimentation. When, for example, they were suspended in the substances usually added to the manometer flasks and incubated at 30° without substrate, there was no measurable loss in ability to oxidize succinate after 30 min. and over 70% of the phosphorylative power remained. Longer incubations emphasized the difference in stability between the oxidative and the phosphorylative reactions (Table 5) and revealed a beneficial action of sucrose on the oxidative enzymes.

Mitochondrial preparations from the leaves of other plants

A sedimentable preparation, which oxidized both succinate and malate, was obtained from the leaves of garden lupins (*Lupinus* sp.) by the usual procedure. The citrate in the extraction and washing media was essential. In one experiment 50 g. of leaves was divided into two equal lots; one lot was ground in the usual medium (70 ml.) and the other in the same medium without the citrate. A mitochondrial preparation from the first had a succinoxidase q_{O_2} (μ l. of oxygen/mg. of protein N/30 min.) of 32 and an endogenous q_{O_2} of 5. That prepared from the second had an endogenous q_{O_2} of 4 and no succinoxidase. A sedimentable succinoxidase could also be prepared from the leaves of greenhouse-grown yams (*Dioscorea* sp.), but with this plant the citrate in the medium had no effect on the activity.

No succinoxidase activity could be prepared from the leaves of French beans (*Phaseolus vulgaris*).

DISCUSSION

The amount of phosphorylation catalysed by the tobacco-leaf preparations (30 μ moles of P/mg. of protein N/hr. in Fig. 1) could doubtless be increased by altering the assay conditions. Thus there was an indication that the phosphate concentration generally used, chosen for convenience of estimation, was not optimum. Nevertheless, the P/O ratios obtained are comparable with those from other green leaves (Smillie, 1956). They suggest that the mitochondria, or mitochondrial fragments, contained in these preparations retain some degree of organization. This conclusion is not at variance with the appearance of these preparations in some (unpublished) electron micrographs. However, the looseness of the connexion between the oxidative and phosphorylative reactions may indicate some functional damage. Rat-liver mitochondria contain a protein which is released on

Table 5. *Stability of oxidative and phosphorylative activities of mitochondrial preparations*

The mitochondrial preparation was divided into two lots before washing, and, after washing, one lot was suspended in water and the other in sucrose (0.25M). Samples (0.5 ml.) were put into the side arms of manometer flasks, whose main compartments contained succinate with the medium described in the Experimental section, and were tipped into this medium after incubation for various times at 30°. Oxygen uptake was followed for 30 min. after it became regular (5 min.), and was corrected for endogenous activity. Phosphate disappearance was measured 40 min. after tipping and was not corrected for adenosine triphosphatase activity.

Time of incubation (min.)	Suspension in sucrose		Suspension in water	
	Oxidative activity (%)	Phosphorylative activity (%)	Oxidative activity (%)	Phosphorylative activity (%)
0	100	100	100	100
40	94	45	96	39
75	93	2	66	0

their disruption and which loosens the dependence of oxidations on phosphorylations in intact mitochondria (Remmert & Lehninger, 1959). Some similar process may have occurred during the grinding of the tobacco leaves. An alternative explanation, that the loose coupling represents the state of the mitochondria in the intact leaf, is possible but less likely.

The chloroplast material contaminating the mitochondrial preparations is unlikely to have any oxidative-phosphorylative activity (James & Das, 1957). Its removal would therefore increase the phosphorylative activity of the preparations, on a protein N basis, about threefold (Pierpoint, 1959). This activity increase, together with increases due to improvements in the method of estimation, may well bring phosphorylation rates to over 100 μ moles of P/mg. of protein N/hr., rates comparable with some of the highest of those reported for non-chlorophyllous plant material (see Allen, Whatley & Arnon, 1958). Whether they would ever reach the rates at which photosynthetic systems esterify phosphate (554–785 μ moles of P/mg. of protein N/hr. at 20°; Allen *et al.* 1958) is more difficult to foretell. Photosynthetic phosphorylation can supply the energy required for starch synthesis in tobacco-leaf disks (Maclachlan & Porter, 1959), and is probably the main source of ATP in the chlorophyllous cells that contain no mitochondria (James & Das, 1957; Weier, 1958). A comparison therefore of the maximal rates of photosynthetic and oxidative phosphorylation in extracts of the same leaves will give an idea of the relative contributions of the two energy-yielding systems in the intact leaf.

The usefulness of the citrate medium in the isolation of a sedimentable succinoxidase from lupin leaves is interesting in view of the results of Brummond & Burris (1954). Their experience with extracts of these leaves prepared in a sucrose solution lead them to question the location of the tricarboxylic acid-cycle complex of enzymes in the mitochondria of green leaves. It seems likely that a citrate- or succinate-containing medium would be helpful in the extraction of mitochondria from plants other than lupin and tobacco. Two recent papers suggest that this conclusion holds for a much wider range of tissues. One (Eichel, 1959) describes the stabilizing effect of citrate and succinate on some of the electron-transport enzymes of *Tetrahymena pyruviformis*. The second deals with the increase in pyruvic oxidase activity of housefly mitochondria that results from including citrate, succinate and pyruvate in the extraction medium (Gregg, Heisler & Remmert, 1959). Ideas about the functioning of the insect mitochondria *in vivo* (Chance & Sacktor, 1958) are based on the properties of preparations made in the absence of these acids and may well need some revision.

SUMMARY

1. Further properties are described of a sedimentable preparation from tobacco leaves which has previously been shown to catalyse many of the oxidations of the tricarboxylic acid cycle.

2. The oxidation of some substrates was accompanied by an evolution of carbon dioxide which varied in amount between 0.3 and 1.5 times the volume of oxygen absorbed.

3. A disappearance of orthophosphate accompanied the oxidations provided that adenosine polyphosphates, glucose and hexokinase were present. The ratio of phosphate disappearing to oxygen absorbed (P/O ratio) was generally between 1 and 2. 2:4-Dinitrophenol completely suppressed the phosphate disappearance and had less effect on oxygen uptake.

4. During the oxidation of substrate acids there was an increase in the amount of glucose 6-phosphate and nucleotide phosphate present. Incubation of the preparation with [32 P]orthophosphate resulted in radioactivity being incorporated into the nucleotide fraction. At least five times as much was incorporated in the presence as in the absence of oxidizable substrate.

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Biochem. J. (1960) **75**, 511

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

3. GLYCOLLIC OXIDASE AND FUMARASE ACTIVITY

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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).