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Polarographic Measurement of Oxygen Uptake by Pea-Root Mitochondria

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Preliminary work on the oxidation of succinate and α -oxoglutarate by pea-root mitochondria showed that the standard manometric method of measuring oxygen uptake was not sufficiently sensitive. A polarographic method was thus developed. It was observed that, whereas the oxidation of α -oxoglutarate as determined manometrically was increased by the addition of diphosphopyridine nucleotide, that measured polarographically was not. This coenzyme also stimulated α -oxoglutarate oxidation (measured polarographically) if the mitochondria were first shaken in a water bath. Similar results with other substrates and cofactors are reported in this paper.

EXPERIMENTAL

Growth of peas and extraction of mitochondria. Peas (*Pisum sativum* L. var. Meteor) were rinsed in ethanol, treated with 10% (w/v) calcium hypochlorite for 30 min. and germinated under sterile conditions in a 5:2 (v/v) vermiculite-water mixture at 26° for 47 hr. Seedlings with roots between 22 and 30 mm. long were selected from those harvested, and a terminal 20 mm. section was excised and placed immediately in ice-water mixture. About 120-140 roots were used in each experiment. Excess of moisture was blotted off the roots, which were weighed and homogenized in a cold glass homogenizer of the Potter-Elvehjem type in 1 vol. of a cold solution containing 0.6M-sucrose and 0.05M-phosphate buffer, pH 6.8. The homogenization was carried out for the minimum length of time needed to ensure disruption of the roots (usually about 10-12 sec.) and in a loose homogenizer (with a clearance of 0.3 mm. round the pestle) which was surrounded by ice.

Unbroken cells, cell walls and nuclei were removed from the homogenate by centrifuging at 1000 *g* for 10 min. at 0°. The supernatant was centrifuged at 15 000 *g* for 15 min.

and the precipitate was taken as mitochondria. It was washed by resuspension in 0.5 vol. of the homogenizing medium and centrifuging at 15 000 *g* for 15 min. The mitochondria were finally resuspended in 0.5 vol. of the medium.

In many experiments part of the mitochondrial suspension was shaken for $\frac{1}{2}$ hr. in a test tube in a Warburg bath at 25°, at a speed of 142 oscillations/min. and through a distance of about $\frac{1}{4}$ in.

Measurement of oxygen concentration. Oxygen concentration in the incubation medium was measured polarographically. The circuit of a simple polarograph suitable for such measurements is shown in Fig. 1. The polarizing circuit is shown on the right side. The voltage divider (R_1) enables an e.m.f., derived from the battery (B_1) and measured on the voltmeter (V), to be applied across the electrodes of the cell. The current which passes through the cell is measured on the galvanometer (G). Some current

Fig. 1. Circuit of polarograph. B_1 , B_2 , 1.5 v dry batteries; C_1 , C_2 , 50 μ F, 50 v condensers; G, Scalamp galvanometer 7893/S (W. G. Pye and Co. Ltd., Cambridge); R_1 , 125 Ω helical potentiometer; R_2 , R_3 , 5 M Ω linear track potentiometer; R_4 , 1 M Ω log track potentiometer; V, 1.5 v d.c. voltmeter.

(the residual current) passes through the cell in the absence of oxygen and in order to ensure that the full sensitivity of the galvanometer is being used for measurement of the oxygen-induced current, it is necessary to incorporate the additional circuit shown on the left of Fig. 1. In this, the battery (B_2) applies an e.m.f. across the galvanometer in opposition to that from B_1 , and the current, varied by potentiometers R_2 , R_3 and R_4 , 'backs off' the residual current. Any small fluctuations in the voltage of the batteries is counteracted by the condensers (C_1 and C_2).

The cell used in this work is shown diagrammatically in Fig. 2, and consisted of Pt (negative) and Ag/AgCl (positive) electrodes. Rods of Pt (0.16 mm. diam.) and Ag (0.20 mm. diam.) with Cu wires soldered to them, were immersed in 4 ml. of *n*-butyl methacrylate (L. Light and Co. Ltd.) containing 1% (w/v) of benzoyl peroxide in a polythene specimen tube with an internal diameter of 12.5 mm. The solution was polymerized at 60° overnight and the polythene tube was then stripped away from the solid plastic cylinder, the end of which was turned down in a lathe to expose the maximum surface area of silver. Insulated wires (I) were then soldered to the exposed ends of the copper wires, and with the former uppermost a section of polythene specimen tube (with the base removed) was placed round the cylinder and the cavity filled with *n*-butyl methacrylate (plus catalyst) and polymerized as before. To obtain an Ag/AgCl electrode, the Ag surface was first exposed to conc. HCl for several minutes, then dipped into saturated KCl solution and an e.m.f. of 1.5v was applied for 10 min. with the Ag electrode positive.

Fig. 2. Section through the complete cell and view of the exposed electrode surface ($\times 1\frac{1}{2}$ approx.). The silver (Ag) and platinum (Pt) electrodes were soldered to copper wires (Cu) and these to insulated wires (I) leading to the polarograph. On the surface of the electrodes was placed a circle of lens tissue (L) and a sheet of polythene (P), held in place by a section of polythene specimen tube (S). Black areas represent solder.

The electrodes were used in a manner similar to those of Bellamy & Bartley (1960). A circle of lens tissue (L; approx. 12.3 mm. diam.) was placed on the electrode surface, moistened with saturated KCl and the whole covered with a disk (22 mm. diam.) of polythene sheet (P) (100 gauge). This polythene sheet was held in place by a section of polythene specimen tube (S), which also formed a vessel for the reactants with a volume of 0.5 ml.

Between 0.5 and 0.8 v the oxygen-induced current was approximately constant (0.59 μ A). A voltage of 0.7 v was selected for further work and the total current at this voltage was 1.04 μ A.

For calibration of the electrodes, the piece of tube (S) was replaced by an inverted whole polythene specimen tube containing two small holes for the passage of gas and a larger hole (blocked by a rubber bung) for addition of liquid. Mixtures of air and oxygen-free nitrogen were passed over 0.5 ml. of water in the chamber for 30–45 min. and the steady current obtained after this time was recorded. A straight-line relationship was obtained between current and oxygen concentration in the water (calculated from the gas composition).

Measurement of oxygen uptake. Since the current took some time to reach a steady value, the cell had to be set up and the polarograph switched on $\frac{1}{2}$ hr. before it was required.

All components of the incubation medium (except mitochondria) were placed in the cell, mixed and left for 2½–3 min. to equilibrate and for the current to become constant. The mitochondrial suspension was then added and the mixture was stirred briskly for 20 sec.; a small square of Perspex was placed over the cell and readings were taken on the galvanometer every 5 sec. for 3 min. Addition of the mitochondria and the stirring caused an increase in the current which lasted for 30–40 sec. Care was taken not to touch the polythene membrane, since this gave a much increased current for up to 2 min., probably due to disturbance of the diffusion shell round the Pt electrode. An initial rate of oxygen uptake was obtained graphically from the readings and is expressed as q_{O_2} (μ l. of O_2 /mg. of N/hr.).

The incubation medium consisted of 0.05M-phosphate buffer, pH 6.8, 0.1 ml. of mitochondrial suspension and substrates (10 mM) and coenzymes as described in the individual experiments, in a total volume of 0.5 ml. All experiments were conducted at room temperature (22–23°).

After each determination the chamber was washed three times with water and mopped out with soft absorbent paper.

Nitrogen estimations. These were done by the Conway dish method of Sunderland, Heyes & Brown (1957) on the 'acid-insoluble matter' obtained according to the procedure of Werkheiser & Bartley (1957).

Preparation of electron micrographs. The mitochondria were fixed and embedded by the method described by Bellamy (1958), except that for the initial step 0.2 ml. of osmic acid medium was added to 1.0 ml. of osmic acid medium and left for 15 min. before centrifuging. Sections were cut and electron micrographs prepared by Mr J. C. W. Crawley.

Special chemicals. The following were used: diphosphopyridine nucleotide (DPN) and thiamine pyrophosphate (L. Light and Co. Ltd., Colnbrook, Bucks.). Cytochrome c, adenosine diphosphate (ADP), adenosine triphosphate

(ATP) and coenzyme A (CoA) (Sigma Chemical Co., St Louis, Mo., U.S.A.); bovine-plasma albumin (The Armour Laboratories).

RESULTS

Effect of cytochrome c. In the absence of any added cofactors, the mitochondria oxidized succinate but not α -oxoglutarate (Table 1). On the addition of cytochrome *c* (10 μ M) the endogenous rate was the same, but there was a small increase in oxygen uptake in the presence of α -oxoglutarate and the rate with succinate was considerably greater.

Cytochrome *c* could be replaced by bovine-plasma albumin (Table 2). With unshaken mitochondria there was oxidation of succinate in the

presence of cytochrome *c*, though this was somewhat less with bovine-plasma albumin. If the mitochondria were shaken in a water bath at 25° for 30 min., the oxygen uptake due to succinate was doubled and was about the same with both cytochrome *c* and bovine-plasma albumin. The protein concentrations in this experiment were 0.2 mg./ml. for cytochrome *c* and 0.5 mg./ml. for bovine-plasma albumin.

Effect of adenosine diphosphate on succinate oxidation. With unshaken mitochondria both the endogenous oxygen uptake and that with succinate were unaffected by the addition of mm-ADP (Table 3). However, ADP stimulated succinate oxidation and, to a lesser extent, the endogenous rate with shaken mitochondria.

Effect of cofactors on α -oxoglutarate oxidation. α -Oxoglutarate was either not oxidized by unshaken mitochondria or, as in Expts. 2, 5 and 6, oxidized only to a small extent (Table 4). However, with shaken mitochondria there was always considerable oxidation of α -oxoglutarate.

DPN (0.1 mm) had no effect on the oxidation of α -oxoglutarate by unshaken mitochondria, but it caused considerable stimulation with shaken mitochondria (Table 4, Expt. 1). A higher DPN concentration (mm) did increase the rate of oxidation of α -oxoglutarate in unshaken mitochondria but decreased that in shaken mitochondria (Table 4, Expt. 2).

Similar effects to that with 0.1 mm-DPN were obtained with ADP (mm), ATP (mm) and thiamine pyrophosphate (0.1 mm) (Table 4, Expts. 3–5). These cofactors caused no increase in α -oxoglutarate oxidation by unshaken mitochondria, but brought about a marked increase with shaken mitochondria.

The effect of CoA was examined at two concentrations. The higher of these (0.1 mm) inhibited the increase in oxygen uptake obtained in the presence of α -oxoglutarate with both unshaken and shaken mitochondria (Table 4, Expt. 6). The lower concentration (0.02 mm) had no effect with unshaken mitochondria, but doubled the total oxygen uptake with shaken mitochondria.

Oxidation of malate and pyruvate. Both L-malate and pyruvate were oxidized either very slowly or not at all when added to unshaken mitochondria (Table 5). As with α -oxoglutarate, however, there was appreciable oxidation of these substrates by shaken mitochondria. If both L-malate and pyruvate were added together, there was an increase in oxygen uptake, small with unshaken mitochondria, but much greater with shaken mitochondria (Table 5, Expt. 1).

DPN had an effect with these substrates similar to that with α -oxoglutarate. It had little or no effect on malate or pyruvate oxidation in unshaken mitochondria, but it brought about a very

Table 1. *Effect of cytochrome c on oxygen uptake*

Incubation medium contained 0.05M-phosphate buffer, pH 6.8, 0.1 ml. of mitochondrial suspension and additions as shown below, in a total volume of 0.5 ml.

Additions	q_{O_2}
None	480
α -Oxoglutarate (10 mm)	480
Succinate (10 mm)	710
Cytochrome <i>c</i> (10 μ M)	480
Cytochrome <i>c</i> (10 μ M) + α -oxoglutarate (10 mm)	510
Cytochrome <i>c</i> (10 μ M) + succinate (10 mm)	810

Table 2. *Replacement of cytochrome c by bovine-plasma albumin*

Incubation medium was as given in Table 1. Concn. of bovine-plasma albumin was 0.5 mg./ml.

Additions	q_{O_2}	
	Unshaken mitochondria	Shaken mitochondria
None	490	400
Succinate (10 mm)	640	720
Cytochrome <i>c</i> (10 μ M)	480	420
Cytochrome <i>c</i> (10 μ M) + succinate (10 mm)	1440	2280
Bovine-plasma albumin	400	390
Bovine-plasma albumin + succinate (10 mm)	1330	1940

Table 3. *Effect of adenosine diphosphate on succinate oxidation*

Incubation medium contained 0.05M-phosphate buffer, pH 6.8, 10 μ M-cytochrome *c*, 0.1 ml. of mitochondrial suspension and additions as shown below in a total volume of 0.5 ml.

Additions	q_{O_2}	
	Unshaken mitochondria	Shaken mitochondria
None	600	600
ADP (mm)	610	790
Succinate (10 mm)	1130	1370
ADP (mm) + succinate (10 mm)	1120	1730

Table 4. *Cofactor requirements for α -oxoglutarate oxidation by unshaken and shaken mitochondria*Incubation medium was as given in Table 3. α -Oxoglutarate concn. was 10 mM.

Expt. no.	Additions	q_{O_2}	
		Unshaken mitochondria	Shaken mitochondria
1	None	770	740
	DPN (0.1 mM)	710	610
	α -Oxoglutarate	740	1080
	α -Oxoglutarate + DPN (0.1 mM)	710	1240
2	None	640	600
	DPN (mM)	640	640
	α -Oxoglutarate	670	850
	α -Oxoglutarate + DPN (mM)	750	750
3	None	600	650
	ADP (mM)	580	650
	α -Oxoglutarate	580	720
	α -Oxoglutarate + ADP (mM)	600	920
4	None	560	500
	ATP (mM)	560	500
	α -Oxoglutarate	550	650
	α -Oxoglutarate + ATP (mM)	560	800
5	None	610	680
	TPP (0.1 mM)	650	710
	α -Oxoglutarate	660	910
	α -Oxoglutarate + TPP (0.1 mM)	660	1320
6	None	870	760
	CoA (0.1 mM)	820	740
	CoA (0.02 mM)	860	810
	α -Oxoglutarate	920	1050
	α -Oxoglutarate + CoA (0.1 mM)	840	820
	α -Oxoglutarate + CoA (0.02 mM)	920	1920

Table 5. *Oxidation of malate and pyruvate by unshaken and shaken mitochondria*

Incubation medium was as given in Table 3. Concn. of L-malate and pyruvate was 10 mM.

Expt. no.	Additions	q_{O_2}	
		Unshaken mitochondria	Shaken mitochondria
1	None	520	470
	L-Malate	540	600
	Pyruvate	540	730
	L-Malate + pyruvate	560	840
2	None	660	860
	DPN (0.1 mM)	660	830
	L-Malate	690	1010
	L-Malate + DPN (0.1 mM)	660	1490
3	None	590	570
	DPN (0.1 mM)	570	590
	Pyruvate	560	710
	Pyruvate + DPN (0.1 mM)	610	890

high increase in oxygen uptake with shaken mitochondria.

Electron micrographs. These were prepared from both shaken and unshaken mitochondria.

The mitochondria which were not shaken were oval, or occasionally circular, bodies, 0.6–0.8 μ long and 0.4–0.6 μ wide. The great majority had considerable internal structure and an apparently intact outer membrane. Those mitochondria which were shaken appeared to be the same in size and

shape. They had as much internal structure, although a small proportion (10%) had lost their external membrane.

DISCUSSION

Unshaken mitochondria showed comparatively little oxidative activity towards several substrates, and cofactors known to be active in these oxidations usually gave no stimulation of oxygen uptake.

However, if the mitochondria were treated in a way that might be expected to damage them, namely by shaking them in a water bath at 25° for $\frac{1}{2}$ hr., then all the substrates added were oxidized and the cofactors gave large increases in oxygen uptake. It seems likely that in the unshaken mitochondria the enzymes were relatively inaccessible to substrates and cofactors, whereas contact could be made once the mitochondria had been shaken, either because the membrane had become more permeable or because the enzymes had leaked out owing to rupture of the mitochondrial membrane. The electron micrographs showed that some of the mitochondria had lost their membrane, but that most had not, and it is thus probable that both these possibilities are involved. Amoore (1958) has shown that isolated rat-liver mitochondria are permeable to succinate, but relatively impermeable to ATP, and Birt & Bartley (1960) found that DPN did not penetrate. This supports both the apparent inaccessibility of ATP and DPN to the intramitochondrial enzymes observed in this paper and also the oxidation of succinate by unshaken mitochondria.

One cofactor, cytochrome *c*, increased the oxygen uptake with succinate and α -oxoglutarate. This suggests that the unshaken mitochondria were themselves disrupted or permeable to the large cytochrome *c* molecule. However, the cytochrome *c* could be replaced by bovine-plasma albumin, which indicates that the cytochrome *c* was probably acting as a protein, and stabilizing the mitochondria, rather than as a coenzyme.

DPN, when employed at the higher concentration, had a different effect from that of the other cofactors. It might have diffused into the unshaken mitochondria from the high external concentration and thus stimulated the oxidation of α -oxoglutarate, whereas with the shaken mitochondria the higher concentration appears to have caused inhibition.

Many workers (see, for example, Bendall & de Duve, 1960; Greville & Chappell, 1959) have recorded this latency of enzymes, mainly with animal mitochondria. Simon (1958) has observed it with mitochondrial cytochrome oxidase from several plants. For this enzyme he found that incubation at 25° for 1 hr. was quite ineffective in increasing the activity: much more drastic treatment, such as exposure to digitonin or hypotonic conditions, was required. He also compared the manometric and spectrophotometric assays of this

enzyme and found that the stimulation of enzyme activity on treatment with digitonin was always less in the manometric assay. It seems possible that this might be due to an increased rate of cytochrome *c* oxidation in the control mitochondria, brought about in a similar way to the stimulations reported for shaken mitochondria in this paper.

SUMMARY

1. A simple polarograph and a cell containing platinum and silver/silver chloride electrodes suitable for measurement of oxygen uptake are described.

2. α -Oxoglutarate, L-malate and pyruvate were either only very slowly oxidized or not oxidized at all by pea-root mitochondria. If the mitochondria were first shaken in a water bath at 25° for $\frac{1}{2}$ hr. they then oxidized these substrates. Succinate was oxidized by the mitochondria, but the rate was increased by shaking.

3. Stimulation of the oxidations by unshaken mitochondria was not obtained by addition of several cofactors (diphosphopyridine nucleotide, adenosine di- and tri-phosphates, thiamine pyrophosphate and coenzyme A) but the oxidations by shaken mitochondria were greatly enhanced.

4. Cytochrome *c* increased the rate of oxidation of succinate and α -oxoglutarate, but could be replaced by bovine-plasma albumin.

5. It is suggested that the action of the shaking is to make the mitochondrial membrane more permeable or to disrupt it and release the enzymes.

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