- Shigematsu, H. (1958). *Nature, Lond.*, **182**, 880.
- Shimura, K., Fukai, H., Suto, S. & Hoshi, R. (1958). *J. Biochem., Tokyo*, **45**, 481.
- Shimura, K., Koide, F., Itabashi, H. & Fukai, H. (1955). *J. Biochem., Tokyo*, **42**, 285.
- Sisakian, A. M. & Kuvaeva, E. B. (1957). *Dokl. Akad. Nauk. S.S.S.R.: Biol. Sci. Sect. (Transl.)*, **113**, 99.
- Takayama, S., Ito, H. & Miura, Y. (1958). *Biochim. biophys. Acta*, **30**, 233.
- Tarver, H. (1954). In *The Proteins*, vol. 2, part B, p. 1199. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
- Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.
- Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L. & Williams, C. M. (1949). *Science*, **109**, 624.

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The Effect of the Incubation Conditions on the Respiration of Mitochondria

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The experiments described arose from the use of the polarographic method to measure the respiration of quantities of mitochondria which were too small to be handled in manometers. In the initial experiments the oxygen uptakes of mitochondria, measured polarographically and manometrically, were different. Such discrepancies have been reported by Chance & Sacktor (1958), who used the two techniques to measure the respiration of insect mitochondria.

In the present work both methods gave the same results with respiring yeast suspensions and with mitochondria after they had been shaken in the medium used for the manometric experiments. More detailed investigation showed that rapid changes in the characteristics of the mitochondrial respiration occurred during the initial period of shaking under the manometric conditions. These changes consisted of a rapid rise and subsequent fall in the oxygen uptake and the transformation of the mitochondria from a state in which the respiration was insensitive to adenosine diphosphate to a state where adenosine diphosphate stimulated the oxygen uptake.

METHODS

Yeast suspensions. Fresh baker's yeast (1 g.) was suspended in 100 ml. of 0.135 M-KCl containing 1% of glucose.

Mitochondria. Rat-liver mitochondria were prepared according to Werkheiser & Bartley (1957), except that the dissolved CO₂ was not removed from the sucrose solution. Rat-brain mitochondria were prepared by the method of Bellamy (1959) and pigeon-breast-muscle mitochondria according to the method of Chappell & Perry (1953). The mitochondria were centrifuged and finally suspended in 0.25 M-sucrose, except in some experiments on rat-liver

mitochondria, which were suspended in the saline medium of Chappell & Perry (1954).

Dry weights. Dry weights were determined as 'acid-insoluble dry matter' according to the method of Werkheiser & Bartley (1957).

Measurement of respiration

(a) **Constant-volume manometry.** The conventional Warburg technique was used with manometer flasks of volume 5.5 or 23 ml. The incubation medium contained 0.01 M-substrate, 0.01 M-K₂HPO₄-KH₂PO₄ buffer (pH 7.4, measured with a glass electrode) and 2 mM-MgCl₂. Adenosine diphosphate (0.01 M) was added where indicated. About 50 or 200 mg. (wet wt.) of mitochondria, according to the size of the manometer cups used, was added, and the solute content adjusted to 0.25 osmolar with sucrose solution. With rat-liver mitochondria suspended in saline the incubations were in 0.1 M-KCl, 0.05 M-2-amino-2-hydroxy-methylpropane-1:3-diol (tris) buffer, pH 7.4, mM-ethylenediaminetetra-acetate and 5 mM-MgSO₄ (Chappell & Perry, 1954). In all experiments the centre well contained 0.2 ml. of 2N-NaOH with filter paper. Incubations were at 20° with air as the gas phase and the oxygen uptake was measured after a 5 min. equilibration period. The initial rates of respiration were expressed as Q_{O₂} values (μl. of O₂ consumed/hr./mg. dry wt.).

(b) **Polarography.** Petering & Daniells (1938) were the first to use a dropping-mercury electrode to measure the respiration of a variety of cell suspensions and rat-liver homogenates, and the method has since been used to measure the respiration of micro-organisms, fibroblasts and mitochondria (Longmuir, 1954; Harris & Barclay, 1955; Chance & Williams, 1955).

When a potential difference of 0.6–0.7 v is applied across a platinum and a reference electrode immersed in an aqueous medium, the current flowing is proportional to the concentration of oxygen in the solution and the area of platinum exposed to the solution. The current is increased when the medium is stirred.

In preliminary experiments stationary electrodes, directly in contact with stirred mitochondrial suspensions, were used. With this type of apparatus there was a progressive decline in the current when the electrodes were immersed in a fluid in contact with room air. The fall in current amounted to about 20% in 1 hr. Another difficulty was that the current produced was reduced when incubation media, containing sucrose, phosphate and MgCl_2 , were used.

However, by far the greatest difficulty was the inactivation of the electrodes on the addition of mitochondria to the incubation medium. The addition of mitochondria resulted in a rapid fall in the current, simulating respiration, which continued for about 4 min. When the suspension was replaced by fresh medium, the initial current was not obtained until the electrodes had been cleaned by rubbing the surfaces with hard filter paper. There are other reports of this type of inactivation (Chance & Williams, 1955; Connelly, 1957). Presumably the inactivation is due to deposition of protein on the electrodes. These problems can be overcome by ensuring that the electrodes have a constant ionic environment and by shielding the electrodes from the incubation medium with thin membranes which are permeable to gases but not to solutions (Clark, 1956). This modification has been described by Reeves, Rennie & Pappenheimer (1957) and the form of apparatus used in the present work is described in Fig. 1.

The base block A was turned from Perspex rod 4 cm. in diameter and drilled to carry a silver and a platinum electrode. The block B was also turned from Perspex rod of the same diameter and threaded to screw tightly down on block A. To assemble the apparatus, a circle of lens tissue (J. B. Green Ltd., Maidstone, England), 0.002 in. thick and 1.7 cm. in diameter, was placed on the top surface of block A covering the two electrodes and moistened with a drop of saturated KCl solution. A circle 2.3 cm. in diameter, cut from a polythene sheet 0.005 in. thick, was placed on the lens tissue and smoothed down to expel any air bubbles between the two. The block B was then screwed down hard so that the rim *a* compressed the outer edges of the lens tissue and polythene against the surface of block A. Thus a circular chamber 2.6 cm. in diameter and about 0.25 cm. deep was formed (*b-b*, Fig. 1). The volume contained, when the liquid level was continuous with the Perspex top, was 0.7 ml., but volumes up to 1.0 ml. could be added without the contents of the chamber spilling over.

Any liquid placed in the chamber was separated from the electrodes by the polythene and lens-tissue membranes forming the bottom of the chamber.

Construction of the electrodes. The main difficulty was to obtain a perfect, water-tight seal between the platinum and the surrounding material. Since the diffusion current is proportional to the area of platinum exposed to the solution, any seepage of the medium between the platinum and the insulating material produced a large current which was insensitive to changes of oxygen concentration in the bulk of the solution.

A platinum wire 2 in. long and 1.5 mm. in diameter was immersed in butyl methacrylate (I.C.I., Ltd.) containing 1% (w/v) of benzoyl peroxide, in a glass specimen tube. The solution was allowed to polymerize for 24 hr. at 45°, and after the glass had been broken the solid cylinder of plastic was machined to form a threaded rod 5 mm. in diameter. A suitable electrode surface was prepared by

rubbing down one end of the rod with fine emery paper till the full cross-sectional area of the platinum was exposed. The thread of the electrode was covered with silicone grease and was screwed into the block A so that the exposed surface of the platinum was flush with the surface of the block.

To prepare the silver electrode, one of the ends of a length of silver wire was filed to a perfect cross-section and dipped in conc. HCl for 10 min., then washed with water. The silver wire was sealed into block A (with Tensal Cement No. 6, I.C.I., Ltd.) flush with the surface.

Measurement of current. A Cambridge pen-recording polarograph (Cambridge Instrument Co. Ltd.) was used to measure the diffusion current. The platinum electrode was connected directly to the negative terminal of the instrument and the silver electrode to the positive terminal. The voltage control was set at 0.65 v. This voltage corresponded to the plateau in the current-voltage curve obtained for the electrode system (Fig. 2).

Fig. 1. The polarograph cell. To assemble the apparatus a circle of lens tissue was placed on the top surface of block A covering the two electrodes and moistened with a drop of saturated KCl solution. A circle of polythene was placed on the lens tissue and smoothed down to expel any air bubbles between the two. The block B was then screwed down hard so that the rim *a* compressed the outer edges of the lens tissue and polythene against the surface of block A. Thus a circular chamber was formed (*b-b*) open at the top and with the polythene membrane forming the bottom.

Calibration of the oxygen electrode. The relationship between the diffusion current and the concentration of oxygen was obtained by calibrating the electrode system with a solution of known oxygen concentration. The apparatus was fitted together with liquid in the chamber and placed in a container which could be evacuated to a fixed pressure; the diffusion current was recorded at intervals until a steady value was obtained. This was repeated at a series of pressures during evacuation and again on admitting air to the system. The current, at zero oxygen concentration, was obtained in the same way by continuously passing oxygen-free nitrogen through the container.

Equilibration of the apparatus with the gas phase was complete in 25 min. Plotting the current at equilibrium against the oxygen concentration of the solution in the chamber (calculated from the air pressure in the container and the solubility of oxygen in the solution) produced a straight line which did not pass through the origin (Fig. 3). The residual current ($0.1 \mu\text{A}$), not related to the oxygen concentration, amounted to one-fifth of the maximum current measured in a solution saturated with air at room temperature and pressure. The maximum current usually remained constant during the experiment. The residual current showed no more than a 10% drift in 5–6 hr.

In most experiments the current corresponding to room air was first measured. The current given in the absence of oxygen was determined after adding sodium dithionite (approx. 20 mg./ml.) to the chamber. Deoxygenation of the fluid took about 5 min.

Operation and stability. Sudden pressure on the membranes covering the electrodes produced fluctuations in the

current, presumably due to disturbance of the diffusion shell around the platinum electrode. For example, when liquid was placed in the chamber there was a sudden upsurge in the current from a steady value. After about 2 min. the current settled down to a slightly higher value and could be recorded over a period of 45 min. with a variation of less than 2%.

To determine respiration rates the usual procedure was as follows. The incubation medium (usually 0.5 ml.) was placed in the chamber and allowed to equilibrate for about 2 min. The polarograph was then adjusted to begin recording a constant current. The mitochondrial suspension (usually 0.1–0.5 ml.) was added, and the contents of the chamber gently mixed with a fine polythene tube. The stirring produced a transient increase in the current generally lasting for 30 sec., after which the current fell linearly as oxygen was consumed until a value correspond-

Fig. 2. Relationship between current and voltage obtained with the oxygen electrode. The apparatus described in Fig. 1 was assembled with 1 ml. of water in the chamber and placed in a container which could be flushed with nitrogen. The electrode system was equilibrated with air at room temperature and pressure (19° , 76.8 cm. Hg) for 15 min. The relationship between current and voltage was then approximately recorded by the polarograph (curve A). Nitrogen (oxygen-free) was passed through the container and polarograms were obtained at intervals of about 20 min. as oxygen was removed from the apparatus. Curve B represents the current-voltage curve after passing nitrogen through the container for about 100 min. The experiment was continued for another hour with no further change in the current-voltage curve. Polarograph settings: sensitivity $\frac{1}{2}$ (80 mm. = $1 \mu\text{A}$); damping 2.

Fig. 3. Relationship between diffusion current and oxygen concn. For experimental details see text. The diffusion current was recorded during evacuation of the container (●), and again on admitting air (■). Polarograph settings: applied p.d. 0.65 v; sensitivity $\frac{1}{2}$ (200 mm. = $1 \mu\text{A}$); damping 1.

Fig. 4. Determination of respiration rates. For details see text. Curve 1: Chamber contained 1.0 ml. of water. At point (a) approximately 20 mg. of sodium dithionite was added. Curve 2: chamber contained 0.5 ml. of incubation medium with α -oxoglutarate described in Table 2. A suspension of rat-liver mitochondria (0.5 ml. in 0.25M-sucrose containing 140 mg. dry wt.) was added at point (b). Curve 3: as curve 2, except that mitochondrial suspension contained 23.3 mg. dry wt. Polarograph settings: applied voltage 0.65 v; sensitivity $\frac{1}{2}$ (200 mm. = $1 \mu\text{A}$); damping 2.

Table 1. *Comparison of the respiration rates of yeast suspensions measured with the oxygen electrode and by constant-volume manometry*

Experiment 1. Five manometer flasks were prepared containing 3.5 ml. of 0.135 M-KCl (with 1% of glucose) in the main compartment and 0.5 ml. of a 1% suspension of baker's yeast in 0.135 M-KCl (containing 1% of glucose) in the side arm. Successively at the stated times, the contents of each side arm were tipped into the main compartment and the flasks were incubated and readings taken every 5 min. for 30 min. at 20°. At the time of mixing the flasks, 0.1 ml. of the yeast suspension incubated at the same temperature was added to 0.7 ml. of 0.135 M-KCl (containing 1% of glucose) in the oxygen electrode chamber.

Experiments 2 and 3. At the stated times, 1.0 ml. of the incubated stock 1% suspension of yeast as above was added to 3.0 ml. of 0.135 M-KCl (containing 1% of glucose) in a manometer vessel and 0.2 ml. was added to 0.6 ml. of the same medium in the oxygen electrode chamber. Expt. 2 was carried out at 18° and Expt. 3 at 20°.

Expt. no.	Times of diluting stock suspension (min.)	O ₂ uptake/min./0.5 ml. of stock suspension		Rate with oxygen electrode Initial manometric rate
		Oxygen electrode (μl.)	Manometer (μl.)	
1	0	2.55	2.47	1.03
	10	2.55	2.81	0.91
	20	2.80	2.93	0.96
	30	3.22	3.38	0.95
	40	3.47	3.42	1.02
2	0	5.93	5.85	1.02
	10	6.55	5.81	1.12
	20	3.15	3.94	0.80
	30	3.10	3.25	0.95
3	0	7.51	7.20	1.04
	10	7.40	8.10	0.92
	20	5.92	5.40	1.10
	30	3.63	3.22	1.12

ing to an oxygen concentration of 4 μM was reached. Then the current fell at a progressively slower rate, becoming asymptotic as it approached the value for zero oxygen concentration. In practice, the amount of respiring tissue was adjusted to consume oxygen at the rate of at least 2 μl./min. (Fig. 4). With respiration of this order, sealing the chamber from the atmosphere did not affect the recorded rate of respiration. The respiration rate of the tissue was calculated from the linear fall in diffusion current during the first 2 min. after addition of the mitochondria and subsequent to the equilibration period. After the recording was completed the contents of the chamber were removed with absorbent paper. The chamber was washed out three times with water and left filled with incubation medium ready to measure the next respiration rate. In some experiments the respiring mitochondrial suspensions with substrate were added to the chamber, and the recording commenced after 1 min. The electrodes usually re-equilibrated with room air in 2 min. and the diffusion current returned to a value within 3% of that before the experiment. In this way an average of six determinations of respiration rates under different conditions could be completed within an hour. Duplicate determinations agreed within 10%.

EXPERIMENTAL AND RESULTS

Comparison of the respiration rates of yeast measured with the oxygen electrode and by constant-volume manometry

Chance & Sacktor (1958) have reported that the oxygen uptake of the mitochondria of insect flight-muscle is about 10 times as great when measured

with the vibrating platinum electrode as it is when measured with the Warburg manometer. Similarly, Leaf & Renshaw (1957) reported that the respiration of frog skin, when measured polarographically, was some 40% higher than that measured manometrically. Before such differences are accepted as significant it is necessary to demonstrate that the two methods do not always differ.

Yeast was chosen as the respiring material for comparing the two methods because it seemed desirable to use suspensions of live organisms rather than more labile homogenates or subcellular tissue fractions. Suspensions of yeast were incubated and samples removed after various times, and their respiratory rate was determined by the polarographic and manometric methods. The rates of respiration as measured in three experiments with slightly different experimental conditions are compared in Table 1.

The rates measured with the oxygen electrode were from -20% to +12% of those measured by the manometric method. Most measurements agreed within 10%. Further, in experiments with mitochondria which were incubated under the conditions used in manometry (see below) the two methods gave the same rate of respiration. Thus it appears that the tenfold difference measured by the two techniques, reported by Chance & Sacktor (1958), is due to a true difference in respiration because of different experimental conditions.

Comparison of the respiration rates of mitochondria measured with the oxygen electrode and by constant volume manometry

Chance & Sacktor (1958) have noted large differences in the rates of respiration of mitochondria when measured polarographically and manometrically. It therefore seemed worth while making a systematic comparison of respiration rates, with both methods, of mitochondria from different sources and testing the effect of different substrates. The results of this comparison are given in Table 2. The first manometric measurement was made after a 5 min. equilibration period and the initial rates were calculated from the rate between 5 and 10 min. incubation. The respiration rate after 40 min. incubation was between 10 and 50 % of that measured between 5 and 10 min., depending on the tissue and the substrate used.

For the polarographic measurements the mitochondrial suspension at 0° (0.5 ml.) was warmed to room temperature (1 min.) and added to 0.5 ml. of the incubation medium in the electrode chamber. The constant rate of respiration was measured over a 2 min. period. Table 2 shows that considerable discrepancies occurred between the methods. With rat liver, the values for the manometric method were uniformly lower than those obtained with the polarographic method. Except when malate or fumarate were substrates, respiration was 50–25 % lower. With rat-brain mitochondria, the manometric values were all higher than polarographic ones, the respiration in the manometer being usually four to five times that measured by the oxygen electrode. With pigeon-breast-muscle mito-

chondria, there was little difference in the two methods except where succinate was present, when the manometric measurements was seven times as high.

Changes in mitochondrial respiration in the early stages of incubation

The experiments in the previous section suggest that large changes in respiration rate occurred during the first few minutes of incubation in the manometers. This possibility was tested by incubating mitochondrial suspensions under conditions similar to those in the manometer vessels and during the first 5 min. incubation transferring samples to the oxygen-electrode chamber. Brain mitochondria in the presence of succinate were used since, as shown in Table 2, the respiration rate in the manometer was much greater than that measured polarographically.

A suspension of rat-brain mitochondria was prepared in the incubation medium described in Table 2, also containing 10 mM-succinate. The mitochondrial suspension was shaken 1 min. at 20° and a sample was transferred as rapidly as possible to a manometer vessel. The initial respiration rate was measured after a 5 min. equilibration period. At 5 min. a further sample of the stock mitochondrial suspension was transferred to a second manometer. This procedure was repeated at 5 min. intervals until five samples of the mitochondrial suspension had been transferred to manometers. Polarographic measurements of oxygen uptake were also made in a similar way.

The experiment was repeated with a preparation of rat-liver mitochondria. The respiration of both

Table 2. *Comparison of the respiration of mitochondria measured by constant-volume manometry and by the oxygen electrode*

Manometric measurements (M). Incubations were carried out at 20° in manometer vessels (total gas volume 5.5 ml.) containing 0.01 M-substrate, 0.01 M-potassium phosphate buffer, pH 7.4, 2 mM-MgCl₂. The tissue suspension (0.5 ml. in 0.25 M-sucrose containing 50 mg. wet wt.) was added to give a total fluid volume of 1 ml. The solute content was adjusted to 0.25 osmolar with sucrose solution. The centre well contained 0.05 ml. of 2N-NaOH with filter paper. Readings were taken every 5 min. after a 5 min. equilibration period and the respiration rate calculated from the initial rate between 5–10 min. incubation and expressed as Q_{O_2} (μ l. of O₂/mg. dry wt./hr.).

Polarographic measurements (P). Incubations were carried out in the polarograph cell under the same conditions as above. Total fluid volume was 1.0 ml. The mitochondrial suspension (0.5 ml.) was warmed to room temperature (1 min.) and added to 0.5 ml. of incubation medium in the electrode chamber. The constant rate of oxygen uptake was recorded over a period of 2 min. and expressed as Q_{O_2} .

		Mitochondrial respiration rate (Q_{O_2} values)								
Tissue ...	...	Rat liver			Rat brain			Pigeon breast		
		P	M	P/M	P	M	P/M	P	M	P/M
Substrate										
Nil		4.1	2.0	2.0	1.1	5.9	0.19	0.6	0.6	1
Citrate		4.3	3.2	1.3	2.2	11.4	0.19	1.7	1.7	1
α -Oxoglutarate		14.6	10.0	1.5	11.1	18.6	0.60	2.0	2.5	0.80
Succinate		23.5	13.1	1.8	3.9	16.7	0.23	1.8	15.0	0.12
Malate		9.7	9.1	1.1	3.7	7.3	0.51	1.4	2.2	0.64
Fumarate		7.7	6.4	1.2	2.9	9.4	0.31	—	—	—
Pyruvate		4.3	2.3	1.9	2.6	11.4	0.23	0.3	0.5	0.60

types of mitochondria (measured in the polarograph cell) increased greatly within the first 5 min. of incubation of the stock suspension (Fig. 5). There was a twofold increase in respiration with brain and a sixfold increase with liver. Thereafter the rate fell very quickly with liver mitochondria but more slowly with brain particles. The first manometric readings were taken between 5 and 10 min. after the stock suspensions had been transferred to the manometer vessels. The oxygen uptake was within 15% of that measured by the polarographic method at the same time. With the brain mitochondria the first manometric rate was about four times as high as that measured polarographically at zero time and three-quarters of the maximum rate after 5 min. incubation. With liver mitochondria the first manometric rate was 60% lower than that measured polarographically at zero

time and one-third of the maximum rate measured polarographically after 6 min. incubation. The stock suspension was incubated for 30 min. and during this time the initial respiration rate of the stock suspension of brain mitochondria transferred to the manometer vessels remained constant. The polarographic rates measured during this time were about 10% higher than the manometric ones. The initial respiration rates of rat-liver particles, measured manometrically, fell slowly between 10 and 30 min. incubation of the stock suspension. The polarographic rates remained constant so that at the end of the incubation the differences between the two methods was about 50%.

After each manometer had been incubated for 30 min. a sample of its contents was transferred to the empty polarographic cell and the respiration measured. At this stage, the rate of oxygen uptake was found to be within 10% of that measured immediately previously in the manometer.

The experiment was repeated with rat-liver mitochondria with α -oxoglutarate as substrate, both with and without adenosine diphosphate, and the same transient increase in respiration occurred. This suggests that the change in respiration is a general phenomenon for all substrates.

These results may be related to the spontaneous increase and decrease in mitochondrial respiration observed by Baltscheffsky (1957) for mitochondria in magnesium-deficient media. Such changes are complete within 10 min. and so cannot be detected by the usual manometric method with a 10 min. equilibration period.

Succinate oxidation in mitochondria pre-incubated without substrate

Fig. 5. Changes in the respiration of mitochondria during incubation. A suspension of rat-brain mitochondria was prepared in the incubation medium described in Table 2, also containing 10 mM-succinate. The suspension was stored at 0° for 10 min. to ensure that substrate was equilibrated with the mitochondria. Manometer vessels and polarograph cell contained the same medium and substrate as the mitochondrial suspension. The suspension was shaken for 1 min. at 20° and a sample was transferred as rapidly as possible to a manometer vessel which was attached to its manometer and shaken in a water bath at 20°. The manometer was read after 5 min. and again at 10 min. and the respiration rate was calculated from these readings. At 5 min. the transfer was repeated and this manometer was read after 10 and 15 min. incubation. This procedure was carried out until five samples of mitochondria had been transferred to the manometers. The experiment was repeated with rat-liver mitochondria suspended in the saline medium of Chappell & Perry (1954). The initial rates of respiration of rat-brain ($\square$) and rat-liver mitochondria ($\blacksquare$) between 5 and 10 min. incubation (expressed as Q_{O_2} values) have been plotted against the time that this rate was measured. Polarographic measurements of oxygen uptake were made at zero time and thereafter with fresh samples of the incubated stock suspension as fast as possible. The polarographic measurements are Q_{O_2} values (measured over a 2 min. period) for rat-brain mitochondria ($\circ$), and rat-liver mitochondria ($\bullet$) pre-incubated for various times.

In the previous section it was shown that the respiration of liver mitochondria at the end of 20–30 min. incubation was lower when particles were transferred to manometers for measurement of oxygen uptake than when measurements were made in the polarograph cell. Although the time of shaking and the proportions of mitochondria to medium were the same in both methods at the time of the test, the conditions during the measurement of respiration were not the same. The sample for the manometric procedure had to be transferred to the incubation medium 5 min. before the initial reading was taken and was thus shaken during this period at only one-quarter of the concentration present in the stock suspension. In contrast, the sample of mitochondria taken for the polarograph cell was diluted only for the 1 or 2 min. during which the measurements were made. The results of these experiments suggested that mitochondria were more susceptible to change when in a dilute suspension than when in a more concentrated one. When mitochondria are incubated without substrate

(usually referred to as aging) they swell and the rate at which they oxidize substrates changes (Siekevitz & Potter, 1955; Dickens & Salmony, 1956). Thus aged mitochondria would be expected to be more susceptible to the effects of shaking in manometer vessels. The possibility was tested as follows.

A stock suspension of rat-liver mitochondria in the saline medium already described, without oxidizable substrate, was prepared at 0°. This suspension was then incubated at 20° and samples were removed over a period of 30 min. and the oxygen uptake was measured by the two methods as in the previous experiments. The respiration rate was measured polarographically by adding a sample to the saline medium containing 10 mM-succinate and simultaneously a sample was placed in a manometer vessel (containing 10 mM-succinate) with the same proportion of medium.

The respiration of the mitochondria measured with the polarograph increased during the first

Fig. 6. Effect of pre-incubation without substrate on the respiration of rat-liver mitochondria in the presence of succinate. The mitochondria were incubated in the saline medium of Chappell & Perry (1954) at 20° with no substrate. Samples of the suspension were transferred to a manometer vessel containing succinate at zero time (○), and at intervals of 9 min. (●), 20 min. (□) and 30 min. (■) and the respiration of each sample was measured over a period of 30 min. after a 5 min. incubation period. The manometric measurements are Q_{O_2} values measured over successive 5 min. periods at the various times of incubation of the stock suspension. Samples of the suspension were also placed at intervals in the polarograph cell containing succinate and the respiration was measured (△). The polarographic measurements are Q_{O_2} measured over a 2 min. period of mitochondria pre-incubated for the various times stated.

15 min. of incubation of the stock suspension to about twice its starting value (Fig. 6) and remained at about this level for a further 35 min. As was expected, the initial rate of respiration measured in the manometer was higher than that measured at the same time in the polarograph cell (Fig. 6). What was not expected was that the divergence between the initial rate measured in the manometer and that measured in the polarograph cell would increase with time. After 40 min. incubation of the stock suspension the initial manometric rate had increased by about 100 %; so that at this time it was about 3.5 times the polarograph rate, whereas initially it had only been about twice as high. Although the initial oxygen uptake was greater in the mitochondria that had been pre-incubated for the longest time, the subsequent fall in respiration was much faster, so that after 20 min. all the manometric samples were respiring at nearly the same rate. It seems clear from these experiments that shaking mitochondria induces changes in the mitochondria which are progressive and that prolonged shaking in dilute suspension without substrate makes these changes more obvious. The reason for the progressive increase in initial oxygen uptake with time of pre-incubation is not clear. It is possible that the results are a reflexion of the increase in permeability and swelling of the mitochondria on incubation without substrate. Alternatively the results may mean that the initial transient rise in respiration (Fig. 5), when mitochondria are incubated with substrate, is progressively delayed, after incubation in the absence of substrate, so that it becomes measurable in the manometers (Fig. 6).

Effect of adenosine diphosphate on respiration

To test whether other properties of the particles depended on the incubation conditions a comparison of the rates of respiration of the mitochondrial suspensions, with and without adenosine diphosphate (ADP), was made by using both methods of measuring oxygen uptake. It was surprising to find (Table 3) that in some circumstances mitochondria did not respond to the addition of ADP, although the same mitochondria could be induced to respond to ADP by shaking in manometer vessels. Thus virtually no effect of ADP on respiration was obtained when the respiration was measured polarographically, whereas in most of the manometric experiments there was a large stimulation of respiration by ADP in manometer vessels which ranged between 7 and 400 % and could be increased a further two- to three-fold on the addition of apyrase.

The comparison of the manometric and polarographic results suggests that the permeability of the mitochondria to ADP is greater after shaking

Table 3. *Effect of adenosine diphosphate and apyrase on the respiration of mitochondrial suspensions*

The incubation conditions were the same as in Table 2. The figures are the Q_{O_2} values when 0.1 M-ADP was present in the medium, measured with the oxygen electrode (P) and by constant-volume manometry (M), and when in addition 0.1 ml. of apyrase (AP) (Lee & Eiler, 1951) was added in the manometric experiments.

Tissue	Substrate	Rat liver			Rat brain			Pigeon breast		
		P	M		P	M		P	M	
			- AP	+ AP		- AP	+ AP		- AP	+ AP
	Nil	4.2	2.0	2.0	1.1	6.1	6.1	0.3	0.3	0.3
	Citrate	4.2	5.9	7.8	2.1	13.6	19.4	1.8	6.8	20.0
	α -Oxoglutarate	14.5	16.4	18.5	11.1	22.2	28.8	2.0	8.4	21.0
	Succinate	23.3	14.1	17.4	4.1	21.5	23.1	2.2	24.0	25.5
	Malate	9.8	9.3	15.1	3.7	7.7	15.6	1.4	8.8	24.0
	Fumarate	7.3	11.2	18.8	2.9	13.9	25.4	—	—	—
	Pyruvate	4.5	3.5	6.4	2.6	13.6	16.7	0.3	1.0	3.2

Fig. 7. Effect of pre-incubation of rat-liver mitochondria on the stimulation of oxygen uptake by adenosine diphosphate. Curves 1 and 2: Fall in diffusion current when a mitochondrial suspension at 0° containing α -oxoglutarate was warmed to 20° and added to the polarograph cell (for composition of medium see Table 2). In curve 1, 1 ml. of suspension was added to 0.1 ml. of water and, in curve 2, to 0.1 ml. of 0.1 M-ADP (no effect of ADP before shaking). Curves 3 to 8: Fall in diffusion current when 1 ml. samples of a mitochondrial suspension shaken at 20° for various times were added to the polarograph cell containing 0.1 ml. of water or 0.1 ml. of 0.1 M-ADP. Curve 3, 1 min. incubation, no ADP. Curve 4, 3.5 min. incubation, plus ADP. Curve 5, 6 min. incubation, no ADP. Curve 6, 9 min. incubation, plus ADP. Curve 7, 12 min. incubation, no ADP. Curve 8, 16 min. incubation, plus ADP (development of ADP stimulation by shaking). Curves 9 and 10, after incubation the initial experiment (curves 1 and 2) was repeated with the same suspension stored at 0°. Curve 9, no ADP. Curve 10, plus ADP (stability of stored suspension). Polarograph settings: applied voltage 0.65 v; sensitivity $\frac{1}{2}$ (200 mm. = 1 μ A); damping 2.

controlling factor, since the respiration measured in the unincubated suspension increased on the addition of substrate.

There are differences in stimulation by ADP with different substrates. With rat liver and brain the smallest effect was obtained when malate was the substrate, whereas in the pigeon-breast muscle the largest stimulation (fourfold increase) was obtained in the presence of malate. The stimulation of the respiration in pigeon-breast-muscle mitochondria was the maximum effect observed with ADP alone in all the mitochondrial suspensions.

In the rat tissues, the stimulation when malate was the substrate was low but the stimulation with fumarate was high (e.g. 2 % increase with malate in rat liver but 75 % increase with fumarate). Bartley & Davies (1954) showed that particulate preparations from sheep kidney in the presence of ATP increased the concentration of fumarate to some seven times that in the medium. Thus the results with rat-liver and brain mitochondria may reflect the increased uptake of fumarate in the presence of ATP.

In the manometric experiments (Tables 2 and 3) the greatest stimulation of rat-liver mitochondria on incubation with ADP occurred in the presence of α -oxoglutarate. An experiment was made to ascertain whether the stimulation of respiration on the addition of ADP could be demonstrated in the polarograph cell. A mitochondrial suspension was shaken at 21° and at intervals the respiration rate of 1 ml. samples was measured in the polarograph cell. A portion of the suspension was stored at 0° (unincubated suspension) and its respiration was measured after warming to 21° (not shaken) both with and without ADP. Experiments were arranged so that the respiration in the presence of ADP could be measured between that of two samples without added ADP. The results show (Fig. 7) that ADP had no effect on the unincubated suspension, and that during the incubation the

the suspension in the manometers than it is in the original suspension, for no stimulation of respiration by ADP occurred in the unshaken suspension. It is likely that differences in permeability to ADP rather than to oxidizable substrates is the main

mitochondria changed from a state where ADP did not affect the respiration to a state where ADP stimulated the oxygen uptake between two- and four-fold.

DISCUSSION

Differences between the polarographic and manometric methods of measuring oxygen uptake

Both manometric and polarographic methods of measuring respiration have limitations which are emphasized by the experiments described in this paper. The equilibration time and the comparatively large amounts of tissue needed are the chief disadvantages of the manometric method, whereas the polarographic method is mainly limited by the amount of oxygen dissolved in the incubation medium. However, the main discrepancy between the two methods appears to result from shaking the mitochondria in a liquid medium, which apparently alters their response to changes in their external environment. This may be illustrated by the differences in response to adenosine diphosphate found with mitochondria before and after incubation in vessels which were shaken. In the latter experiments the respiration was stimulated by adenosine diphosphate, but no such effect was observed with uncubated mitochondria.

Changes in the respiration of mitochondria

Changes in the solute content of mitochondria have already been demonstrated by Siekevitz & Potter (1955) and Bartley & Amore (1958), who showed that mitochondria lost materials absorbing at 260 m μ , either on storage at 0°, or more rapidly on incubation. As judged by the phosphate levels in the medium in which the mitochondria are stored (see Werkheiser & Bartley, 1957; Amore & Bartley, 1958; and Bartley, unpublished work) an equilibrium is set up between the concentration of nucleotide in the medium and that in the mitochondria. The attainment of this equilibrium will be hastened by shaking, since diffusion will no longer limit the rate of transfer of nucleotides from the mitochondrial surface to the medium. The level of cofactors in the mitochondria will be lower in a dilute suspension than in a concentrated suspension, since relatively more of the cofactors will be lost in raising their level in the medium to the equilibrium value. In some instances the rate of respiration of mitochondria is dependent on the level of internal cofactors, because aged or water-treated mitochondria require addition of cofactors such as diphosphopyridine nucleotide and adenosine triphosphate for maximal rates of respiration. Further, the enzymes of washed mitochondria are not saturated with substrate, since the addition of

oxidizable substrate causes an increase in respiration. Therefore the rise and subsequent fall in respiration observed in the first few minutes when mitochondria were shaken with substrate can be explained if it is assumed that substrate penetrates to the enzyme sites at a faster rate than the endogenous cofactors leak out.

Intact and damaged mitochondria

Most workers believe that the mitochondria they use are 'intact' or 'undamaged' and they often tacitly assume that differences in behaviour of mitochondria prepared by others are due to the use of 'damaged' mitochondria. Various logical but often conflicting criteria have been applied to the behaviour of mitochondria to add support to an author's view that the mitochondria used in his experiments are 'intact'. Such criteria are the rate of respiration (Aldridge, 1957), the effect of added substrate (Ziegler & Linnane, 1958), the effect of added phosphate acceptor (Chance & Williams, 1955), the stimulation of respiration by dinitrophenol (Aldridge, 1957), the degree of 'respiratory control' (Chance & Baltscheffsky, 1958), the solute and water content (Werkheiser & Bartley, 1957), and the appearance under the electron microscope (Witter, Watson & Cottone, 1955). All these criteria, apart from the last, involve a consideration of the permeability of the mitochondrial membrane. Some authors believe that the mitochondrial membrane in 'intact' mitochondria is permeable and others that it is impermeable. In our experiments a comparatively gentle procedure such as the type of shaking which occurs in the manometer vessel can change the mitochondria from a state that some authors would consider 'damaged' (lack of response to adenosine diphosphate) to an 'undamaged' state (responding to adenosine diphosphate). The change in permeability which could account for this alteration in behaviour may be related to the controlling mechanism which governs the behaviour of mitochondria in the intact cell.

If it is conceded that a change in the permeability of the mitochondrial membrane reflects the state of metabolism of these particles, the terms 'damaged' and 'intact' become meaningless. Thus it is possible that the whole range of mitochondrial preparations of different workers, from the 'tightly coupled' to the 'uncoupled', are all 'intact' and 'undamaged', in the sense that they represent possible states of the mitochondria within the cell. We feel that the term 'damaged' as applied to mitochondria should be reserved for the cases where it is known that the mitochondria have been physically disrupted into fragments or have been treated so that they lose their soluble enzymes.

SUMMARY

1. A simple cell for measuring the respiration of tissue suspensions polarographically is described, in which a stationary platinum electrode and a silver electrode are shielded from the incubation medium by a polythene membrane.

2. Q_{O_2} values at 20° for mitochondria from rat liver, rat brain, and pigeon-breast muscle in the presence of citric acid-cycle intermediates, and for yeast in the presence of glucose, have been measured both manometrically and by the polarographic method.

3. Whereas yeast suspensions showed the same respiration when measured under these different conditions, the mitochondrial suspensions showed differences of two- to three-fold. For instance, when rat-liver and -brain mitochondria at 0°, suspended in a medium containing succinate, were warmed to 20° and incubated, the respiration (as measured by the polarographic method) rose during the first 3 min. to values two to five times those measured initially (maximum Q_{O_2} values of 30 and 15 respectively). Subsequently the respiration rate fell and after 6 min. reached a steady value roughly corresponding to the initial value. This steady rate of respiration corresponded with the initial rate measured manometrically.

4. The respiration of mitochondria shaken in the manometer vessel was stimulated by the addition of adenosine diphosphate. Maximum increases were rat liver 84%, rat brain 52% and pigeon-breast-muscle 400%. The oxygen uptake with adenosine diphosphate was usually increased a further two- to three-fold on the addition of apyrase. No stimulation by adenosine diphosphate occurred when respiration was measured in the polarograph cell unless the suspension had previously been shaken.

5. It is suggested that shaking a mitochondrial suspension is a procedure which allows a rapid equilibration of intramitochondrial solutes with solutes in the incubation medium. The effects of shaking are more pronounced when the mitochondrial suspension is dilute and when substrate is omitted.

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REFERENCES

- Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
 Amooore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
 Baltscheffsky, H. (1957). *Biochim. biophys. Acta*, **25**, 382.
 Bartley, W. & Amooore, J. E. (1958). *Biochem. J.* **69**, 348.
 Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
 Bellamy, D. (1959). *Biochem. J.* **72**, 165.
 Chance, B. & Baltscheffsky, M. (1958). *Biochem. J.* **68**, 283.
 Chance, B. & Sacktor, B. (1958). *Arch. Biochem. Biophys.* **76**, 509.
 Chance, B. & Williams, G. R. (1955). *Nature, Lond.*, **175**, 1120.
 Chappell, J. B. & Perry, S. V. (1953). *Biochem. J.* **55**, 586.
 Chappell, J. B. & Perry, S. V. (1954). *Nature, Lond.*, **173**, 1094.
 Clark, L. C. (1956). *Trans. Amer. Soc. artif. intern. Org.* **2**, 41.
 Connelly, C. M. (1957). *Fed. Proc.* **16**, 681.
 Dickens, F. & Salmony, D. (1956). *Biochem. J.* **64**, 645.
 Harris, H. & Barclay, W. R. (1955). *Brit. J. exp. Path.* **36**, 592.
 Leaf, A. & Renshaw, A. (1957). *Biochem. J.* **65**, 82.
 Lee, K. H. & Eiler, J. J. (1951). *Science*, **114**, 393.
 Longmuir, I. S. (1954). *Biochem. J.* **57**, 81.
 Petering, G. H. & Daniells, F. (1938). *J. Amer. chem. Soc.* **60**, 2796.
 Reeves, R. B., Rennie, D. W. & Pappenheimer, J. R. (1957). *Fed. Proc.* **16**, 693.
 Siekevitz, P. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 221.
 Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
 Witter, R. F., Watson, M. L. & Cottone, M. (1955). *J. biophys. biochem. Cytol.* **1**, 127.
 Ziegler, D. M. & Linnane, A. W. (1958). *Biochim. biophys. Acta*, **30**, 53.