Reconstruction of the Succinic Oxidase System from Two Preparations, One Deficient in Succinic Dehydrogenase, the Other Deficient in Cytochrome Oxidase

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The best material for the study of the succinic oxidase system, which is bound to the insoluble structural elements of the cell, is the cell-free heart muscle preparation of Keilin & Hartree (1947). It consists probably of fragmented sarcosomal membranes (Cleland & Slater, 1953). Preparations of similar nature can be obtained from a variety of aerobic cells. It is believed that each particle contains all the catalysts necessary for the oxidation of succinate, but to what extent the enzymes present in one particle can react with enzymes of another particle in the course of hydrogen transfer from succinate to oxygen is not known. One way to study this problem of interaction of particles is to mix two preparations, each one inactive in the oxidation of succinate by being deficient in a different component of the enzyme system, thus trying to reconstruct the complete succinic oxidase system. Although such preparations have been obtained in the past (Keilin & Hartree, 1940), the treatments used to destroy some of the enzymes (for instance incubation with 45% aqueous ethanol for 10 min. to destroy cytochrome oxidase) undoubtedly modify the colloidal structure. It is, therefore, not surprising that the attempts made in this direction have so far failed.

While the classical succinic oxidase system, as in heart muscle, is found in wild type *Neurospora crassa*, preparations from the mutant strains designated *C 117* and *poky* are deficient in succinic oxidase and cytochrome oxidase activities, though succinic dehydrogenase is as active as in the wild type (Haskins, Tissières, Mitchell & Mitchell, 1953; Tissières & Mitchell, 1954). On the other hand, a heart muscle or wild-type *Neurospora* preparation deficient in succinic dehydrogenase can easily be obtained by incubation at alkaline pH (Keilin & Hartree, 1940).

In this paper, spectroscopic and manometric experiments on the succinic oxidase system as reconstructed from these two preparations, one deficient in cytochrome oxidase, the other deficient in succinic dehydrogenase, will be described and discussed.

MATERIAL AND METHODS

The origins of the *Neurospora* strains *poky* and *C 117* have been described by Mitchell & Mitchell (1952) and Mitchell, Mitchell & Tissières (1953), respectively. The mould was cultivated in aerated liquid medium (Haskins *et al.* 1953) and the enzyme preparation isolated according to Tissières & Mitchell (1954). Heart muscle preparation was made from horse heart according to the procedure of Keilin & Hartree (1947). Preparations deficient in succinic dehydrogenase were obtained as described by Keilin & Hartree (1940) with the following modifications: it was found necessary to bring the pH to 9·3–9·4 with 0·1 N-NaOH and let the preparation stand for 75 min. at room temperature (18–20°) to destroy succinic dehydrogenase activity almost completely. With wild-type *Neurospora*, the same result was obtained by incubation at pH 9·1–9·2 for 60 min. The preparations were neutralized by addition of 0·2 M-KH_2PO_4. Estimations of cytochrome oxidase, succinic oxidase and succinic dehydrogenase were carried out in Barcroft differential manometers at 35°, in a reaction mixture including the enzyme, of total volume 3 ml., with 0·075 M phosphate buffer, pH 7·0.

Cytochrome oxidase. The manometric flask contained 4×10^{-5} M cytochrome *c* and, in a dangling tube, neutralized ascorbic acid (5%, w/v, 0·3 ml.). The dangling tube was dislodged after temperature equilibration, at zero time.

Succinic oxidase. The dangling tube contained 0·3 ml. 0·4 M succinate; cytochrome *c* was omitted in some experiments.

Succinic dehydrogenase. The flask contained no cytochrome *c*, but 0·001 or 0·002 M methylene blue and 0·003 M cyanide were present, and the dangling tube contained 0·3 ml. 0·4 M succinate.

The cytochrome spectrum was examined by means of a Zeiss microspectroscope mounted on a microscope, as described by Keilin & Hartree (1946). Fat-free dry weight was obtained according to the procedure used by Slater (1949).

RESULTS

Spectroscopic observations

C117 *preparation.* The cytochrome system in *C 117* preparations has been studied in some detail by Tissières & Mitchell (1954). On addition of a small amount of succinate, the absorption bands of cytochromes *b* and *e*, the only components found in

this strain of *Neurospora*, became quite strong. Their intensity was not modified by aeration, which indicated that these components could not be oxidized by air. Exogenous oxidized cytochrome *c*, added to the *C 117* preparation, was reduced at once in the presence of succinate.

Poky *preparation*. A study of cytochrome in this strain was made by Haskins *et al.* (1953). The high concentration of cytochrome *c* was mostly in the reduced form, since its strong α-band was only slightly intensified on addition of succinate. The bands of cytochromes *b* and *a* were not visible. Cytochrome *c* could not be oxidized by aeration.

Alkali-treated heart muscle and wild-type Neurospora *preparations*. Cytochromes *a*, *b* and *c* were present in the oxidized form and their absorption bands were hardly visible. They remained oxidized on addition of succinate, both in the absence and in the presence of exogenous cytochrome *c*; they were reduced by sodium dithionite.

Reconstruction of the succinic oxidase system. In the following experiments, the absorption bands of cytochrome, in either *C 117* or *poky* preparations, were observed after the addition of a very small amount of alkali-treated preparation from heart muscle or wild-type *Neurospora*. The amount of alkali-treated preparation was such that even on addition of sodium dithionite the absorption bands of its cytochrome were not visible under the spectroscope, and thus did not modify the spectrum of *C 117* or *poky* preparations. For instance, 0·05 ml., 10 times diluted alkali-treated heart muscle, or 4 times diluted alkali-treated wild-type *Neurospora* preparation, were added to 0·4 ml. *C 117* or *poky* preparation and 0·05 ml. 0·2 M sodium succinate in a flat-bottomed glass tube of diameter 14 mm., giving a depth of about 3 mm.

The addition of alkali-treated heart muscle or wild-type *Neurospora* preparation, in the presence of a small amount of succinate (0·02 M), to a *C 117* preparation did not modify the absorption spectrum of the latter, even after aeration. However, when exogenous oxidized cytochrome *c* was added (e.g. 0·05 ml. 2×10^{-4} M cytochrome *c* in a reaction mixture of 0·5 ml.), it was reduced at once, and intermittent aeration of the mixture led to oxidation and subsequent reduction of cytochromes *b* and *e* belonging to *C 117*, and of exogenous cytochrome *c*.

On addition of a small amount of alkali-treated heart muscle or wild-type *Neurospora* preparation to the *poky* preparation in the absence of succinate, the strong cytochrome *c* band of the latter disappeared at once, thus showing that the endogenous cytochrome *c* of the *poky* particles had been oxidized by the oxidase of heart muscle or wild-type *Neurospora* particles. In the presence of succinate alone (0·02 M), cytochrome *c* remained reduced on shaking in air, but with succinate and 0·01 M malonate, the absorption band of cytochrome *c* disappeared almost completely on aeration, and slowly came back on standing, which showed that the succinic oxidase system had been reconstructed.

The possibility that the two kinds of particles from *poky* and from alkali-treated preparation had reacted together because of the presence of soluble cytochrome *c*, extracted from the *poky* particles in the suspending buffer, was examined in the following way. A *poky* preparation was made up as usual (Tissières & Mitchell, 1954) but was washed 3 times in buffer by high-speed centrifuging, instead of once. To 5 ml. of this preparation, 0·15 ml., 10 times diluted alkali-treated heart muscle, 0·25 ml. 0·4 M succinate and 0·05 ml. M malonate, were added, and after the spectroscopic observations, the mixture was centrifuged at high speed and the clear supernatant was examined spectroscopically in a long flat-bottomed tube at a depth of 12 cm., after addition of sodium dithionite. No cytochrome *c* was visible under these conditions which allow the detection of 5×10^{-8} M cytochrome *c*. The supernatant was also examined for its ability to replace cytochrome *c* in a succinic oxidase system built up from *C 117* and alkali-treated heart muscle preparations, but it was found to be ineffective. The fact that it was ineffective showed that the supernatant did not contain any significant amount of cytochrome *c*. It is therefore not likely that the reaction between the two kinds of particles, from *poky* and alkali-treated preparations, was due to the presence of a small amount of soluble cytochrome *c*.

Manometric experiments

Effect of alkali treatment. With both sources of enzymes, heart muscle or wild-type *Neurospora*, alkali treatment abolished succinic dehydrogenase activity almost completely, while cytochrome oxidase activity retained roughly half its original value. The results of a typical experiment are presented in Table 1.

Reconstruction of the succinic oxidase system. As shown in Fig. 1, the succinic oxidase system was reconstructed by mixing two inactive preparations, one from *C 117* providing succinic dehydrogenase, the other (alkali-treated wild-type *Neurospora*) providing cytochrome oxidase. The system was only active in the presence of exogenous cytochrome *c* and the activity was proportional to the concentration of this substance. In this experiment, the oxygen uptake did not increase when 0·3 ml. *C 117* preparation was used instead of 0·2 ml., which showed that the cytochrome oxidase of the alkali-treated wild-type preparation was the limiting factor. Under these conditions, the same amount of alkali-treated wild-type *Neurospora* preparation took up about 15 % less oxygen when

Table 1. *Succinic oxidase and cytochrome oxidase activities of alkali-treated and untreated heart muscle and wild-type* Neurospora *preparations*

	$-Q_{O_2}$			
	Heart muscle preparation		Wild-type *Neurospora* preparation	
	Untreated	Treated at pH 9·4	Untreated	Treated at pH 9·2
Succinic oxidase	405	6	63	0
Cytochrome oxidase	1120	520	102	62

Succinic oxidase determinations: each flask contained 4×10^{-5} M cytochrome *c*, 0·04 M Na succinate in 0·075 M phosphate buffer, pH 7·0, and the enzyme. Total volume 3·0 ml.

Cytochrome oxidase: 4×10^{-5} M cytochrome *c*, 0·5% (w/v) Na ascorbate, 0·075 M phosphate buffer, pH 7·0, and the enzyme. Total volume 3·0 ml. Gas phase air; 35°. Values of O_2 uptake as μl./mg. fat-free dry wt./hr.

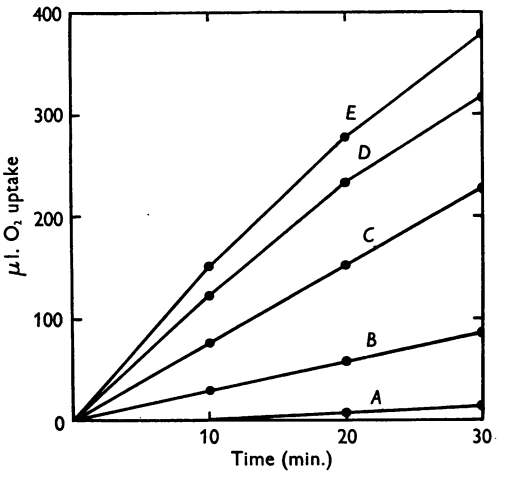

Fig. 1. Reconstruction of the succinic oxidase system by mixing, in presence of varying amounts of cytochrome *c*, two inactive preparations: one from the *Neurospora* strain *C 117* providing succinic dehydrogenase, the other, an alkali-treated wild-type preparation (AWT), providing cytochrome oxidase. The estimations of succinic oxidase activity (curves *A*, *B*, *C* and *E*) were carried out in Barcroft manometers, as described in Methods with 0·2 ml. *C 117* preparation and 0·3 ml. AWT, in the presence of the following concentrations of cytochrome *c*. Curve *A*: no cytochrome *c* added; curve *B*: 4×10^{-6} M; curve *C*: 1×10^{-5} M; curve *E*: 4×10^{-5} M. Curve *D* represents the estimation of cytochrome oxidase activity of 0·3 ml. AWT, as described in the text.

cytochrome oxidase activity in the presence of ascorbate and the same amount of cytochrome *c* was being measured.

In the experiments reported in Tables 2 and 3, the succinic oxidase system was reconstructed by mixing an alkali-treated heart muscle preparation with the *C 117* enzymes. Alkali-treated heart muscle was added in small excess, so that the *C 117* preparation was limiting. The activities of *C 117*, in terms of succinic dehydrogenase and of its ability to reconstruct the succinic oxidase system, were compared with the succinic dehydrogenase and succinic oxidase activities of the wild type (Table 3). In the case of the reconstructed system (with *C 117* and heart muscle preparation), as in the case of the wild type, the succinic dehydrogenase activity was found to be about 50–60% (depending on the concentration of methylene blue) of that of the succinic oxidase system. It should be noted that the measurement of succinic dehydrogenase activity in manometers in presence of methylene blue and cyanide is only a relative one, as it is limited by the concentration of methylene blue (Slater, 1949). The oxygen uptake given by these measurements should not therefore be compared directly with that of the succinic oxidase activity.

Poky preparations, in the presence or in the absence of added cytochrome *c*, were found to be completely ineffective in replacing the *C 117* enzyme in manometric experiments on the reconstruction of the succinic oxidase system. There is evidence that this is due to the presence in *poky* of an inhibitor. Thus, the succinic oxidase activity of either untreated wild-type or heart muscle preparations, or that of the reconstructed system with *C 117* and alkali-treated enzymes, was found to be almost completely inhibited by the addition of a small amount of *poky* preparation. That the effect of this inhibitor was not observed in spectroscopic experiments on the reconstructed system is not inconsistent with the manometric results. On the one hand, the spectroscopic method is much more sensitive than the manometric and, on the other hand, the initial reading in manometric experiments takes place several minutes after the two types of particle have been mixed, that is after the postulated inhibitor has had time to act. By contrast, the reaction observed with the spectroscope is practically instantaneous.

Table 2. *Reconstruction of the succinic oxidase system with alkali-treated heart muscle and* C 117 *preparations*

	O_2 taken up in 10 min. (μl.)			
			Succinic dehydrogenase	
	Cytochrome oxidase	Succinic oxidase	0·001 M methylene blue	0·002 M methylene blue
Alkali-treated heart muscle	172	0	0	—
C 117	—	7	75	85
Alkali-treated heart muscle + *C 117*	—	145	—	—

Cytochrome oxidase and succinic oxidase determinations as in Table 1. Succinic dehydrogenase: each flask contained 0·04 M succinate, 0·003 M cyanide and 0·001 or 0·002 M methylene blue in 0·075 M phosphate buffer pH 7·0, and the enzyme. Total volume 3·0 ml.

Table 3. *Activities of a* C 117 *preparation in the reconstruction of the succinic oxidase system, and of its succinic dehydrogenase, compared with the activities of the same enzymes in the wild type*

	$-Q_{O_2}$				
		Succinic dehydrogenase		Succinic dehydrogenase as % of succinic oxidase	
	Succinic oxidase	0·001 M methylene blue	0·002 M methylene blue	0·001 M methylene blue	0·002 M methylene blue
C 117	164 (Reconstructed system with alkali-treated heart muscle preparation)	85	98	52	60
Wild type	138	70	83	51	60

For experimental conditions see Tables 1 and 2.

DISCUSSION

The results of spectroscopic experiments show that *C 117* particles (which have normal succinic dehydrogenase, are deficient in cytochrome oxidase and lack cytochrome *c*) were unable to react with cytochrome oxidase from heart muscle or wild-type *Neurospora* preparations to reconstruct the succinic oxidase system, unless exogenous cytochrome *c* was present. *Poky* particles, on the other hand, which also have normal succinic dehydrogenase activity and are deficient in cytochrome oxidase, but contain a high concentration of cytochrome *c*, were found to react directly with cytochrome oxidase from heart muscle or wild-type preparations. Thus the α-band of reduced cytochrome *c* in *poky* preparations was not modified by aeration but it disappeared at once on addition of a small amount of heart muscle or wild-type enzyme; in the presence of 0·02 M succinate and 0·01 M malonate, cytochrome *c* was oxidized on aeration and subsequently became reduced on standing. This suggests that in the absence of a soluble component the succinic oxidase system had been reconstructed from two different types of particle. It is unlikely that the reaction was due to a small amount of cytochrome *c* extracted from the particles into the suspending buffer, as this substance was not found in the supernatant after centrifuging down the particles at high speed, and as the supernatant was ineffective in replacing cytochrome *c* in spectroscopic experiments to reconstruct the succinic oxidase system from *C 117* and alkali-treated preparations.

The fact that manometric experiments did not confirm spectroscopic observation with *poky* particles has already been explained by the inhibition that a *poky* preparation produced in the succinic oxidase system. On the other hand, the sensitivity of spectroscopic experiments performed with thick suspensions of particles is very much greater than that of manometric techniques. It is therefore not surprising that the effect noticed with the spectroscope was not detected while measuring the oxygen uptake of the succinic oxidase system. Furthermore, it is obvious that interaction of particles is a much slower process in the absence than in the presence of a soluble component, cytochrome *c*, acting as a link.

The study of the activity of the reconstructed system with *C 117* and heart muscle enzymes (Table 2) suggests that when a suitable amount of preparation from each source was used, the oxygen taken up was of the order expected from measurements of the full activity of succinic dehydrogenase in *C 117* and cytochrome oxidase in heart muscle preparations. In this connexion it is interesting to note that in Fig. 1 the amount of oxygen taken up by the reconstructed succinic oxidase system exceeded that of the alkali-treated preparation alone in presence of ascorbate.

SUMMARY

1. The succinic oxidase system was reconstructed, in presence of exogenous cytochrome *c*, from two inactive preparations, one, from the *Neurospora* strain *C 117*, providing succinic dehydrogenase, the other from alkali-treated wild-type *Neurospora* or heart muscle preparations, providing cytochrome oxidase.

2. It was found in spectroscopic experiments that particles from the *Neurospora* strain *poky* reacted directly with wild-type or heart muscle particles to reconstruct the succinic oxidase system in the absence of a soluble component.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

3. THE DISSOCIATIONS OF THE MAGNESIUM SALTS OF PHOSPHORIC ACID, GLUCOSE 1-PHOSPHORIC ACID AND GLYCEROL 2-PHOSPHORIC ACID

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A considerable body of evidence exists indicating that the magnesium salts of many divalent acids such as sulphate (Jones & Monk, 1952), malonate (Evans & Monk, 1952), orthophosphate (Greenwald, Redish & Kibrick, 1940), glycerol 1-phosphate (Tabor & Hastings, 1943) and glucose 1-phosphate (Trevelyan, Mann & Harrison, 1952) are incompletely dissociated in aqueous solution. Only in the cases of magnesium sulphate and magnesium malonate have the thermodynamic dissociations been measured at several temperatures and the thermodynamic quantities associated with the dissociations calculated. As magnesium is known to activate a number of enzyme systems concerned with phosphate metabolism, it is of interest to obtain detailed information about the dissociations of the magnesium salts of those phosphoric acids for which the thermodynamic acid dissociation constants are known.

THEORY

Jones & Monk (1952) have pointed out that the addition of magnesium or other salts of incompletely dissociated acids to cells without liquid junction of the type Pt, H_2 | HCl | AgCl, Ag will yield information concerning the dissociation of these salts. Accordingly phosphate or phosphate ester buffers containing $MgCl_2$ were examined potentiometrically in cells without liquid junction of the type

Pt, H_2 (1 atm.) | MHA (m_a). M_2A (m_b). $MgCl_2$(m_s) | AgCl. Ag,

where M is an alkali metal.

The e.m.f. of such a cell is given by

$$E = E_0 - \frac{2{\cdot}3026RT}{F} \log a_{H^+} . a_{Cl^-} . \qquad (1)$$

From this equation in terms of molalities and the approximation for the activity coefficients proposed by Güntelberg (1926), the use of which has been discussed previously (Ashby, Crook & Datta, 1954*a*) we have:

$$\log m_{H^+} = -\frac{(E - E_0)\,F}{2{\cdot}3026RT} - \log m_{Cl^-} + \frac{2AI^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}} . \qquad (2)$$

This equation, together with equation (3), the expression for the dissociation constant K_a of the acid HA^-:

$$-\log K_a = -\log \frac{m_{H^+} . m_{A^{2-}}}{m_{HA^-}} + \frac{4AI^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}} \qquad (3)$$

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