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Comparison of Polarographic and Spectrophotometric Assays for Cytochrome c Oxidase Activity*

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The rate of oxygen uptake measured polarographically in the system: ascorbate → cytochrome c → cytochrome c oxidase → oxygen was compared with one-fourth the initial rate of oxidation of ferrocytochrome c measured spectrophotometrically in the system: ferrocytochrome c → cytochrome c oxidase → oxygen, using identical mixtures except for the presence of ascorbate in the polarographic measurements. In contrast with the conclusions of other investigators, our data show that the two methods can be brought into agreement when certain conditions are fulfilled. Thus, it is possible to use the polarographic (or manometric) method for assaying cytochrome c oxidase activity without measurable interference from ascorbate.

Cytochrome c oxidase activity is usually assayed (a) by measuring the rate of oxidation of soluble ferrocytochrome c (spectrophotometric method) or (b) by measuring the rate of oxygen uptake when the cytochrome c is continuously reduced by an appropriate reducing agent such as ascorbic acid[1] (polarographic or manometric method). The following equations summarize the passage of electrons in the two systems:

(a) ferrocytochrome c → cytochrome c oxidase → O_2
(b) reducing agent → cytochrome c → cytochrome c oxidase → O_2

Reaction (a) is first order with respect to ferrocytochrome c, but the rate constant varies with the total concentration of cytochrome c in the reaction mixture (Smith and Conrad, 1956).

If the reduction of cytochrome c by ascorbate in system (b) is rapid compared to the rate of oxidation of ferrocytochrome c by the oxidase, the cytochrome c should be nearly completely reduced during the reaction, and the rate of oxygen uptake in system (b) should be equal to one-fourth the initial rate of oxidation of ferrocytochrome c in system (a) when the total concentration of cytochrome c is the same.

Several studies indicate that the above condition does not hold in the reaction mixtures usually employed with system (b) (see Minnaert, 1961; Yonetani, 1962). Thus we have tested the variables concerned to ascertain whether conditions could be found where the rate of oxygen uptake in system (b) is the same as the calculated corresponding rate in system (a). The data show that the two rates are the same when the concentrations of ascorbic acid, cytochrome c, and enzyme are adjusted so that the cytochrome c is more than 85% reduced during the reaction and provided that there has been no aggregation of the particulate oxidase preparations.

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[1] The advantages of ascorbic acid have been discussed by Minnaert (1961) and Yonetani (1962).

Methods and Reagents

Enzyme Preparations.—As a source of cytochrome c oxidase, the insoluble membrane fragments bearing the respiratory chain system were prepared from beef heart according to the method of Keilin and Hartree (1947), modified by disintegrating the mince in a Waring Blendor (Chance, 1952). Some preparations of particles were made deficient in cytochrome c by extracting the mince with buffer as described by Tsou (1952). Beef heart mitochondria were prepared by the method of Crane *et al.* (1956).

Cytochrome c was either prepared from beef heart by the Keilin-Hartree (1945) and Margoliash (1954) procedures, or was purchased from the Sigma Chemical Co. (type III). The cytochrome c was reduced with hydrogen and palladium, as previously described (Smith, 1954). The total concentration of cytochrome c was assessed from the optical density at 550 mμ of the compound reduced with $Na_2S_2O_4$, using 27.6 as the mM extinction coefficient (Margoliash, 1954).

Ascorbate.—A solution of ascorbic acid (Fisher reagent grade) was neutralized to *p*H 7.0 with NaOH and made 0.001 M with EDTA and 0.5 M with respect to ascorbate. This mixture was either used immediately or stored in the frozen state; in either case the same results were obtained.

Spectrophotometric Assay of Cytochrome c Oxidase.—The assay was performed at 25° in 0.05 M buffer made from a mixture of NA_2HPO_4 and KH_2PO_4 as described by Smith (1954), the first-order rate constant being calculated in each case. The initial rates were then obtained by multiplying the rate constant by the concentration of cytochrome c.

Polarographic Measurement of Cytochrome c Oxidase Activity.—The rate of oxygen uptake was measured with the Clark oxygen electrode (Clark, 1956) calibrated for each experiment with buffer saturated with air at 25°. The reagents were incubated in a bath at 25°;

TABLE I

COMPARISON OF CYTOCHROME OXIDASE ACTIVITY MEASURED SPECTROPHOTOMETRICALLY AND WITH OXYGEN ELECTRODE[a]

Preparation	μMolar O_2 Uptake sec^{-1}/mg Protein in Test Mixture (a) Calcd. from Rate of Oxidation of Cytochrome c	(b) Measured in O_2 Electrode
Keilin-Hartree[b]	0.74	0.71
	0.72	0.76
	0.91	0.93
	1.39	1.27
	0.96	0.96
Tsou-type	0.86	0.91
Beef-heart mitochondria	2.10	2.10

[a] Ascorbate conc. = 33 or 50 mM; temperature = 25°.
[b] The data with Keilin-Hartree preparations were obtained with five different preparations. The concentration of cytochrome c ranged from 19 to 33 μM in the test mixture.

there was no measurable temperature change during the time of the measurement of the rate of oxygen uptake.

The oxygen electrode was fitted into a plastic cup so that only a small opening was exposed to the air. This insured that air was not pulled into the reaction mixture, which was stirred by means of a small Teflon-coated bar magnet. The recorded rates of oxygen uptake were never in excess of 1 μM oxygen/second.

The oxygen uptake rates are expressed as μM oxygen decrease/second divided by the total protein in the reaction mixture, which was always 3 ml. (These figures multiplied by 242 give the rates as Q_{O_2}).

For comparison of the two methods, the oxygen uptake was measured with exactly the same mixture of buffer, cytochrome c, and oxidase-bearing particles as in the spectrophotometric method; the only difference was the presence of ascorbate in the polarographic method.[2] The rate of autooxidation of ascorbate in the presence of cytochrome c was sometimes immeasurably small; when it was measurable, it was subtracted from the rate in the presence of the oxidase. Replicate determinations with the polarographic method agree to within 5%, and these measurements were found to be in agreement with duplicates run with the usual Warburg manometry.

Since cytochrome c showed a tendency to adhere to the membrane of the oxygen electrode, the electrode was cleaned by suspension in HCl (around 0.5 N) for several minutes after each determination.

Measurement of the Extent of Reduction of Cytochrome c During the Reaction.—The extent of reduction of cytochrome c in a polarographic reaction mixture was measured by recording the optical density at 550 mμ of an identical mixture while it was aerobic, and again after the oxygen in solution had been exhausted. The blank cuvet contained the identical mixture except for the cytochrome c. The per cent reduction of the cytochrome c in the steady state is equal to

$$\frac{(27.6\ OD_{aerobic} - 9.1\ OD_{anaerobic}) \times 100}{(27.6 - 9.1)(OD_{anaerobic})}$$

[2] The increase in ionic strength resulting from this concentration of ascorbate did not affect the rate significantly, as shown by adding an equivalent concentration of NaCl at an ascorbate concentration giving a nearly maximal rate. Further increases in ionic strength, however, resulted in a decrease in rate.

TABLE II

EFFECT OF HOMOGENIZATION ON CYTOCHROME OXIDASE ACTIVITY ASSAYED SPECTROPHOTOMETRICALLY AND POLAROGRAPHICALLY[a]

Preparation	Cytochrome c (μM)	μMolar O_2 Uptake sec^{-1}/mg Protein in Test Mixture Spectrophotometrically	O_2 Electrode
Keilin-Hartree	21 (Sigma)	0.37	0.52
two weeks old	21 (K-H, Margoliash)	0.44	0.64
Same prep. rehomogenized	20	0.73	0.71

[a] Ascorbate conc. = 33.3 mM; temperature = 25°.

Protein Determination.—The protein content of the suspensions of heart muscle particles and mitochondria was measured with the biuret reaction (Gornall *et al.*, 1949), in the presence of 0.1% deoxycholate. An equivalent concentration of sucrose was added to the blank when measuring the protein content of mitochondria.

RESULTS

Table I compares the measured rates of oxygen uptake in system (b) with the corresponding rates calculated from the initial rates of oxidation of cytochrome c in system (a). The reaction mixtures were identical in the two assays, except for the addition of ascorbate in the polarographic method.[2] Under the conditions used there is quite good agreement. These conditions were such that the cytochrome c was more than 85% reduced during the reaction. This extent of reduction was obtained with final concentrations of 33 mM ascorbate, cytochrome c between 7 and 50 μM, and heart muscle particles containing around 0.05 μM cytochrome a_3 or less (see following paper).

Occasionally heart muscle preparations which had been stored at 4° for several days gave rates of oxygen uptake in the polarographic method greater than the corresponding calculated rates from the spectrophotometric assay. However, when the particle suspensions were redispersed by treatment with a Teflon homogenizer, the rates obtained with the two methods fell into agreement. The difference in rates thus could result from clumping of the particles on storage in the cold, then dispersal of the clumps by rapid stirring in the polarographic method but not in the cuvet during the spectrophotometric measurements. Illustrative data are found in Table II, which also shows that the rates obtained with a commercial preparation of cytochrome c are somewhat lower than those with cytochrome c prepared by the Keilin-Hartree, Margoliash procedures. The heart muscle preparations described in Table I were either freshly prepared or rehomogenized immediately before assaying. The addition of phospholipid sols, prepared as described by Wharton and Griffiths (1962), to the reaction mixture did not affect the oxidase activity of these preparations when measured with the polarographic method.

Figures 1 and 2 show the changes in rate of oxygen uptake in the polarographic method with variations in the concentrations of cytochrome c and ascorbate with constant oxidase. The data are plotted according to the method of Lineweaver and Burk (1934). In these experiments the rate of oxygen uptake showed a linear relationship to the amount of the particulate oxidase in the test mixture up to about 1 mg protein

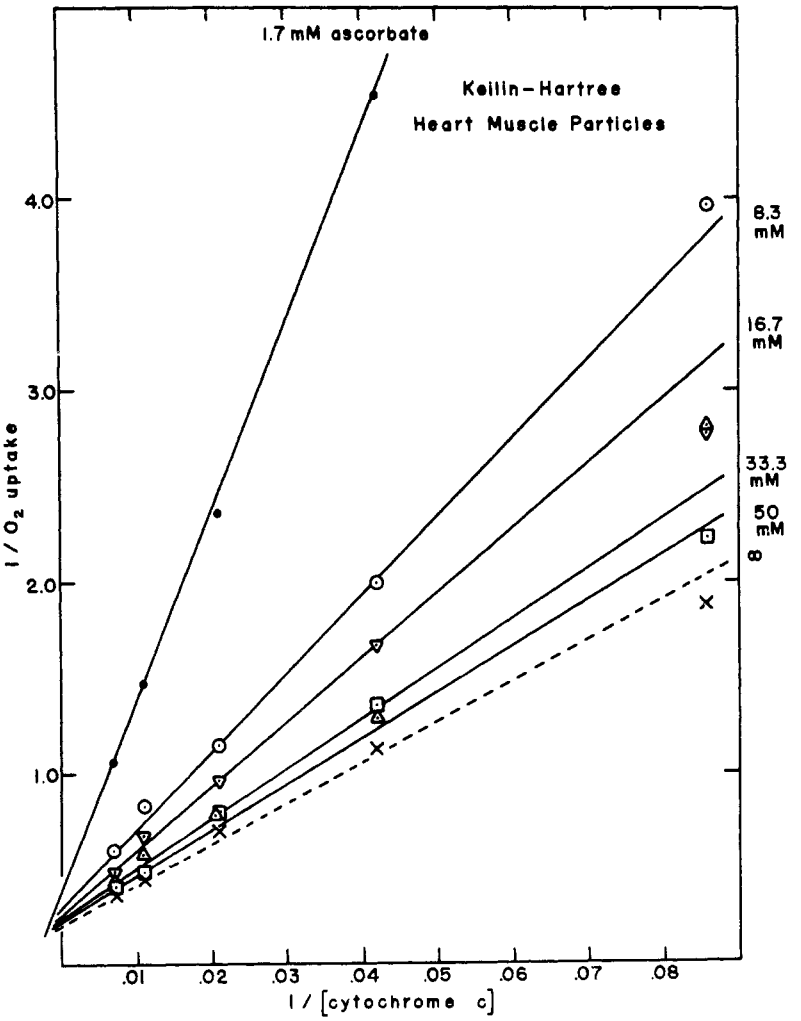

FIG. 1.—Plot of oxygen uptake, measured polarographically, at different concentrations of cytochrome c and ascorbate with a constant amount of Keilin-Hartree particles. The data are plotted according to the method of Lineweaver and Burk (1934). Calculated values at infinite ascorbate concentrations are indicated by X- - -X.

with cytochrome c concentrations between 12.5 and 150 μM and 1.67–50 mM ascorbate.

The plot of the reciprocals of the oxygen uptake values against the reciprocals of the ascorbate concentrations calculated at infinite cytochrome c concentration is nearly parallel to the abscissa. Because of the variability of the values it is difficult to decide whether the plots of the reciprocals of oxygen uptake values against reciprocals of the concentrations of cytochrome c at different concentrations of ascorbate intersect at the ordinate.

DISCUSSION

All investigations of system (a)—ferrocytochrome c → cytochrome c oxidase → oxygen—now seem to agree that the reaction is first order with respect to the concentration of ferrocytochrome c and that the rate constant decreases with increasing concentration of total cytochrome c in the reaction mixture. It is this effect of cytochrome c (oxidized or reduced) on the rate constant which is responsible for the hyperbolic curve observed on plotting oxygen uptake against cytochrome c concentration in system (b): reducing agent → cytochrome c → cytochrome c oxidase → oxygen. Numerous observations of oxygen uptake in system (b), including a detailed study by Slater (1949), have led to documentation of this hyperbolic relationship. A plot of the inverse of activity against the inverse of the concentration of total cytochrome c gives straight lines, such as those of Figures 1 and 2. Within the limit of error of our measurements, there is no evidence for an interference by ascorbate in the oxidase reaction. The plot of the inverse of oxygen uptake against the inverse of the ascorbate concentration at infinite cytochrome c is almost parallel with the ordinate.

If the nonenzymatic reduction of cytochrome c in system (b) is rapid compared to the oxidation of ferrocytochrome c by the oxidase, the rate of oxygen uptake should equal one-fourth the rate of oxidation of the same concentration of ferrocytochrome c, measured in system (a). This would be expected when the concentrations of cytochrome c, ascorbate, and oxidase are such that the cytochrome c is nearly completely reduced in the reaction mixture.

Minnaert (1961) recently demonstrated that under conditions "ordinarily used" in the manometric measurement of the oxidase activity of heart muscle particles, the cytochrome c is not "largely reduced." In spite of this, he found the rate of oxygen uptake in system (b) with 20 mM ascorbate slightly higher than the rate calculated from reaction (a). From his observation that the rate of oxygen uptake at infinite cytochrome c concentration was not independent of

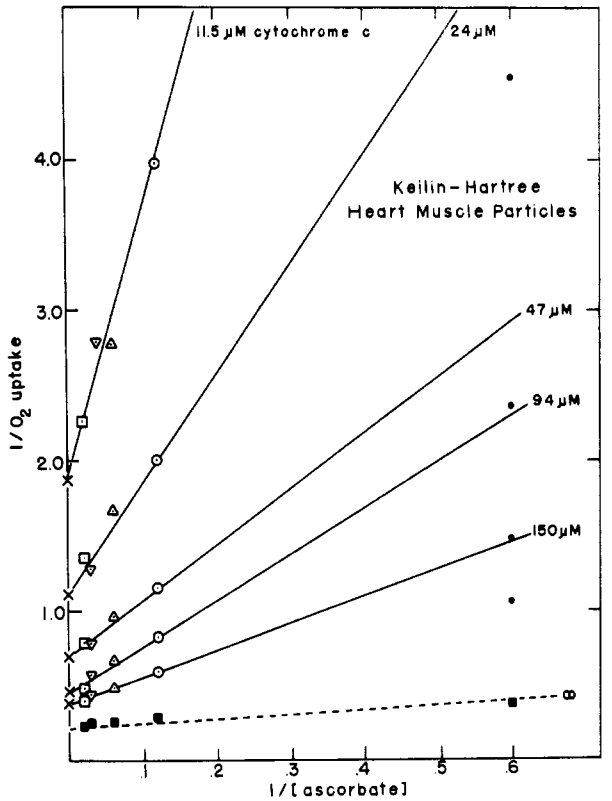

FIG. 2.—Data of Fig. 1 replotted to show the changes in oxygen uptake with increasing ascorbate concentrations at the levels of cytochrome c indicated on the curves. Calculated values at infinite cytochrome c concentration are indicated by ■- - -■.

the ascorbate concentration and his calculations of the assumed reactions, he concluded that the ascorbate not only reduces the cytochrome, but, in addition, that the "ascorbate interferes with the cytochrome oxidase reaction." This work thus questions the validity of employing the manometric (or polarographic) method for cytochrome c oxidase assay in the presence of ascorbate.

Yonetani (1962) studied system (b) polarographically, using a purified preparation of cytochrome c oxidase "activated" with a nonionic detergent. He obtained maximum turnover numbers with 10–60 μM cytochrome c, 40 mM ascorbate, and 0.025 μM oxidase (expressed on a heme basis), and these were about the same as the values obtained by extrapolating to infinite cytochrome c concentration. With the purified preparation, Yonetani found the same turnover number at infinite cytochrome c concentration, independent of the concentration of oxidase. He also found conditions where the cytochrome c was more than 90% reduced in the reaction mixture. Yonetani did not compare the polarographic with the spectrophotometric method.

Wharton and Griffiths (1962) compared reaction (a), measured spectrophotometrically, with reaction (b), measured both manometrically and polarographically. They worked with a purified oxidase preparation containing deoxycholate and "activated" with a phospholipid sol. The latter acted to prevent the aggregation of the purified oxidase in dilute suspensions, and in its presence they claim that the velocities obtained with the three methods could be brought into "reasonably good" agreement. They did not use the same conditions for the three methods, and they give data only on comparisons of the three methods at infinite cytochrome c concentration. The observations of Wharton and Griffiths are in agreement with those of Yonetani (1962) that the maximum rate of oxygen uptake in system (b) at infinite cytochrome c concentration is independent of the concentration of the oxidase. It is not clear what kind of inhibition of cytochrome oxidase by cytochrome c they refer to in addition to the demonstrated effect of cytochrome c concentration on the velocity constant.

The data reported here show that with identical conditions except for the presence of ascorbate in the polarographic method, the spectrophotometric and polarographic assays can be brought into good agreement when the cytochrome c is more than 85% reduced in the reaction. This condition must always be established experimentally. The appropriate amount of oxidase preparation to be used depends upon the concentration of cytochrome a_3 and upon the reactivity of the preparation with soluble cytochrome c (see following paper).

Our data do not agree with Minnaert's (1961) finding that the measured oxygen uptake in the manometric method is greater than the corresponding rate calculated from the rate of oxidation of ferrocytochrome c. In our experiments, whenever the polarographic method gave higher rates, the two methods could be brought into agreement by homogenization of the particulate oxidase suspension. Thus higher rates in the polarographic method, where the mixture is stirred, seemed in our case to result from the dispersal of particles which had aggregated when the strong suspension was stored at 4°. There is no stirring in the spectrophotometric method. The aggregation of the oxidase in dilute suspensions, observed by Wharton and Griffiths (1962) with their purified oxidase, does not seem to occur with the enzyme on heart muscle particles.

Our observations on the effects of cytochrome c and ascorbate concentration on the rate of oxygen uptake, as well as the good agreement of the two methods, show that in our system ascorbate does not interfere with the oxidase reaction to a measurable extent. We cannot explain the difference between our observations and those of Minnaert (1961), but it is clear that caution is necessary in employing the manometric (or polarographic) method.

The significant observation is that under the proper conditions the observed and calculated rates in the two systems are in agreement. It was necessary to establish this relationship in order to compare the reaction of the particulate oxidase with added soluble cytochrome c and with the endogenous cytochrome c of the particles, and to relate these reactions to the maximal possible turnover of the oxidase. These experiments are described in the following paper.

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The Reaction of Particle-bound Cytochrome c Oxidase with Endogenous and Exogenous Cytochrome c*

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A systematic study has been made of the reaction of different kinds of particulate preparations from heart muscle with soluble ferrocytochrome c, *p*-phenylenediamine, and DPNH, and with combinations of these. The effect of treatment of the preparations with deoxycholate on these reactions was also studied.

The data show that the turnover rates of the cytochrome c oxidase of the different preparations in the reaction with soluble ferrocytochrome c are highly variable and always less than the maximal turnover rate possible, even when the rates are extrapolated to an infinitely large concentration of cytochrome c. The turnover rate of cytochrome a_3 in this reaction can always be increased by initiating electron transport from DPNH or by treatment with deoxycholate under specific conditions. Large consistent maximal turnover rates are observed in the deoxycholate-treated preparations at cytochrome c concentrations greater than 150 μM. This maximal rate is similar to the turnover of cytochrome a_3 in its reaction with oxygen. Conditions are described for a reproducible assay for heart muscle cytochrome c oxidase which appears to measure the maximal turnover rate possible.

The exogenous (soluble) cytochrome c has been found to react directly with the cytochrome a (or a_3) of the respiratory chain system of the heart muscle particles rather than with the endogenous cytochrome c.

The DPNH oxidase activity of Keilin-Hartree preparations is increased somewhat (as much as doubled) by addition of a low concentration of cytochrome c (2 μM); then further increases in the concentration of cytochrome c do not give further increases in the rate of oxygen uptake in the presence of DPNH.

Cytochrome c oxidase is the terminal part of the membrane-bound respiratory chain system where oxygen is reduced. The sequence of hydrogen and electron transport through this multienzyme system is usually represented as follows (Keilin and Hartree, 1938; Keilin and Slater, 1953; Chance, 1961):

DPNH → flavoprotein
↘
(ubiquinone) cytochrome b → cyto. c_1 → cyto. c → (cytochrome c oxidase) cyto. a → cyto. a_3 → O_2
↗
succinate → flavoprotein

The particle-bound oxidase can also oxidize purified soluble ferrocytochrome c. It is this reaction that is most often used as an assay for cytochrome c oxidase (Slater, 1949; Smith and Conrad, 1956). The kinetics of the oxidation of soluble ferrocytochrome c are unusual in that the rate constant decreases with increasing concentration of cytochrome c (oxidized plus reduced) in the reaction mixture (Smith and Conrad, 1956). This effect is observed with the oxidase on swollen mitochondria or heart muscle particles, as well as with purified preparations. Because of this decrease in rate constant with increasing concentration of cytochrome c a hyperbolic curve is observed on plotting the rate of the reaction against the total concentration of soluble cytochrome c added. This has led to the procedure of calculating the activity extrapolated to an infinitely large concentration of cytochrome c (Slater, 1949; Yonetani, 1962; Wharton and Griffiths, 1962), even though the rate constant for the reaction falls to quite low values at high concentrations of cytochrome c. The relationship of the cytochrome c oxidase activity of different preparations with a finite or at infinitely large concentration of added cytochrome c to the maximum activity attainable has not been investigated. It is also uncertain how the rate of oxidation of soluble cytochrome c reflects the reaction of the particle-bound oxidase with the endogenous cytochrome c. Turnover rates reported for the reaction of the oxidase with high concentrations of soluble cytochrome c (Yonetani, 1962) are low compared with some values reported for the enzyme in intact cells (Chance, 1952a, 1955).

A perusal of the literature on the subject reveals a great variability in the reactivity (usually expressed as Q_{O_2}) of different kinds of oxidase preparations with constant and with increasing concentrations of cytochrome c (Wharton and Griffiths, 1962; Smith and Conrad, 1961; Griffiths and Wharton, 1961; Yonetani, 1962). Although swollen mitochondria have a very active cytochrome c oxidase (Smith and Conrad, 1961), some particulate preparations derived from beef heart mitochondria were observed to react only slowly with soluble cytochrome c unless they were first treated with deoxycholate (Mackler and Green, 1956).

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