containing 1 ml. 15% glycerol solution, 1·0 ml. 0·1 M-sodium acetate, 0·5 ml. 0·3 M-bicarbonate solution and 0·5 ml. of the enzyme solution. Occurrence of the back reaction would have given rise to an uptake of CO_2 from the system, but in fact no such uptake was observed. Under these conditions, therefore, no back reaction takes place, so that the incompleteness of the de-esterification cannot be due to the existence of an equilibrium between the back and forward reactions.

A crude acetone-powder preparation from liver, which contained an active esterase, had no pectin-esterase activity when tested by the manometric method. Consequently, liver esterase has no pectin-esterase activity under these conditions, although the enzyme preparation from *Ps. prunicola* has an esterase action on glycerol esters. This could, however, be due to the presence of esterases in the crude enzyme preparation, although the non-inhibition by di*iso*propyl fluorophosphonate does not support this view (see below).

Inhibitors. The rate of CO_2 output remained unchanged in the presence of 0·01 M-copper sulphate, potassium cyanide, ferrous sulphate, sodium azide and iodoacetate. A final concentration of 0·0001 M-di*iso*propyl fluorophosphonate, which was sufficient to cause 100% inhibition of liver esterase, had a negligible effect on the reaction towards pectin and tributyrin. Tannic acid (0·001 M), which is said to inhibit the action of pectinases (Kertesz, 1936), caused no inhibition of the pectin-esterase reaction. The enzyme and pectin solutions were submitted to dialysis against tap water and then distilled water, and the activity determined in the presence and absence of sodium chloride and sodium oxalate, which have been reported as being necessary for activation of the enzyme from tomatoes (Hills & Mottern, 1947). The velocity of the reaction was increased 20% by 0·05 M-sodium chloride, but was unaffected by 0·002 M-oxalate.

Further work is being carried out on the pectinase enzymes produced by *Ps. prunicola*, and on the separation of the pectin esterase and pectinase enzymes.

SUMMARY

1. A manometric method is described for the determination of pectin-esterase activity, and has been used to investigate some of the properties of such an enzyme obtained from *Ps. prunicola*.

2. The exo-enzyme, produced by *Ps. prunicola*, is formed adaptively in response to growth in the presence of pectin, pectate, and galacturonic acid.

3. The enzyme preparation splits off 75% of the methoxyl groups (estimated by the micro-Zeisel apparatus) of various pectins, and also hydrolyzes tributyrin, triacetin, diacetin and monoacetin, to the extent of approximately 70%. It has no action on ethyl acetate, ethyl oxalate or the methyl ester of α-methylgalacturonic acid.

I wish to express my thanks to Dr M. Stephenson, F.R.S., Dr R. Hill, F.R.S., Dr E. F. Gale and Dr W. J. Dowson for their interest and advice, and to the Agricultural Research Council for a personal grant.

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The Measurement of the Cytochrome Oxidase Activity of Enzyme Preparations

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Cytochrome oxidase has been defined by Keilin & Hartree (1938) as the enzyme responsible for the oxidation of reduced cytochrome *c*. The activities of oxidizing enzymes are usually measured by determining the rate of O_2 uptake in the presence of excess substrate. This method is, however, scarcely applicable in the case of cytochrome oxidase, since the amount of reduced cytochrome *c* required to give a measurable uptake of O_2 in the types of manometers usually employed is too great (thus 3·3 ml. of 0·1% reduced cytochrome *c* requires only 1·1 μl. O_2 for complete oxidation). The rate of oxidation of cytochrome *c* can be measured spectrophotometrically (e.g. Albaum, Tepperman & Bodansky, 1946), but the apparatus required is not always available. Consequently an indirect method is usually employed in which the rate of O_2 uptake of the system

reducing agent → cytochrome *c* → cytochrome oxidase → O_2

(the arrows indicating the direction of hydrogen or electron transfer) is measured. It is generally believed that, under the conditions of the experiment, cytochrome *c* is reduced by the reducing agent as soon as it is oxidized by the cytochrome oxidase oxygen, and so the rate of O_2 uptake is a measure of the cytochrome oxidase activity. This method has frequently been employed in the study of the action of inhibitors on the cytochrome oxidase system, and it is often assumed, without further examination, that any decrease in the rate of O_2 uptake is due to inhibition of the enzyme.

It has been shown (Slater, 1948*a*, 1949*a*) that treatment of heart-muscle preparation with BAL (2:3-dimercaptopropanol) causes the complete inactivation of the succinic oxidase system. As cytochrome oxidase is a part of this system, the effect of BAL on this enzyme was also studied. It was found that there was little if any inhibition when ascorbic acid or hydroquinone was used as the reducing agent in the measurement of the cytochrome oxidase activity, but a considerable inhibition (30–40 %) if *p*-phenylenediamine was the reducing agent. To investigate this further, a study has been made of the factors involved in the measurement of cytochrome oxidase activity.

The present paper reports the results of this study. Under the conditions usually employed in making this measurement, the rate of O_2 uptake was found to depend not only on the cytochrome oxidase activity, but also on the concentrations of cytochrome *c* and reducing agent, and on factors which affect the catalytic activity of the cytochrome *c*. A procedure suitable for studying the true activity of cytochrome oxidase, independently of these factors, is described. A preliminary account of some of the findings of this investigation has appeared elsewhere (Slater, 1948*b*).

METHODS

Cytochrome oxidase. The heart-muscle preparation of Keilin & Hartree (1947*b*) was used.

Cytochrome c, containing 0·34 % Fe, was prepared by the method of Keilin & Hartree (1945). The solution contained $6{\cdot}7 \times 10^{-4}$ M-cytochrome *c* in 0·5 % NaCl.

Phosphate buffer. A Sorensen phosphate buffer, pH 7·3, was used.

Manometric experiments were carried out in Barcroft differential manometers at 38°. The reducing agent was added (by displacement of a dangling tube) at zero time, after temperature equilibration. Readings were begun 3 or 5 min. after the addition. In all experiments the gas phase was air.

RESULTS

Reducing agents

Table 1 lists various reducing agents commonly employed for the manometric estimation of cytochrome oxidase activity, together with their oxidation-reduction potentials at pH 7·3. It also shows the proportion of cytochrome *c* in the reduced form, at equilibrium, when the initial concentration of reducing agent was 0·05 M, that of oxidized cytochrome *c* was 6×10^{-5} M, and the reducing agent has been oxidized by the absorption of 100 μl. O_2, the total volume of the solution being 3·3 ml. It is obvious that catechol and adrenaline are not satisfactory reducing agents. It was found, in fact, that the rate of O_2 uptake, corrected for blank (see p. 307), of 0·05 M-catechol, 6×10^{-5} M-cytochrome *c* and enzyme preparation, was only 21 % that of the similarly corrected figure obtained for the same concentration of hydroquinone in the presence of the same concentration of cytochrome *c* and the same enzyme preparation. Unfortunately, the oxidation-reduction potential of *p*-phenylenediamine, one of the most useful reagents for the determination of cytochrome oxidase activity, cannot be determined at pH 7·3. Thus Clark, Cohen & Gibbs (1926) stated that 'attempts to measure the system by which *p*-phenylenediamine is the reductant were frustrated by the extreme instability of the system'. Fieser (1930) reported no success with measurements in solutions less acid than pH 5. Barron (1939) calculated, by extrapolation of the data of Fieser at acid reactions, that the E_0' at pH 7·0 of *p*-phenylenediamine would be + 0·381 V., i.e. in the same region as catechol and adrenaline. Stotz, Sidwell & Hogness (1938*b*), on the other hand, state that *p*-phenylenediamine possesses a much lower potential than hydroquinone and is even capable of reducing cytochrome *b* ($E_0' = -0{\cdot}04$ V.). However, neither Borei (1945) nor the author has been able to confirm this reduction of cytochrome *b*.

Table 1. *Capacity of various reducing agents to reduce cytochrome* c

(E_0' of cytochrome $c = 0{\cdot}262$ V. at pH 7·3, 30° (Stotz, Sidwell & Hogness, 1938*a*). Initial concentrations: reducing agent, 0·05 M; oxidized cyt. *c*, 6×10^{-5} M. The figures in the third column correspond to the equilibrium state after the reducing agent has been oxidized by the absorption of 100 μl. O_2. Total vol. 3·3 ml.)

Reducing agent	E_0' at pH 7·3, 30° (V.)	Cyt. *c* in reduced form (%)
Ascorbic acid	0·049 (Ball, 1937)	99·9
Hydroquinone	0·255	84·4
Catechol	0·354 (calculated from Ball & Chen, 1933)	9·4
Adrenaline	0·364 (calculated from Ball & Clark, 1931)	7·6

It must be emphasized that the figures given in Table 1 are theoretical, calculated from the oxidation-reduction potentials, assuming a simple oxidation of the reducing agent, without further reaction of the oxidized form. Ball & Chen (1933)

pointed out that the substance with the higher oxidation-reduction potential can reduce that with the lower if the oxidized state of the former is unstable. For example, adrenaline can reduce 2:6-dichlorophenolindophenol at pH 7, although originally only about 0·1 % of the dye would be reduced, and the equivalent amount of adrenaline oxidized.

Boss & Friedenwald (1946) reported that cytochrome *c* had no effect on the oxidation. These discrepancies are possibly due to differences in the amounts of impurities in the cytochrome *c* preparations used in the different investigations. The cytochrome *c*-catalyzed oxidation of ascorbic acid is probably not inhibited by heart-muscle preparation, since, according to Borei (1945), it is not affected by diethyldithiocarbamate, a copper-binding reagent which strongly inhibits the copper-catalyzed oxidation of ascorbic acid.

Table 2. *Rates of oxidation of reducing agents in the absence of enzyme*

(Phosphate buffer, 0·15 M, pH 7·3; total vol. 3·3 ml.)

Reducing agent	Concentration (M)	Initial rate of O_2 uptake (μl./hr.) Cyt. *c* absent	With 2×10^{-5} M-cyt. *c*	With 4×10^{-5} M-cyt. *c*	With 6×10^{-5} M-cyt. *c*	'Blank' obtained by extrapolation* in presence of 6×10^{-5} M-cyt. *c*
Hydroquinone	0·017	107	—	—	125	—
	0·05	294	—	—	303	300
Catechol	0·017	42	—	—	51	—
	0·05	177	—	—	193	196
p-Phenylenediamine	0·05†	27	—	—	43	27
Ascorbic acid	0·0017	24	—	—	16	—
	0·0085	80	—	—	53	—
	0·017	122	—	—	81	76
	0·026	156‡	91	93	101	—

* See Fig. 1 and text, p. 308.

† Correction for lower concentrations is negligible.

‡ Rate of O_2 uptake in presence of heart-muscle preparation (0·3 mg. dry wt./ml.) = 80 μl./hr.

The oxidized adrenaline quickly decomposes and, in order to restore the equilibrium, more of the dye must be reduced. In this way the reduction of the dye can proceed to completion. A similar situation may exist with respect to the reduction of cytochrome *c* by *p*-phenylenediamine or adrenaline. The reaction with adrenaline is very complex (see p. 308).

The reducing agents listed in Table 2 are oxidized to a certain extent in the absence of the enzyme. This so-called 'auto-oxidation' is due to catalysis by traces of heavy metals present in the reagents, and its magnitude depends on the purity of the reagents and on the pH. It must be determined as a 'blank' and subtracted from the total O_2 uptake in order to obtain a measure of the cytochrome oxidase activity. However, this 'blank' may not be the same in the presence of heart-muscle preparation as in the buffer solution alone, e.g. the oxidation of ascorbic acid, which is catalyzed by traces of heavy metals (Barron, De Meio & Klemperer, 1935–6), is inhibited by heart-muscle preparation (see footnote to Table 2, and also Borei, 1945), probably because —SH groups in the latter react with traces of Cu^{++} present in the solution. Table 2 shows that it is also inhibited by small concentrations of cytochrome *c*, although larger concentrations of the latter catalyze the oxidation. Borei (1945) reported catalysis of the oxidation of ascorbic acid by cytochrome *c*, but did not find any inhibition by small concentrations; Herrmann,

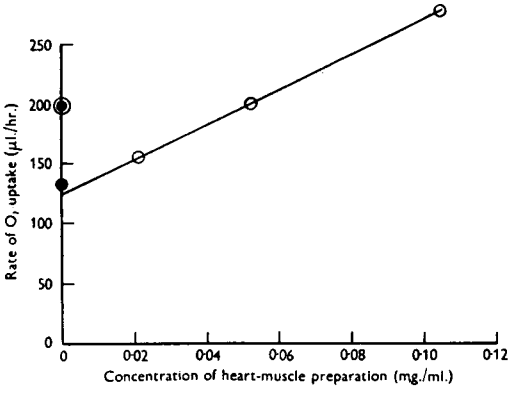

Fig. 1. Method of calculating 'blank' by extrapolation to zero enzyme concentration. 0·017 M-Ascorbic acid (neutralized), 0·15 M-phosphate buffer, vol. 3·3 ml. Ascorbic acid introduced from dangling tube after equilibration. ○, rate of oxidation in presence of 6×10^{-5} M-cytochrome *c* and varying amounts of heart-muscle preparation; ●, 6×10^{-5} M-cytochrome *c*, no enzyme; ⊙, no cytochrome or enzyme. (Note that the higher rates of O_2 uptake compared with the figures given in Table 2 are due to the use of a different phosphate buffer.)

Probably the best method of determining the true 'blank' oxidation is that of Schneider & Potter (1943), in which the rates of O_2 uptake in the presence of a constant concentration of reducing agent and cytochrome *c*, but with varying concentrations of enzyme, are extrapolated to zero enzyme concentration. An example of this method is shown in Fig. 1, and the values obtained are included in Table 2. With all four of the reducing agents studied, these values are only slightly different from those obtained by direct measurement in the presence of cytochrome *c* and in the absence of enzyme. All measurements reported in this paper have been corrected for the blank.

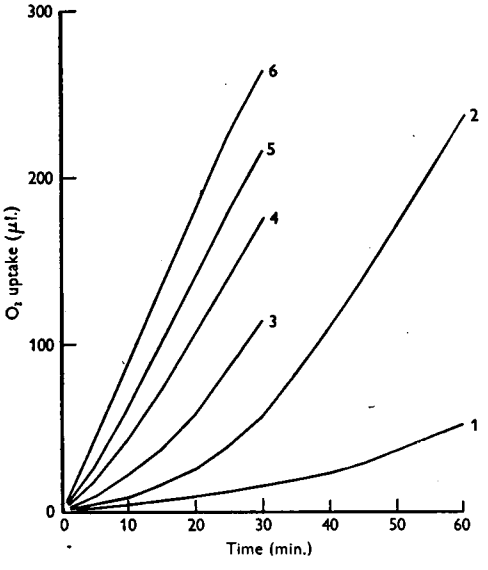

Fig. 2. O_2 uptake of adrenaline. 0·15M-Phosphate buffer, 3·3 ml. total vol. 10 mg. solid adrenaline added at (zero–5) min. by dislodging dangling tube; manometric measurements commenced at zero time.

Curve	Concentration of cytochrome *c* (M)	Concentration of heart-muscle preparation (mg. fat-free dry wt./ml.)
1	0	0
2	6×10^{-5}	0
3	6×10^{-5}	0·019
4	6×10^{-5}	0·07
5	6×10^{-5}	0·14
6	6×10^{-5}	0·28

Adrenaline is only slowly oxidized in the absence of cytochrome *c* or of cytochrome oxidase (Fig. 2). The oxidation is, however, strongly catalyzed by cytochrome *c*, and the course of this reaction shows that the oxidation products of adrenaline strongly catalyze the latter's oxidation at a rate which masks that due to the cytochrome oxidase system, when the latter is added. Thus, addition of cytochrome oxidase decreases the initial lag of the oxidation which occurs in the presence of cytochrome *c* alone, but does not appreciably increase the maximum rate of oxidation. This is clearly seen in Fig. 3, where the uptakes in the first 5 min. and the

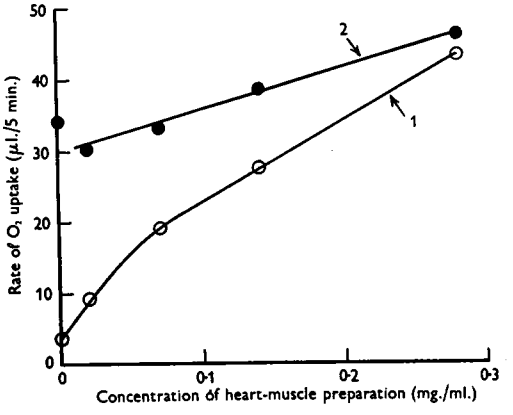

Fig. 3. Rate of O_2 uptake of adrenaline in the presence of different concentrations of heart-muscle preparation (calculated from Fig. 2). 6×10^{-5}M-cytochrome *c*; 0·15M-phosphate; total vol. 3·3 ml.; 10 mg. adrenaline. Curve 1, oxygen uptake between zero time (5 min. after adding adrenaline) and 5 min; curve 2, maximum rate of O_2 uptake.

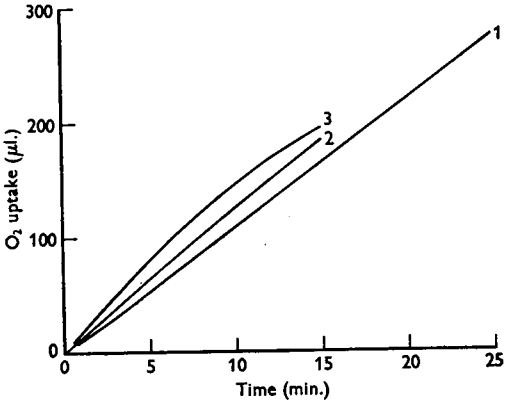

Fig. 4. Course of the O_2 uptake of 0·017M neutralized ascorbic acid (curve 1), 0·05M-*p*-phenylenediamine (curve 2), 0·05M-hydroquinone (curve 3), each in the presence of 6×10^{-5}M-cytochrome *c* and heart-muscle preparation (0·28 mg./ml.). Total vol. 3·3 ml.; 0·15M-phosphate buffer. Reducing agents added at (zero–5) min.

maximum rates of O_2 uptake are plotted against the enzyme concentration. Thus, although in the presence of cytochrome *c* and enzyme, adrenaline, after an initial lag, shows a constant rate of O_2 uptake, this measurement is useless for the measurement of cytochrome oxidase activity. The fact that this rate of O_2 uptake is about the same as that obtained with other reducing agents in the presence

of cytochrome *c* and the amount of heart-muscle preparation usually employed in these studies is purely a coincidence; it is the reason why this effect of cytochrome *c* and the oxidation products of adrenaline on the latter's oxidation has not previously been observed.

The course of the O_2 uptake with the other reducing agents used is shown in Fig. 4. In the case of hydroquinone and *p*-phenylenediamine, the rate of O_2 uptake decreased with time, and the initial rate, obtained by extrapolation to zero time, was used as the measure of activity. This decrease with time is presumably due to the toxic action of the reducing agent or of its oxidation product. Ascorbic acid showed a slight lag, and the maximum uptake was obtained 10–15 min. after the addition, and was then constant for the next 10–15 min. This constant maximum rate was used as the measure of activity.

Effect of cytochrome c *concentration*

As has been pointed out by Keilin & Hartree (1940), the amount of cytochrome *c* in the heart-muscle preparation is nearly sufficient to oxidize succinate at the maximal rate; the addition of a large excess of cytochrome *c* increases the rate of oxidation of succinate by only about 20%. This shows that the endogenous cytochrome *c* of the heart-muscle preparation can be rapidly reduced by succinate (through intermediate carriers) and, more important in the present connexion, can be rapidly oxidized by the cytochrome oxidase. Keilin & Hartree (1938) showed, however, that, with the exception of *p*-phenylenediamine, none of the reducing agents studied was oxidized by heart-muscle preparation at an appreciable rate, unless cytochrome *c* was added. Since the oxidation of cytochrome *c* is common to both the oxidation of succinate and of these reducing agents, the difference between the rates of oxidation of succinate and of the reducing agents must lie in the relative rates of reduction of the endogenous cytochrome *c*. It is interesting to note, in this connexion, that the reduction by succinate is an enzymic reaction, while that by ascorbic acid is non-enzymic.

The rate of reduction of the endogenous cytochrome *c* of the heart-muscle preparation by 0·025 M-ascorbic acid was determined under completely anaerobic conditions in the presence of 0·01 M-KCN. The heart-muscle preparation contained $2{\cdot}3 \times 10^{-5}$ M-cytochrome *c* (Slater, 1949*b*), i.e. $2{\cdot}3 \times 10^{-2}$ micromole/ml. and this was half reduced in about 25 sec. at 20°. If we assume that the reduction of cytochrome *c* is monomolecular it can be calculated that the initial rate of reduction is $3{\cdot}8 \times 10^{-2}$ micromole cytochrome *c*/min./ml. of heart-muscle preparation. Such a reaction would cause the uptake of 13 μl. O_2/hr./ml. of heart-muscle preparation. Owing to the low concentration of the cytochromes and their high catalytic activity, spectroscopic observations are usually made with the undiluted heart-muscle preparation, while only 0·02 ml. of the preparation is taken in the manometric experiments. Thus the rate of O_2 uptake in the manometric experiments due to this reaction would be $0{\cdot}02 \times 13 = 0{\cdot}26$ μl./hr., which is negligible even when allowance is made for the higher temperature in the manometric experiments. Under the same conditions, 0·05 M-*p*-phenylenediamine reduced the endogenous cytochrome *c* almost instantaneously (< 5 sec.).

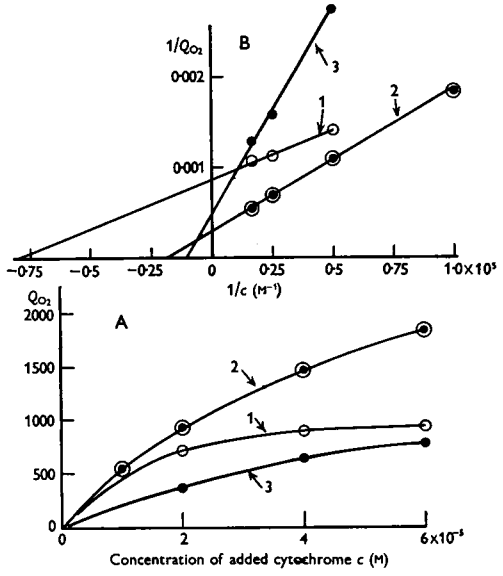

Fig. 5. A, effect of concentration of cytochrome *c* on the rate of oxidation of ascorbic acid in the presence of heart-muscle preparation. Ascorbic acid, 0·025 M; NaCl, 0·0008 M; 0·51 mg. heart-muscle preparation (wt. of fat-free dried material); total vol. 3·3 ml. Curve 1, 0·01 M-phosphate buffer; curve 2, 0·065 M-phosphate buffer; curve 3, 0·146 M-phosphate buffer. B, figures in A, plotted according to the procedure of Lineweaver & Burk (1934).

The rate of reduction of the cytochrome *c* of the heart-muscle preparation by ascorbic acid is much less than that of the same concentration of pure cytochrome *c*, which was reduced almost instantaneously. In this respect, ascorbic acid behaves in the same way as cysteine, already studied by Keilin (1930).

The effect of the concentration of cytochrome *c* on the rate of O_2 uptake of ascorbic acid (corrected for 'blank') in the presence of heart-muscle preparation at different concentrations of phosphate buffer is shown in Fig. 5A. In Fig. 5B these figures have been plotted according to the procedure of

Lineweaver & Burk (1934), i.e. the inverse of the activity against the inverse of the cytochrome *c* concentration. In Fig. 6A, B, similar data are given for *p*-phenylenediamine oxidation in 0·15 M-phosphate. The difference between ascorbic acid and *p*-phenylenediamine is most clearly seen in Fig. 7, which shows the result of parallel experiments obtained with the same heart-muscle preparation in 0·065 M-phosphate.

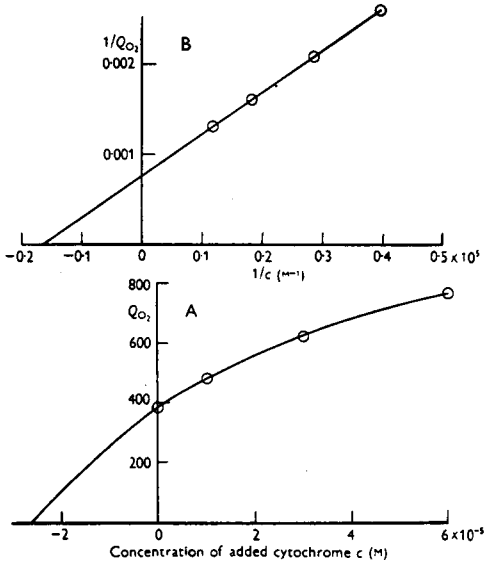

Fig. 6. A, effect of concentration of cytochrome *c* on the rate of oxidation of *p*-phenylenediamine in the presence of heart-muscle preparation. *p*-Phenylenediamine, 0·05 M; phosphate buffer, 0·15 M; 1·02 mg. heart-muscle preparation (wt. of fat-free dried material); total vol. 3·3 ml. The curve has been extrapolated to the abscissa by assuming that the points fall on a rectangular hyperbola. B, figures in A, plotted according to the procedure of Lineweaver & Burk (1934).

The endogenous cytochrome *c*, which can be reduced rapidly by *p*-phenylenediamine but not by ascorbic acid, is much more active catalytically than added cytochrome *c*. Thus, by the extrapolation shown in Fig. 6A, it was found that in 0·15 M-phosphate, the endogenous cytochrome *c* had a catalytic activity equal to that of $2{\cdot}5 \times 10^{-5}$ M added cytochrome *c*. The actual concentration of endogenous cytochrome *c* was $2{\cdot}8 \times 10^{-7}$ M, i.e. the endogenous cytochrome *c* was about 100 times as active as that in solution (cf. Keilin, 1930; Keilin & Hartree, 1940, 1945). The corresponding ratio of activities in 0·065 M-phosphate, calculated from Fig. 7, was 40. It is not surprising that the endogenous cytochrome *c* should be so much more active than that added, since the cytochrome *c* firmly bound to the particles of the heart-muscle preparation is probably in the most favourable position for the transfer of electrons to cytochrome oxidase.

The relative slopes of the two curves in Fig. 7B show that when *p*-phenylenediamine is the reducing agent, not only is the endogenous cytochrome *c* more effective than is the case when ascorbic acid is used as a reducing agent, but added cytochrome *c* is also more effective.* It is important to note, however, that the activity at infinite cytochrome *c* concentration is independent of the reducing agent.

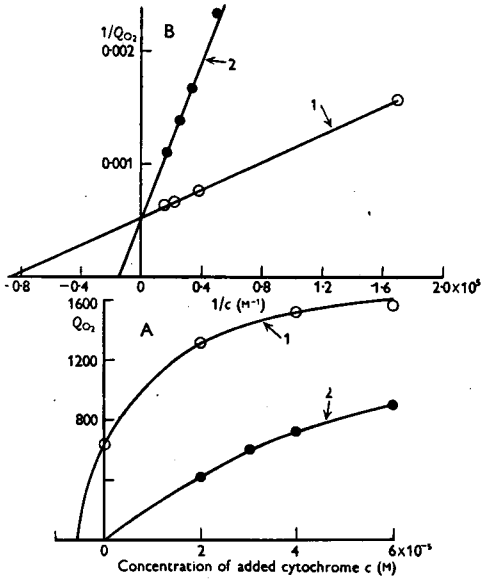

Fig. 7. A, effect of different concentrations of cytochrome *c* on the rate of oxidation of ascorbic acid and *p*-phenylenediamine. Phosphate buffer, 0·065 M; 0·41 mg. (fat-free dry wt.) heart-muscle preparation; total vol. 3·3 ml. Curve 1, *p*-phenylenediamine (0·05 M), extrapolated as in Fig. 6A; curve 2, ascorbic acid (0·025 M). B, figures in A, plotted as in Figs. 5A and 6A.

These experiments show that very large concentrations of cytochrome *c* are required for maximal activity of the cytochrome oxidase, especially when ascorbic acid is the reducing agent and high concentrations of phosphate buffer are employed. Thus it can be calculated from Fig. 5B that, in 0·146 M-phosphate, 10^{-3} M-cytochrome *c* (= 16 mg./ml.) is required to give 90% of the maximum activity. This is about 100 times the concentration of total protein contributed by the heart-muscle preparation itself, and obviously it would be quite impossible to have such concentrations of cytochrome *c* in the cell itself. This finding is another example of

* This effect of reducing agent on the activity of the added cytochrome *c* is much more marked at 0·065 M-phosphate than at 0·15 M.

the poor catalytic activity of cytochrome *c* in solution compared with that in the cell. In 0·01 M-phosphate, $1{\cdot}2 \times 10^{-4}$ M-cytochrome *c* is necessary for 90% activity. It follows that it is impossible to measure directly the full activity of the cytochrome oxidase present in 0·5 mg. heart-muscle preparation by determining the rate of oxygen uptake in the presence of excess cytochrome *c*. This can be done only by measuring the activity at different cytochrome *c* concentrations and extrapolating to infinite concentration. The concentrations of cytochrome *c* found in the present study to be necessary for maximal activity of the cytochrome oxidase are very much greater (about 10 times) than those found by Stotz, Altschul & Hogness (1938) and Borei (1945). This is probably due to the much greater activity of the heart-muscle preparation used in the present study. The inhibitory effect of high cytochrome *c* concentrations on the oxidation of *p*-phenylenediamine by heart-muscle preparation, reported by Borei, was not found in the present study.

Effect of phosphate concentration

The effect of the concentration of the phosphate buffer on the rate of oxidation of ascorbic acid in the presence of a fixed concentration of cytochrome *c* and ascorbic acid is shown in Fig. 8. The amount of ascorbic acid used was the same as that in Fig. 5, and the amount of cytochrome *c* was the highest concentration used in Fig. 5; the same enzyme preparation was used. From Fig. 5B, the maximum activity (i.e. at infinite cytochrome *c* concentration) can be calculated from the point at which each straight line intersects the ordinate, which equals the inverse of the maximum activity. The point at which the line intersects the abscissa equals $-1/(\text{cyt. } c)_{\frac{1}{2}}$, where $(\text{cyt. } c)_{\frac{1}{2}}$ is the concentration of cytochrome *c* required for half maximal activity. These quantities, calculated from Fig. 5B, are given in Table 3, which also includes the activities in the presence of 6×10^{-5} M-cytochrome *c*. The difference in the slopes of the lines in Fig. 5B at different phosphate concentrations shows how dangerous it is to measure the activity at only one cytochrome *c* concentration. Thus, at all concentrations of cytochrome *c* shown in Fig. 5, the activity in 0·146 M-phosphate was less than that in 0·01 M-phosphate, but the curves show that, if excess cytochrome *c* was used, the activity of the cytochrome oxidase at 0·146 M-phosphate would be nearly double that at 0·01 M-phosphate (Table 3). In order to measure the true activity of the cytochrome oxidase it is necessary to measure the activity at different cytochrome *c* concentrations and extrapolate to infinite cytochrome *c* concentration.

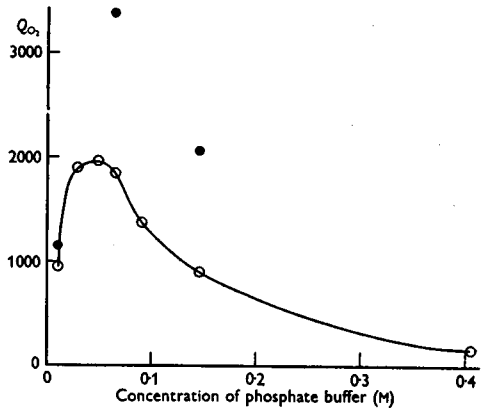

Fig. 8. Effect of phosphate concentration on rate of oxidation of ascorbic acid (0·025 M) in the presence of cytochrome *c* (6×10^{-5} M) and heart-muscle preparation (0·154 mg. fat-free dry wt./ml.); total vol. 3·3 ml. ○, activities measured under these conditions; ●, activities at infinite cytochrome *c* concentration, calculated from Fig. 5B.

Keilin & Hartree (1949) (see also Slater, 1949*c*) have shown that the activity of the succinic oxidase system of the heart-muscle preparation is dependent on the concentration of the phosphate buffer used. Figs. 5 and 8 show that the concentration of the

Table 3. *Effect of phosphate concentration on the cytochrome oxidase activity of heart-muscle preparation*

(Q_{O_2} measured at 38°; based on wt. of fat-free dried material.)

$(\text{Cyt. } c)_{\frac{1}{2}}$ = concentration of cytochrome *c* required for half maximal activity.

Phosphate concentration (M)	Q_{O_2}: At infinite cyt. *c* concentration (*A*)	Q_{O_2}: In presence of 6×10^{-5} M-cyt. *c* (*B*)	$\frac{B}{A} \times 100$	$(\text{Cyt. } c)_{\frac{1}{2}}$ (M)
0·01	1140	950	83	$1{\cdot}25 \times 10^{-5}$
0·065	3360	1840	55	$5{\cdot}4 \times 10^{-5}$
0·146	2040	890	44	$9{\cdot}3 \times 10^{-5}$

buffer is also very critical so far as the cytochrome oxidase activity is concerned. When ascorbic acid is the reducing agent, the concentration of phosphate buffer has two separate effects: (1) on the activity of the cytochrome oxidase itself, measured at infinite cytochrome *c* concentration; the concentration of phosphate buffer which gives optimal activity is 0·065M; (2) on the catalytic activity of added cytochrome *c*; this catalytic activity may be measured either by the inverse of the concentration of cytochrome *c* required for half-optimal activity or by the inverse of the slopes of the straight lines in Fig. 5B. The catalytic activity of added cytochrome *c* decreases rapidly with increase of phosphate concentration, and is optimal at low concentrations. Because the optimal phosphate concentration for this second effect is different from that required for the first, it follows that, as has already been mentioned, the effect of phosphate concentration depends upon the amount of cytochrome *c* used.

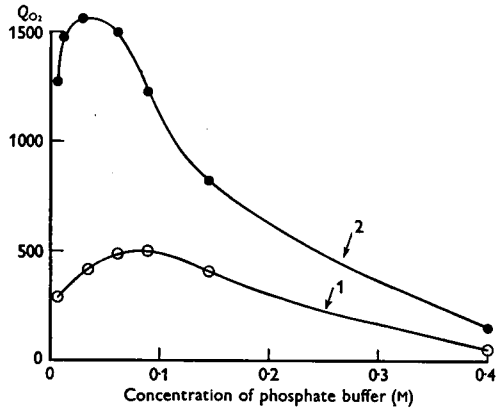

Fig. 9. Effect of phosphate concentration on the rate of oxidation of *p*-phenylenediamine (0·05M), by heart-muscle preparation in the absence of cytochrome *c* (curve 1) and in the presence of 6×10^{-5}M-cytochrome *c* (curve 2). 0·29 mg. fat-free dry wt. heart-muscle preparation/ml.; total vol. 3·3 ml.

When *p*-phenylenediamine is the reducing agent, in the absence of added cytochrome *c*, the second effect is no longer operative, but the effect of phosphate on the catalytic activity of the endogenous cytochrome *c* must be considered. In this case, this factor is probably all important, except perhaps at high phosphate concentrations, since the system operates far below its full cytochrome oxidase activity. The effect of phosphate concentration on the rate of oxidation of *p*-phenylenediamine by heart-muscle preparation, both in the presence and absence of cytochrome *c*, is shown in Fig. 9. In the absence of added cytochrome *c*, the optimum phosphate concentration is 0·08M, and the curve is much flatter than those already considered. In the presence of added cytochrome *c* (6×10^{-5}M) the curve is similar to that obtained with ascorbic acid.

Keilin & Hartree (1949) found that the addition of denatured globin to heart-muscle preparation caused a considerable increase in the succinic oxidase activity, especially at low phosphate concentrations; in fact, the inhibiting effect of low phosphate concentrations is completely removed by the addition of globin. It was found in the present study, however, that denatured globin had little effect (it actually caused a slight inhibition) on the rate of oxidation of *p*-phenylenediamine by heart-muscle preparation at any of the phosphate concentrations studied, either in the presence or absence of cytochrome *c*.

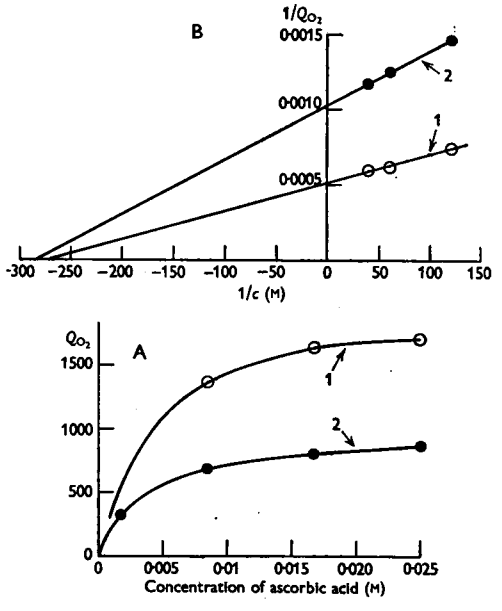

Fig. 10. A, effect of ascorbic acid concentration on the rate of its oxidation by heart-muscle preparation (0·15 mg. fat-free dry wt./ml.) in the presence of cytochrome *c* (6×10^{-5}M); total vol. 3·3 ml. Curve 1, 0·065M-phosphate; curve 2, 0·146M-phosphate. Same heart-muscle preparation as Figs. 5, 6. B, figures in A, plotted according to the procedure of Lineweaver & Burk (1934).

Effect of concentration of reducing agent

The effect of the concentration of ascorbic acid on the rate of O_2 uptake at two phosphate concentrations is shown in Fig. 10. The activity (Q_{O_2}) at infinite ascorbic acid concentration is 980 in 0·146M-phosphate and 1990 in 0·065M-phosphate. The effect of different concentrations of *p*-phenylene-

diamine is shown in Fig. 11. In this case, the inverse of the *p*-phenylenediamine concentration is not proportional to the inverse of the activity.

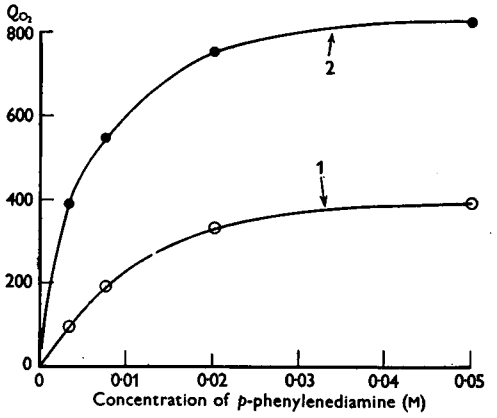

Fig. 11. Effect of *p*-phenylenediamine concentration on the rate of its oxidation by heart-muscle preparation (0·31 mg. fat-free dry wt./ml.) in the absence of cytochrome *c* (curve 1) and in the presence of 6×10^{-5}M-cytochrome *c* (curve 2). 0·15M-phosphate concentration.

These results show that, even in the presence of the highest concentrations of cytochrome *c* used in this study, the activity depends on the concentration of the reducing agent, and higher activities would be obtained by using higher concentrations of reducing agent.* However, it should be noted that in the presence of 6×10^{-5}M-cytochrome *c* the activity at infinite ascorbic acid concentration is considerably less than the activity at infinite cytochrome *c* concentration, in the presence of a definite amount of ascorbic acid. The concentration of cytochrome *c* is therefore the limiting factor.

* There are practical disadvantages in using too high concentrations of reducing agents, viz. the high 'blanks' often obtained and, in some cases, the toxicity of the reducing agents.

Effect of enzyme concentration

The effect of two different enzyme concentrations on the rate of oxidation of ascorbic acid in the presence of different amounts of cytochrome *c* is shown in Fig. 12.

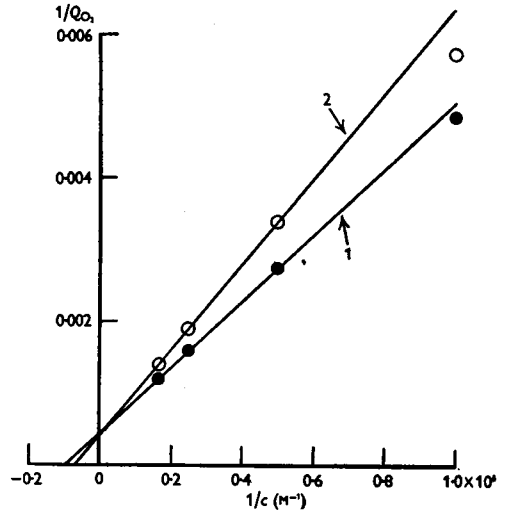

Fig. 12. Effect of concentration of heart-muscle preparation on the rate of oxidation of ascorbic acid (0·025M) in the presence of different concentrations of cytochrome *c*, plotted according to the procedure of Lineweaver & Burk (1934). Phosphate, 0·146M; total vol. 3·3 ml. Curve 1, 0·18 mg. (fat-free dry wt.) heart-muscle preparation/ml.; curve 2, 0·36 mg. heart-muscle preparation/ml.

Recommended procedure for measuring cytochrome oxidase activity

As a result of the experiments described above, the following procedure for measuring the cytochrome oxidase activity of the Keilin & Hartree (1947*b*) preparation has been adopted. This procedure should not be used with other preparations,

Table 4. *Recommended procedure for measuring cytochrome oxidase activity. Contents of right-hand flasks of the differential manometers*

Reagent	Volume (ml.)							
Flask no. ...	1	2	3	4	5	6	7	8
Heart-muscle preparation diluted 10-fold with 0·1M-phosphate buffer, pH 7·3	0·2	0·2	0·2	0·2	—	—	—	—
Phosphate buffer, 0·1M	2·0	2·0	2·0	2·0	2·2	2·2	2·2	2·2
Cytochrome *c*, $6{\cdot}7 \times 10^{-4}$M in 0·5% NaCl	0·1	0·15	0·2	0·3	0·1	0·15	0·2	0·3
0·5% NaCl	0·2	0·15	0·1	—	0·2	0·15	0·1	—
Water	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Ascorbic acid (50 mg./ml. neutralized) in dangling tube	0·3	0·3	0·3	0·3	0·3	0·3	0·3	0·3
Total vol.	3·3	3·3	3·3	3·3	3·3	3·3	3·3	3·3

without first examining the effect of the various factors influencing the rate of O_2 uptake.

One complete estimation requires eight manometers; four of these are, however, used for determining the 'blank' oxidation at each level of cytochrome *c*, and these measurements need only be made once for each batch of phosphate buffer and cytochrome *c*. The right-hand flasks of the differential manometers are filled as shown in Table 4. The left-hand flasks each contain 3·3 ml. 0·065M-phosphate buffer, pH 7·3. KOH papers are not used.

The manometers, with flasks attached, are placed in a bath at 38° and gently shaken for 15–25 min. for temperature equilibration. It is important that this shaking should not be too prolonged as it causes inactivation of the enzyme. The dangling tubes are then dislodged, and the manometers rapidly shaken (at about 140 shakes/min.). Readings are taken every 5 min. for 35 min. The maximum rate of O_2 uptake, which usually occurs 10–15 min. after adding the reducing agent and is maintained for a further 10–15 min., is calculated. If the maximum rates from the eight manometers are $R_1, R_2, \ldots, R_8$, the activities (v) may be calculated as shown in Table 5.

Table 5. *Calculation of cytochrome oxidase activities*

(c = cytochrome *c* concentration (M); v = cytochrome oxidase activity (corrected).)

(c) (M)	$1/c$ (M^{-1})	Activity (uncorr.)	Blank	Activity (corr.) (v)	$1/v$
2×10^{-5}	$0{\cdot}5\times10^{5}$	R_1	R_5	R_1-R_5	$1/(R_1-R_5)$
3×10^{-5}	$0{\cdot}33\times10^{5}$	R_2	R_6	R_2-R_6	$1/(R_2-R_6)$
4×10^{-5}	$0{\cdot}25\times10^{5}$	R_3	R_7	R_3-R_7	$1/(R_3-R_7)$
6×10^{-5}	$0{\cdot}167\times10^{5}$	R_4	R_8	R_4-R_8	$1/(R_4-R_8)$

The values for $1/v$, when plotted against $1/c$, should lie on a straight line; if this is extrapolated until it meets the $1/v$ axis at X, then the cytochrome oxidase activity $= 1/X$.

DISCUSSION

When a reducing agent (RH)* and cytochrome *c* are added to a cytochrome oxidase preparation, two consecutive reactions occur, viz.

$$Fe^{+++}\text{ cyt. } c + RH \rightarrow Fe^{++}\text{ cyt. } c + R + H^+, \quad (1)$$

$$Fe^{++}\text{ cyt. } c + \tfrac{1}{4}O_2 + H^+ \xrightarrow{\text{cyt. ox.}} Fe^{+++}\text{ cyt. } c + \tfrac{1}{2}H_2O. \quad (2)$$

Net reaction: $RH + \tfrac{1}{4}O_2 \rightarrow R + \tfrac{1}{2}H_2O$,

in which Fe^{+++} cyt. *c* and Fe^{++} cyt. *c* are the oxidized and reduced forms of cytochrome *c*. Reaction (1) is the non-enzymic reduction of cytochrome *c*, and reaction (2) is the enzymic oxidation.

Cytochrome oxidase preparations, to which cytochrome *c* has been added, will catalyze the oxidation of any substance which can reduce cytochrome *c* sufficiently rapidly. Keilin (1930) showed that cysteine is such a substance, and other thiols would be expected to behave similarly. This has, indeed, been shown to be the case with the dithiol, 2:3-dimercaptopropanol (Webb & van Heyningen, 1947) and with sodium diethyldithiocarbamate (Keilin & Hartree, 1940). Ames & Elvehjem (1945) have found that cytochrome *c* + kidney homogenate catalyzes the oxidation of glutathione, but believe that this is due to the presence of a glutathione dehydrogenase. Since pure glutathione rapidly reduces pure cytochrome *c*, there does not seem any need to postulate the existence of such a dehydrogenase. In a later paper, Ames, Ziegenhagen & Elvehjem (1946) claim to have shown that inhibitors act differently on the systems involved in the oxidation of glutathione and ascorbic acid respectively. This question requires further investigation, since Ames *et al.* measured the rate of oxidation of glutathione by determining the rate of O_2 uptake, and used as enzyme an unwashed kidney homogenate which has an appreciable endogenous respiration and probably also contains an appreciable concentration of metals which might catalyze the oxidation of glutathione and ascorbic acid. Moreover, many of the inhibitors used, e.g. diethyldithiocarbamate, are themselves rapidly oxidized by tissue preparations in the presence of cytochrome *c*, and the degree of inhibition calculated will depend largely on the manner of correcting for this oxidation. The method of making this correction is not stated by these workers. There seems to be no more evidence for the existence of a glutathione dehydrogenase than for dehydrogenases activating *p*-phenylenediamine, hydroquinone, cysteine, ascorbic acid, etc.

It has been known for many years that tissue preparations are able to catalyze the oxidation of *p*-phenylenediamine. Keilin & Hartree (1938) showed that the addition of cytochrome *c* to the tissue preparation resulted in a greatly increased

* Since the catalyzed rate of oxidation of a reducing agent is actually measured, the latter is often termed the 'substrate' of the reaction. Such a terminology has been responsible for a confusion of thought, since the only enzyme substrate in the two reactions is cytochrome *c* which is oxidized by its specific oxidase, cytochrome oxidase. The reducing agent is not the substrate of an enzyme; its function is to reduce cytochrome *c* by a non-enzymic reaction.

rate of oxidation, and suggested that the oxidation in the absence of added cytochrome *c* was due to the cytochrome *c* present in the tissue preparation; it was considered that this cytochrome *c* was accessible to *p*-phenylenediamine but not to the other reducing agents, since the latter were not oxidized unless cytochrome *c* was added. Keilin & Hartree's experiments did not exclude the possibility that enzyme preparations contained an alternative pathway for the oxidation of *p*-phenylenediamine, independent of cytochrome *c* and cytochrome oxidase. Stotz *et al.* (1938*b*) suggested that cytochrome *b* offered such a pathway, since it could be reduced by *p*-phenylenediamine and the oxidation of this compound by tissue preparations was, in contrast to that of hydroquinone, not completely inhibited by cyanide. Since Borei (1945), Laki (1938) and the writer have failed to confirm these findings, this explanation seems unlikely. Another possible catalyst for the oxidation of *p*-phenylenediamine by heart-muscle preparation is a copper-containing enzyme, like laccase. It is, however, most unlikely that heart-muscle preparation contains such an enzyme, since substances such as catechol, which are readily oxidized by laccase, are not oxidized by heart-muscle preparation. The present investigation has provided strong evidence in favour of Keilin & Hartree's view, viz. that *p*-phenylenediamine is oxidized in the heart-muscle preparation only by the cytochrome *c*-cytochrome oxidase system. This evidence is: (1) the spectroscopic observation that the cytochrome *c* in the heart-muscle preparation is much more rapidly reduced by *p*-phenylenediamine than by other reducing agents, which explains the ability of the preparation to oxidize *p*-phenylenediamine in the absence of added cytochrome *c*; (2) an abnormal heart-muscle preparation, which contained very little cytochrome *c*, did not catalyze the oxidation of *p*-phenylenediamine, unless cytochrome *c* was added; (3) a kidney preparation, which did not rapidly oxidize succinate unless cytochrome *c* was added, also required the addition of cytochrome *c* for the oxidation of *p*-phenylenediamine; (4) the activity of the cytochrome oxidase, measured by extrapolation to infinite cytochrome *c* concentration, was the same when *p*-phenylenediamine was used as the reducing agent as when ascorbic acid was used.

Stotz *et al.* (1938) and Borei (1945) have concluded that cytochrome *c* forms with cytochrome oxidase a dissociable complex of the same type as postulated by Michaelis to occur between a soluble enzyme and its substrate. The only evidence on which they base this conclusion is that the effect of cytochrome *c* on the activity can be described by the Michaelis-Menten equation, which, when expressed in the modified form of Lineweaver & Burk (1934), postulates that the inverse of the activity bears a linear relationship to the inverse of the substrate concentration. However, it does not necessarily follow from such a relationship, which has also been obtained in the present work, that a dissociable complex is formed; the only conclusion which can be drawn with validity is that the relationship velocity-substrate concentration is a rectangular hyperbola. There are, in fact, several objections to the view of Stotz *et al.* and Borei, viz. (1) An assumption made in deriving the Michaelis-Menten equation is that the rate of formation and dissociation of the complex is rapid compared with its decomposition into the products of the enzymic reaction. It seems unlikely that such a rapid equilibrium would be established between the insoluble cytochrome oxidase attached to the particles of the heart-muscle preparation and cytochrome *c* of molecular weight 16,500.

(2) This view does not explain the fact that the rate of O_2 uptake depends upon the particular reducing agent and on its concentration as well as on the concentration of cytochrome *c*. Since the sole function of this reducing agent is to reduce the cytochrome *c*, it follows that the rate of reduction of cytochrome *c*, as well as its oxidation, is important. The rate of reduction would be augmented by increases in the concentration both of reducing agent and of cytochrome *c*. It is also much greater when *p*-phenylenediamine is used as the reducing agent than when other reducing agents are used.

(3) According to the Michaelis-Menten equation, the effect of substrate concentration (i.e. $1/(\text{cyt.}c)_{\frac{1}{2}}$) should be independent of the enzyme concentration. Fig. 12 shows, however, that $1/(\text{cyt. } c)_{\frac{1}{2}}$ is decreased 45% by doubling the enzyme concentration.

The effect of the cytochrome *c* concentration is therefore much more complicated than that postulated by Stotz *et al.* and by Borei, even though, at any one enzyme concentration, the activities obey the Michaelis-Menten equation. But this equation, which is that of a rectangular hyperbola, merely expresses the finding that at low cytochrome *c* concentrations the rate of O_2 uptake is proportional to the cytochrome *c* concentration, while at high concentrations the rate of O_2 uptake becomes independent of the cytochrome *c* concentration and is dependent upon the activity of the cytochrome oxidase. Such a relationship would be expected, for example, if the rate of reduction of cytochrome *c* was the limiting factor at low cytochrome *c* concentrations. Indeed, at a fixed cytochrome *c* concentration the relationship between the rate of O_2 uptake and the ascorbic acid concentration is a rectangular hyperbola, and this can be explained by assuming that the rate of reduction of cytochrome *c* by ascorbic acid is the limiting factor at

low ascorbic acid concentrations. This cannot, however, be the complete explanation of the effect of cytochrome *c* on the rate of O_2 uptake, since the activity at a definite ascorbic acid concentration and infinite cytochrome *c* concentration is considerably greater than that at a definite cytochrome *c* concentration and infinite ascorbic acid concentration.

It is probable that the rate of diffusion of cytochrome *c* to and from the cytochrome oxidase is a limiting factor at low concentrations of the former. It has already been mentioned that the ascorbic acid is unable to reduce rapidly the cytochrome *c* in the heart-muscle preparation, although this cytochrome *c* can be rapidly reduced by succinate and also, to a considerable degree, by *p*-phenylenediamine. It is likely then, that the cytochrome *c* in the immediate vicinity of the cytochrome oxidase can be readily oxidized by the oxidase, but cannot be easily reduced by ascorbic acid. The oxidized cytochrome *c* diffuses away and is replaced by a neighbouring cytochrome *c* molecule, which has been reduced by the ascorbic acid in solution. If the rate of this diffusion is a limiting factor, it would be understandable that the nearness of available molecules, which would depend on the cytochrome *c* concentration, would be important. This would also explain why the rate of reduction would be important; the rate of reduction must be sufficiently rapid so that the few molecules of cytochrome *c* in the immediate vicinity of the cytochrome oxidase are rapidly reduced; the turn-over rate would have to be very much more rapid than that which might be calculated from the observed O_2 uptake and the concentration of cytochrome *c* in the solution as a whole. The rate of reduction of cytochrome *c* would be augmented by increases in the concentration of both reducing agent and cytochrome *c*. The importance of the reducing agent is shown in Fig. 7B; thus the apparent Michaelis constant was equal to $1{\cdot}2 \times 10^{-5}$ M of added cytochrome *c* with *p*-phenylenediamine and $6{\cdot}1 \times 10^{-5}$ M with ascorbic acid. The maximum activity, at infinite cytochrome *c* concentration, was, however, independent of the reducing agent. The rate of reduction of cytochrome *c* in solution by the concentrations of ascorbic acid and *p*-phenylenediamine used is very rapid, and there is no evidence that *p*-phenylenediamine reduces such cytochrome *c* more rapidly than does ascorbic acid. The greater effectiveness of low concentrations of added cytochrome *c* in the presence of *p*-phenylenediamine is probably due to the ability of the latter to penetrate more closely than ascorbic acid to the added cytochrome *c* in the immediate neighbourhood of the cytochrome oxidase. One must postulate some such difference between these two reducing agents to explain the relative rates of reduction of the endogenous cytochrome *c* of heart-muscle preparation.

When the rate of reaction is dependent, not only on the activity of the enzyme system responsible for the oxidation of the cytochrome *c*, but also on the rate of supply of the reduced cytochrome to the system, it is not difficult to see that doubling the enzyme concentration might not double the rate of O_2 uptake (Fig. 12), since the extra cytochrome oxidase 'molecules' would compete for the supply of reduced cytochrome *c*. At infinite cytochrome *c* concentration, however, the rate of oxidation is exactly doubled.

The suggestion that it is the cytochrome *c* in the immediate vicinity of the heart-muscle particles which is concerned in the reaction, not the cytochrome *c* in the bulk of the solution, explains the apparently conflicting observations that (1) the O_2 uptake depends on the ascorbic acid concentration, suggesting that the rate of reduction is a limiting factor, and (2) when ascorbic acid is added to a solution of oxidized cytochrome *c* containing a suspension of heart-muscle preparation, the cytochrome *c* is immediately reduced and remains reduced even when the solution is vigorously aerated. According to the above explanation, the cytochrome *c* in the bulk of the solution would remain reduced, although that in the immediate vicinity of the particles of the heart-muscle preparation was largely in the oxidized form. When succinate is added instead of ascorbic acid, the bulk of the cytochrome *c* remains oxidized even though the succinic oxidase activity is less than that of cytochrome oxidase. This is because, in this case, the reduction of cytochrome *c* occurs not in the bulk of the solution, but on the particles of the heart-muscle preparation.

The catalytic activity of the added cytochrome *c*, expressed by the value of $1/(\text{cyt. } c)_{\frac{1}{2}}$, is affected by (1) the rate of diffusion of cytochrome *c* to the oxidase, (2) the rate of reduction of cytochrome *c* by the reducing agent, and (3) the accessibility of the reducing agent to the added cytochrome *c* in the neighbourhood of the cytochrome oxidase. Phosphate concentration has a very marked effect on $1/(\text{cyt. } c)_{\frac{1}{2}}$, but little if any effect on $1/(\text{asc. acid})_{\frac{1}{2}}$ (at least between 0·065 and 0·146 M). This suggests that it has little influence on the rate of reduction of cytochrome *c* by ascorbic acid or on the accessibility of ascorbic acid to the cytochrome *c*; consequently its effect on $1/(\text{cyt. } c)_{\frac{1}{2}}$ must be due to an action on the rate of diffusion of cytochrome *c* to the oxidase, caused probably by an alteration of the colloidal particles of the heart-muscle preparation.

The figures in the present paper show that the true activity (Q_{O_2}) of the cytochrome oxidase in the heart-muscle preparation, viz. 3400, is much higher than that previously reported by Keilin & Hartree (1947*a*), viz. 1420. The reasons for this discrepancy are: (1) the activity measured by Keilin & Hartree's

method ($5{\cdot}4 \times 10^{-5}$M cytochrome *c*, 0·1M-phosphate) is less than half the maximal activity, and (2) the activities calculated by Keilin & Hartree are based on the weight of the dried whole preparation, while the figures in the present paper are based on the weight of the fat-free dried preparation, which is 30% less than the total weight of the dried preparation. It should be noted that the 1947 preparation of Keilin & Hartree* is very much more active than the 1938 preparation, which, according to Keilin & Hartree (1938), has a Q_{O_2} of 181 with *p*-phenylenediamine, and 128 with hydroquinone. This greatly increased activity is due to the introduction, in the method of preparation in 1947, of precipitation with acid at about 0°. Most of the published work on cytochrome oxidase has been performed with preparations the same as, or similar to, the 1938 Keilin & Hartree preparation.

It has been pointed out above that faulty conclusions regarding the effect of treatments of the enzyme preparation on the cytochrome oxidase may be drawn if the activity of the latter is measured at only one cytochrome *c* concentration. An inhibition of the rate of O_2 uptake measured at one cytochrome *c* concentration may be due either to an inhibition of the cytochrome oxidase or to an effect on the catalytic activity of the added cytochrome *c*. Which of these two factors is affected can be determined only by carrying out the experiment at different cytochrome *c* levels. Such a procedure may also be necessary to detect a partial inhibition of the oxidase since, at low concentrations of cytochrome *c*, this concentration and not the activity of the cytochrome oxidase may be the factor limiting the rate of O_2 uptake. Keilin & Hartree (1938) pointed out that this was the explanation for the small effect of carbon monoxide on the rate of oxidation of *p*-phenylenediamine in the absence of added cytochrome *c*.

Borei (1945) has made a comprehensive study of the action of fluoride on the cytochrome oxidase system. He found that fluoride, in fairly high concentration, inhibited the rate of O_2 uptake of various reducing agents in the presence of heart-muscle preparation and cytochrome *c*, but that this inhibition disappeared on extrapolation to infinite cytochrome *c* concentration. Borei concluded that sodium fluoride inhibits the rate of O_2 uptake by combining with the cytochrome *c* and so competing with the oxidase for its substrate. However, he was unable to produce any direct evidence for the formation of such a compound, since there was no alteration of the absorption spectrum of cytochrome *c* or of its rate of reduction by reducing agents when fluoride was added. It is clear from the above discussion that an alternative explanation of the inhibiting effect of fluoride on the rate of O_2 uptake must be considered, viz. that fluoride reduces the catalytic activity of cytochrome *c* in the system, not by combining with cytochrome *c* but by an effect on the enzyme preparation, in much the same manner as that of phosphate. It may be significant that, according to Borei, the inhibition due to fluoride is increased by high phosphate concentrations.* The question whether the effect of phosphate and fluoride is due to combination at a specific point in the cytochrome oxidase system, e.g. with magnesium as is the case with enolase (Warburg & Christian, 1942), or is due to a non-specific effect on the particles of the heart-muscle preparation, must await further investigation.

Most studies of enzyme kinetics have been made with a simple system consisting of the enzyme protein and its substrate of small molecular weight. The system reducing agent + cytochrome *c* + heart-muscle preparation provides a useful method of studying reactions between a substrate which is itself a protein and its enzyme, which is part of the macromolecular complex constituting the particles of the heart-muscle preparation. Two conclusions of general interest may be drawn from the present work: (1) that in such a system the rate of diffusion of the protein substrate to and from its enzyme may limit the rate of the reaction, and (2) that the protein substrate when attached to its enzyme may not be readily accessible to other reagents.

SUMMARY

1. It has been found that, under the conditions usually employed in determining cytochrome oxidase activity, the rate of O_2 uptake depends not only on the cytochrome oxidase activity but also on the concentrations of cytochrome *c* and reducing agent and on the catalytic activity of the cytochrome *c*.

2. A procedure suitable for studying the true activity of cytochrome oxidase is described. It is necessary to measure the rate of oxidation at different cytochrome *c* concentrations and extrapolate to infinite concentration.

3. Most of the reducing agents used for the estimation of cytochrome oxidase activity reduce the cytochrome *c* present in the heart-muscle preparation so slowly that their rate of oxidation is negligibly small, unless cytochrome *c* is added. *p*-Phenylenediamine, however, rapidly reduces this cytochrome *c*, and is rapidly oxidized by the heart-muscle preparation; this oxidation is, however, also strongly catalyzed by the addition of cytochrome *c*. The heart-muscle preparation does not possess a pathway for the oxidation of *p*-phenylenediamine additional to the cytochrome oxidase system.

* This improved method of preparation, although not described until 1947, was used by Keilin & Hartree in 1940, when they reported a cytochrome oxidase activity of 1400.

* Borei also found the inhibiting effect of increasing the phosphate concentration in the absence of fluoride.

4. Catechol and adrenaline, which are sometimes used as reducing agents for the measurement of cytochrome oxidase activity, are not satisfactory for this purpose.

5. The concentration of phosphate buffer has a very important effect on the rate of oxidation of *p*-phenylenediamine and ascorbic acid by heart-muscle preparation.

6. It is probable that the added cytochrome *c* when attached to its enzyme cannot be readily reduced by ascorbic acid. It is suggested that the effect of the concentrations of cytochrome *c* and reducing agent on the rate of O_2 uptake can be explained by assuming that the rate of diffusion of cytochrome *c* to and from its enzyme may limit the rate of the overall reaction.

7. The effect of fluoride on the rate of O_2 uptake found by Borei is probably due not to combination of cytochrome *c* with fluoride, as suggested by Borei, but to an effect of fluoride on the particles of the enzyme preparation, causing an impairment of the catalytic activity of added cytochrome *c*.

8. The true cytochrome oxidase activity of Keilin & Hartree's heart-muscle preparation is expressed by a Q_{O_2} (based on a fat-free dry weight) of 3400 at 38°, which is much higher than previously reported.

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Studies on the Absorption of Proteins: the Amino-acid Pattern in the Portal Blood

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Although dietary protein is certainly the source of body protein, the steps by which it is transferred from the ingested food to the tissues are still incompletely known. In particular, there is considerable doubt about the form in which protein is absorbed from the gut into the portal blood stream, and the possible effect of the liver in altering the products of protein digestion on their way to the tissues. The tacitly accepted view is that the protein is completely broken down into free amino-acids in the gut. These are absorbed into the blood stream and carried to the tissues, each of which withdraws such amino-acids from the blood as it may require for the synthesis of its own proteins. This view is based

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