Complex-Formation between Cytochrome *c* and Cytochrome *c* Peroxidase

KINETIC STUDIES

By PETER NICHOLLS AND EUGENE MOCHAN*

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K., and State University of New York at Buffalo, Buffalo, N.Y. 14214, U.S.A.

(Received 18 June 1970)

1. The kinetics of ferrocytochrome *c* peroxidation by yeast peroxidase are described. Kinetic differences between the older and more recent preparations of the enzyme most probably arise from differences in intrinsic turnover rates. 2. The time-courses of cytochrome *c* peroxidation by the enzyme follow essentially first-order kinetics in phosphate buffer. Deviations from first-order kinetics occur in acetate buffer, and are due to a higher enzymic turnover rate in this medium accompanied by a greater tendency to autocatalytic peroxidation of cytochrome *c*. 3. The kinetics of ferrocytochrome *c* peroxidation by yeast peroxidase are interpreted in terms of a mechanism postulating formation of reversible complexes between the peroxidase and both reduced and oxidized cytochrome *c*. Formation of these complexes is inhibited at high ionic strengths and by polycations. 4. Oxidized cytochrome *c* can act as a competitive inhibitor of ferrocytochrome *c* peroxidation by peroxidase. The K_i for ferricytochrome *c* is approximately equal to the K_m for ferrocytochrome *c* and thus probably accounts for the observed apparent first-order kinetics even at saturating concentrations of ferrocytochrome *c*. 5. The results are discussed in terms of a possible analogy between the oxidations of cytochrome *c* catalysed by yeast peroxidase and by mammalian cytochrome oxidase.

Altschul, Abrams & Hogness (1940) reported the presence of a peroxidase in yeast rather specific for the oxidation of cytochrome *c*. It was a protohaematin enzyme, the spectrum of which was similar to that of other peroxidases, and it oxidized cytochrome *c* in a first-order process with the following stoichiometry:

It differed somewhat from other peroxidases not only in its specificity but also in being readily inhibited by high salt concentrations as well as by classical iron enzyme inhibitors such as cyanide. Subsequently Abrams, Altschul & Hogness (1942) prepared the enzyme in a purer form (mol.wt. approx. 60 000) and showed that it formed a 1:1 molar complex with hydrogen peroxide, resembling the (secondary) peroxide compound of horseradish peroxidase, in a reaction showing high affinity (K_d for H_2O_2 was $1 \mu\text{M}$).

Beetlestone (1960) confirmed the kinetic ob-

servations and extended them to show that the first-order characteristic could be due to product inhibition of the enzyme by ferricytochrome *c*. He interpreted his results in terms of the theory of Smith & Conrad (1956), who found that both ferrous and ferric cytochrome *c* inhibit cytochrome oxidase. Although other interpretations are possible (Minnaert, 1961), cytochrome *c* peroxidase undoubtedly resembled cytochrome oxidase in its kinetic behaviour.

The similarities between the peroxidase and the oxidase, and the different behaviour of horseradish peroxidase, were later extended by Nicholls (1964), who proposed the existence of an 'endogenous donor' in all three enzymes, the oxidation of which mediated the oxidation of cytochrome *c*. This resembled the ideas of Chance (1951) and Lemberg (1949), although it differed from the former in postulating complex-formation between cytochrome *c* and yeast peroxidase but not horseradish peroxidase.

Yonetani (1965) and Yonetani & Ray (1965, 1966) subsequently developed a greatly improved method for the preparation of the enzyme and showed that the enzyme had a much higher turnover number than had been previously reported,

* Present address: Department of Biochemistry, University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

and different kinetics. Thus time-courses of cytochrome *c* oxidation that approached zero order in their kinetics were observed. The K_i for ferri-cytochrome *c* was considerably larger than the K_m for ferrocytochrome *c*, suggesting that the two forms of the substrate did not react in analogous ways with the enzyme. Yonetani (1966a) also showed that the nature of the peroxide reaction differed from that with other peroxidases and catalase in that no intermediate corresponding to the primary compound occurred, the reaction with cytochrome *c* involving the reduction of a peroxide compound of the secondary type together with the reduction of a peroxide-induced free radical species (Yonetani, Schleyer & Ehrenberg, 1966).

With the new preparation of the enzyme, therefore, questions arose as to the possible existence of different forms of yeast peroxidase and the physiological significance of the differences. In addition, two analogies were apparently lost: (a) the analogy between the kinetics of cytochrome *c* peroxidase and cytochrome *c* oxidase (Nicholls, 1964), and (b) the analogy between the mechanisms of action of the various hydroperoxidases (Keilin & Nicholls, 1958).

The present paper attempts to show that kinetic differences between the new and classical preparations of enzyme most probably arise from differences in intrinsic turnover rates rather than in affinity for cytochrome *c*, and that the new and older observations may be reconciled in a kinetic mechanism that preserves a central role for reversible complexes between enzyme and both reduced and oxidized cytochrome *c*.

The following paper (Mochan & Nicholls, 1971) presents some direct evidence for the existence of such complexes, and discusses their role in cytochrome *c* peroxidase activity, and their relationship to the complexes formed between the haem of cytochrome *c* peroxidase and peroxides.

MATERIALS AND METHODS

Horse heart cytochrome *c* (Sigma type III) (Sigma Chemical Co., St Louis, Mo., U.S.A.) was employed either directly or after chromatography on Amberlite CG-50. Both samples behaved similarly. The oxidized form was prepared by the addition of a tenfold excess of potassium ferricyanide followed by dialysis against 0.5% NaCl. Ferrocytochrome *c* was prepared by the anaerobic gel-filtration method of Yonetani (1966b).

Cytochrome *c* peroxidase was prepared by slight modifications of both the method of Yonetani & Ray (1965) and that of Abrams *et al.* (1942). The enzyme prepared by the former method (method Y) had a purity index (E_{408}/E_{280}) of 1.0, was homogeneous in the analytical ultracentrifuge, and gave one band on disc electrophoresis. The latter method (method A) gave rise to a less pure enzyme with a purity index of 0.2. Spectroscopic properties of the enzyme as prepared in our laboratory are

given in the following paper (Mochan & Nicholls, 1971). The maximum velocity of cytochrome *c* peroxidation for both enzymes was a linear function of peroxidase concentration in the ranges studied.

Reactions were initiated by rapidly mixing 5 μ l of the enzyme into reaction mixtures containing ferrocytochrome *c* and H_2O_2 at 25°C. No appreciable initial oxidation of ferrocytochrome *c* was observed in the absence of enzyme or peroxide. The reaction was followed by measuring the decrease in extinction at 550 nm. The apparent first-order rate constant was obtained from the slope of the line extrapolated to zero time in a plot of the logarithm of the change in extinction against time. The initial velocities were calculated as the product of the first-order rate constant and initial ferrocytochrome *c* concentration. These initial velocities were a linear function of enzyme concentration in the range studied. The maximum turnover number (TN_{max}) was obtained by dividing V_{max} (mol of cytochrome *c* oxidized/s per mol of enzyme) at infinite cytochrome *c* concentration by the enzyme concentration.

In the inhibitor studies, the initial velocity of the reaction was measured in the presence and absence of inhibitor at various ferrocytochrome *c* concentrations. The K_i was then estimated from double-reciprocal plots (Lineweaver & Burk, 1934) or from plots of reciprocals of initial velocity against inhibitor concentration (Dixon, 1953).

In some cases kinetic constants were obtained from secondary plots according to the method of Dalziel (1957). Stopped-flow measurements were made with a Gibson stopped-flow apparatus (Durrum Instruments).

RESULTS

Comparison of the two types of enzyme preparation. The enzyme prepared by both method A (Abrams *et al.* 1942) and method Y (Yonetani & Ray, 1965) was examined kinetically, as described above. Fig. 1 illustrates the oxidation of reduced cytochrome *c* in phosphate buffer catalysed by the enzyme prepared by method A.

The oxidation follows first-order kinetics for at least 90% of the reaction. The time-course remains first-order at other phosphate concentrations (from 5 mM to 0.5 M) although the overall rate varies considerably. This is in agreement with previous observations (Altschul *et al.* 1940; Beetlestone, 1960; Chance, 1951). Fig. 2 gives the double-reciprocal plots obtained for reaction rates measured as in Fig. 1 at four concentrations of buffer. A dependence of both V_{max} and K_m on ionic strength is evident, and the values obtained are shown in Table 1.

No inhibition by excess of cytochrome *c* is seen up to 30 μ M-cytochrome *c*; the maximum turnover number for this preparation of peroxidase, however, is low (approx. $13s^{-1}$) even under 'optimum' conditions.

Fig. 3 shows that hydrogen peroxide does not inhibit at concentrations below 200 μ M. At 500 μ M-hydrogen peroxide, however, a decrease in reaction

Fig. 1. Time-course for cytochrome *c* oxidation by 'low turnover' cytochrome *c* peroxidase in phosphate buffer. The oxidation of cytochrome *c* was followed by observing the decrease in extinction at 550nm on the addition of 50nm-peroxidase (prepared by method A) to a solution containing the indicated concentration of ferrocytochrome *c* and 27 μM - H_2O_2 in 0.01M-potassium phosphate buffer, pH 7.0, at 25°C. No appreciable initial oxidation occurred in the absence of enzyme. Log ΔE corresponds to the logarithm of the change in extinction of cytochrome *c* at a given time minus the extinction of completely oxidized cytochrome *c*. The initial concentrations of ferrocytochrome *c* were: (a) 25.8 μM ; (b) 19.3 μM ; (c) 12.8 μM ; (d) 6.15 μM ; (e) 3.14 μM .

rate is observed. Table 2 summarizes the results of varying the hydrogen peroxide concentration in the presence of a fixed concentration of cytochrome *c* at both low and high phosphate concentrations. The increase in apparent affinity is due to the

Fig. 2. Lineweaver-Burk plots for cytochrome *c* oxidation by 'low turnover' cytochrome *c* peroxidase preparation. Initial velocities (*v*) at the indicated phosphate concentration, pH 7.0, were determined by multiplying the observed first-order rate constant (obtained from the slopes of plots as in Fig. 1) by the initial concentration of ferrocytochrome *c*. Solutions contained 27 μM - H_2O_2 and 50 nm-peroxidase (prepared by method A) at 25°C in: $\blacksquare$, 0.1M; $\circ$, 0.005M; $\blacktriangle$, 0.05M; $\bullet$, 0.5M-potassium phosphate buffer. The kinetic parameters from these plots are summarized in Table 1.

Table 1. *Effect of buffer concentration on the Michaelis constant and apparent V_{max} for cytochrome *c* reacting with 'low turnover' peroxidase*

The K_m and $\text{TN}(V_{\text{max}}/e)$ at the indicated concentrations of potassium phosphate buffer, pH 7.0, were estimated from Lineweaver-Burk plots similar to those illustrated in Fig. 2, where TN = turnover in mol of *c* oxidized/s per mol of enzyme at infinite [*c*]. Solutions contained 27 μM - H_2O_2 and 0.05 μM -cytochrome *c* peroxidase (prepared by method A) at 25°C.

Potassium phosphate concn. (M)	TN (s^{-1})	K_m (μM)	TN/K_m ($\text{M}^{-1} \text{s}^{-1}$)
0.005	9.0	1.7	5.3×10^6
0.01	13.3	2.0	6.7×10^6
0.03	12.6	6.7	1.9×10^6
0.05	6.2	8.0	0.8×10^6
0.1	2.8	18.0	0.2×10^6
0.3	1.7	20.0*	0.08×10^6
0.5	0.6	25.0*	0.02×10^6

* As in Fig. 2; other determinations at these high phosphate concentrations gave even greater K_m values (about 40 μM in 0.3M-phosphate and 50 μM or more in 0.5M-phosphate).

Fig. 3. Effects of peroxide concentration on cytochrome *c* oxidation. Rates of oxidation of $25\mu\text{M}$ -ferrocytochrome *c* (followed as described in Fig. 1) were observed at various concentrations of H_2O_2 , in 0.01M -potassium phosphate buffer, pH 7.0, at 25°C . The final concentration of peroxidase (prepared by method A) was 50nm . The turnover number (TN) was determined by dividing the initial velocity of ferrocytochrome *c* oxidation (first-order rate constant $\times$ initial concentration of ferrocytochrome *c*) by the final concentration of peroxidase.

Fig. 4. Effects of phosphate concentration on cytochrome *c* oxidation by 'low turnover' cytochrome *c* peroxidase. Turnover numbers (TN) and Michaelis constants (K_m) were obtained at various phosphate concentrations determined from plots in Fig. 2. The individual values are plotted as a function of the logarithm of potassium phosphate buffer concentration, pH 7.0; $\square$, $-\log K_m$; $\circ$, $\log \text{TN}$; Δ , $\log \text{TN}/K_m$. Reactions were observed with $27\mu\text{M}$ - H_2O_2 and 50nm -peroxidase (prepared by method A) at 25°C .

Table 2. Effect of buffer concentration on the Michaelis constant and apparent $V_{\max}$ for hydrogen peroxide reacting with 'low turnover' cytochrome *c* peroxidase

Solutions contained $0.05\mu\text{M}$ -peroxidase (prepared by method A, Table 1) and $27\mu\text{M}$ -ferrocytochrome *c* in the indicated concentration of potassium phosphate buffer, pH 7.0, at 25°C . The K_m and TN were obtained from Lineweaver-Burk plots, where $\text{TN} = \text{turnover in mol of cytochrome } c \text{ oxidized/s per mol of enzyme at infinite } [\text{H}_2\text{O}_2]$.

Potassium phosphate concn. (M)	K_m (μM - H_2O_2)	TN (s^{-1})
0.01	24.0	14.2
0.3	7.8	0.8

decrease in turnover of the enzyme. Raising the ionic strength has a greater effect on the reaction with cytochrome *c* than on that with hydrogen peroxide, as previously postulated (Beetlestone, 1960; Nicholls, 1964). This is borne out by direct measurements of k_1 at various ionic strengths with the 'high turnover' enzyme, described below. In Fig. 4 the values of both $V_{\max}$ and K_m obtained from primary plots such as those in Fig. 2 are plotted logarithmically against buffer concentration. Both $1/K_m$ and $V_{\max}$ decrease as the phosphate concentration increases, and the function $V_{\max}/K_m$ therefore decreases proportionately to the square of the buffer concentration.

As with cytochrome oxidase, the characteristic

Fig. 5. Direct demonstration of the inhibitory effects of ferri-cytochrome *c*. Initial velocities of ferrocytochrome *c* oxidation in the presence and absence of $17\mu\text{M}$ -ferri-cytochrome *c* were determined directly at $27\mu\text{M}$ - H_2O_2 , 50nm -peroxidase (prepared by method A) in 0.01M -potassium phosphate buffer, pH 7.0, at 25°C . $\bullet$, -Ferri-cytochrome *c*; $\blacksquare$, $+17\mu\text{M}$ -ferri-cytochrome *c*.

first-order appearance of the time-course (Fig. 1) seems attributable to an inhibition of the enzyme, otherwise saturated, by the product ferri-cytochrome *c*. Fig. 5 illustrates the competitive nature of this inhibition, with ferri-cytochrome *c* added before commencing the reaction.

The turnover of the enzyme prepared by method

Fig. 6. Time-course for cytochrome *c* oxidation by 'high turnover' cytochrome *c* peroxidase in (a) phosphate and (b) acetate buffer. (a) The oxidation of cytochrome *c* was followed by observing the decrease in extinction at 550 nm on the addition of 1.7 nm-peroxidase (prepared by method Y) to a solution containing the indicated concentration of ferrocytochrome *c* and $27 \mu\text{M}$ - H_2O_2 in 0.01 M-potassium phosphate buffer, pH 7.0, at 25°C. No appreciable initial oxidation occurred in the absence of enzyme. $\log \Delta E$ corresponds to the logarithm of the change in extinction of cytochrome *c* at a given time minus the extinction of completely oxidized cytochrome *c*. The initial concentrations of ferrocytochrome *c* were: (a) $45 \mu\text{M}$; (b) $23 \mu\text{M}$; (c) $10.7 \mu\text{M}$; (d) $3.4 \mu\text{M}$; (e) $1.83 \mu\text{M}$. (b) Time-courses were followed as described above except that $170 \mu\text{M}$ - H_2O_2 was used in 0.05 M-sodium acetate, with 0.42 nM-peroxidase, pH 6.0, 25°C. Initial concentrations of ferrocytochrome *c* were: (a) $49.3 \mu\text{M}$; (b) $35.9 \mu\text{M}$; (c) $26.4 \mu\text{M}$; (d) $10.4 \mu\text{M}$; (e) $2.58 \mu\text{M}$.

Y is known to be much higher. It was therefore of interest to examine its kinetic behaviour towards ionic strength and ferricytochrome *c* accumulation. Fig. 6(a) shows the time-course of cytochrome *c* oxidation by this preparation of the enzyme. Deviations from first-order behaviour are very small. However, if the same enzyme is assayed in acetate buffer, as recommended by Yonetani & Ray (1966), the results of Fig. 6(b) are obtained. In phosphate buffer, whether at pH 6 or at pH 7, the oxidation of cytochrome *c* remains first order for at least 70% of the reaction. In acetate, however, the reaction is of a mixed order (between zero- and first-order), mimicking first-order behaviour for no longer than 40% of the reaction. A partial explanation of this phenomenon, based on the observations of Mochan & Degn (1969), is advanced below (see the Discussion section). As found by Yonetani & Ray (1966), the initial enzymic reaction rate is considerably higher in an acetate buffer than in a phosphate buffer of the same pH.

Fig. 7 shows that the initial oxidation kinetics given in phosphate buffer are very similar to those

given by the enzyme prepared by method A (Fig. 2), and the K_m values are also closely similar (Table 3). However, the maximal turnover numbers obtained are some 30 times greater under all the conditions used (cf. Tables 1 and 3).

In both acetate and phosphate buffers there is evidence for binding of ferricytochrome *c*, although in the former case the affinity may be slightly lower than that shown for the substrate (ferrocytochrome *c*). Table 4 lists the calculated values for both K_m and K_i illustrating the inhibitory action of the product. In phosphate buffer the K_i for ferricytochrome *c* is equal to the K_m for ferrocytochrome *c*. In acetate buffer, K_i is somewhat greater than K_m , a finding which indicates a second reason for the differences in turnover and time-course between the two assay media, in addition to the autocatalytic peroxidation discussed below.

As with the enzyme prepared by method A, inactivation by excess of peroxide does not occur at concentrations of the latter up to $200 \mu\text{M}$. Below that concentration, a linear relationship is obtained by plotting the reciprocal of velocity against the

Fig. 7. Effects of phosphate concentration on cytochrome *c* oxidation. (a) Lineweaver-Burk plots. Initial velocities (v) at the indicated phosphate concentrations, pH 7.0, were determined by multiplying the observed first-order rate constant (obtained from the slopes of plots in Fig. 6) by the initial concentration of ferrocytochrome *c*. Solutions contained $27 \mu\text{M}$ - H_2O_2 and 1.7 nM -peroxidase (prepared by method Y) at 25°C in: $\circ$, 0.01 M ; $\square$, 0.03 M ; $\triangle$, 0.1 M ; $\bullet$, 0.3 M ; $\blacksquare$, 0.5 M -potassium phosphate buffer. The kinetic parameters from these plots are summarized in Table 3. (b) Secondary plots. Turnover number (TN) and Michaelis constant (K_m) at various phosphate concentrations were determined from the primary plots in Fig. 7(a), and listed in Table 3. Values are represented as a function of the logarithm of potassium phosphate concentration: $\blacksquare$, $-\log K_m$ (i.e. $\text{p}K_m$); $\bullet$, $\log \text{TN}$; $\triangle$, $\log \text{TN}/K_m$.

Table 3. Kinetic constants for cytochrome *c* oxidation obtained with 'high turnover' cytochrome *c* peroxidase

The K_m and TN ($V_{\text{max.}}/e$) were determined from Lineweaver-Burk plots. Solutions contained 1.7 nM -peroxidase (prepared by method Y) and the indicated concentrations of H_2O_2 and buffer at 25°C .

	Buffer concn. (M)	H_2O_2 (μM)	TN (s^{-1})	K_m (μM -cytochrome <i>c</i>)	TN/K_m ($\text{M}^{-1} \text{s}^{-1}$)
Potassium phosphate buffer (pH 7.0)	0.005	27	305	2.2	138×10^6
	0.01	27	460	2.3	200×10^6
	0.03	27	320	3.0	107×10^6
	0.05	27	215	5.7	37.9×10^6
	0.1	27	85	9.5	8.7×10^6
	0.3*	27	28	16.8	1.8×10^6
	0.5*	27	25	28.0	0.9×10^6
	0.01	170	445	1.5	296×10^6
	0.05	170	288	11.8	244×10^6
	0.3*	170	45	19.0	2.4×10^6
Sodium acetate buffer (pH 6)	0.01†	170	480	10.0	48.0×10^6
	0.05†‡	170	1500	7.0	214×10^6

* 42 nM -enzyme; † 0.42 nM -enzyme; ‡ pH 6.0.

reciprocal of peroxide concentration. The intercept of such a plot on the $1/v$ axis is a function of cytochrome *c* concentration, but the slope of the plot is independent of the concentration of cytochrome *c*. Yonetani & Ray (1966) have used the methods of Dalziel (1957) to analyse such data. The present observation that the value of the $1/V_{\text{max.}}$ intercept in the plots of v^{-1} against $[\text{hydrogen peroxide}]^{-1}$ approaches a finite value as the concentration of cytochrome *c* is increased confirms their finding that

a ternary complex is formed involving both cytochrome *c* and peroxide.

Direct and indirect measurement of kinetic constants. Two methods are available for calculating the individual rate constants in this reaction, the analysis of the steady-state equations with catalytic quantities of enzyme, and direct measurement of formation and decomposition of the enzyme-substrate complexes. The former calculations (see the Discussion section) lead to a value of $2.7 \times$

Table 4. *Inhibitory effects of ferricytochrome c on the oxidation of ferrocytochrome c by cytochrome c peroxidase*

Solutions contained 1.7 nM-peroxidase (prepared by method Y, Table 3) and the indicated concentrations of H₂O₂ and buffer at 25°C. The *K_i* values were determined by the indirect method described in the text. *K_m* values were obtained from Lineweaver-Burk plots under the same conditions (hence showing slight variations from the values in Table 3).

Buffer	Concentration (M)	H ₂ O ₂ (μM)	<i>K_m</i> (μM-cytochrome c ²⁺)	<i>K_i</i> (μM-cytochrome c ³⁺)	<i>K_i/K_m</i>
Potassium phosphate (pH 7.0)	0.005	27	2.9	2.4	0.8
	0.01	27	2.5	3.0	1.2
	0.03	27	6.4	6.0	0.9
	0.05	27	7.2	9.0	1.3
	0.1	27	12.7	7.0	0.6*
	0.3‡	27	25.0	25.0	1.0
	0.5‡	27	25.0	38.0	1.5
	0.01	170	1.4	2.0	1.4
	0.05	170	11.2	16.0	1.4
	0.3‡	170	17.0	27.0	1.6
	0.01†	170	11.5	10.0	0.9
	0.5§	170	8.8	26.0	2.95
Sodium acetate (pH 6.0)					

* Time-course curves up; † pH 6.0; ‡ 4.2 nM-enzyme; § 0.42 nM-enzyme.

Table 5. *Estimated values of rate constants for cytochrome c oxidation by peroxidase from steady-state analysis (according to the mechanism of eqn. 3)*

Reactions were carried out with 2.1 nM-cytochrome *c* peroxidase in 0.01 M-potassium phosphate buffer, pH 7.0, at 25°C, with both the H₂O₂ and ferrocytochrome *c* concentrations being varied. Comparable data obtained by Yonetani & Ray (1966) with horse cytochrome *c* (0.05 M-sodium acetate buffer, pH 6.0) and yeast cytochrome *c* (0.2 M-sodium acetate buffer, pH 6.0) are also listed: *k*₁, bimolecular rate of interaction of peroxidase with H₂O₂; *k*₂, bimolecular rate of interaction of peroxidase with cytochrome *c*; *k*₃, rate-limiting conversion of ternary complex into product; *k*₄, rate of dissociation of product (oxidized cytochrome *c*).

Conditions			<i>k</i> ₁	<i>k</i> ₂	<i>k</i> ₋₂	<i>k</i> ₃	<i>k</i> ₄	Reference
Enzyme	Cytochrome <i>c</i>	Buffer	(M ⁻¹ s ⁻¹)	(M ⁻¹ s ⁻¹)	(s ⁻¹)	(s ⁻¹)	(s ⁻¹)	
Method A	Horse	Phosphate*	—	3 × 10 ⁸	(600)¶	7	600	Present study
Method Y	Horse	Phosphate†	2.7 × 10 ⁷	3 × 10 ⁸	(600)	375	600	
Method Y	Horse	Acetate‡	—	2 × 10 ⁸	(600)	750	>600	
Method Y	Horse	Acetate‡	7 × 10 ⁷	3 × 10 ⁸	(600)	1300	>600	Yonetani & Ray (1966)**
Method Y	Yeast	Acetate§	[6 × 10 ⁷	2.8 × 10 ⁸	(~600)	7000	≥600	
Method Y	Yeast	Acetate	6 × 10 ⁷	2.8 × 10 ⁸	(~600)	≥600	7000	

* *k*₄ ≃ *k*₋₂ > *k*₃; *K_m* = *k*₋₂/*k*₂; *V*_{max.} = 2*k*₃ *e*.† *k*₄ ≃ *k*₋₂ > *k*₃; *K_m* = *k*₋₂/*k*₂; *V*_{max.} = $\frac{2k_3k_4}{k_3+k_4}$ *e*.‡ *k*₄ > *k*₃ > *k*₋₂; *K_m* = (*k*₋₂ + *k*₃)/*k*₂; *V*_{max.} = 2*k*₃ *e*.§ *k*₄ > *k*₃ > *k*₋₂; *K_m* = *k*₃/*k*₂; *V*_{max.} = 2*k*₃ *e*.|| *k*₃ > *k*₄ > *k*₋₂; *K_m* = *k*₄/*k*₂; *V*_{max.} = 2*k*₄ *e*.¶ Assuming *K_m* = *k*₋₂/*k*₂ at low turnover rates, where *k*₂ = 0.5 × 'apparent *k*₂' and *k*₃ = 0.5 × 'apparent *k*₃'.** The values are half those given by Yonetani & Ray (1966) because 2 molecules of cytochrome *c* are oxidized each complete enzyme cycle.

10⁷ M⁻¹ s⁻¹ for the reaction of peroxidase and hydrogen peroxide at pH 7.0 and 25°C (see Table 5). Fig. 8 illustrates the formation of the cytochrome *c* peroxidase-hydrogen peroxide compound measured in the stopped-flow apparatus.

The half-time for complex-formation was found

to be inversely proportional to the peroxide concentration, with a rate constant of 3.2 × 10⁷ M⁻¹ s⁻¹ (see Table 6). Under these conditions, the rate constant obtained at 425 nm was identical with that at 380 nm, indicating that any intermediate of the compound I type must have a half-life less than

Fig. 8. Reaction of cytochrome *c* peroxidase with peroxide (stopped-flow oscilloscope trace). A solution containing $3.7\mu\text{M}$ -peroxidase (prepared by method Y) in buffer was mixed with the same buffer containing the indicated concentration of H_2O_2 . The reactions were followed at 20°C in the stopped-flow apparatus, measuring the formation of the enzyme-peroxide compound at 425nm and the disappearance of free enzyme at 380nm . In (a) traces are presented for the reaction of $1.85\mu\text{M}$ -peroxidase with $2\mu\text{M}$ - H_2O_2 in 0.01M -potassium phosphate buffer, $\text{pH} 7.0$, at both wavelengths. In (b) traces are presented for the reaction of $1.85\mu\text{M}$ -peroxidase and $4\mu\text{M}$ - H_2O_2 in 0.01M - (—) and 0.3M - (·····) potassium phosphate buffer, $\text{pH} 7.0$, and in 0.05M -sodium acetate buffer, $\text{pH} 6.0$ (----), at 425nm .

1 ms. Chance (1967) has reported evidence for the existence of such an intermediate with a half-life of $30\mu\text{s}$.

Table 6 also shows that the effects of ionic strength and buffer composition that are described above are not associated with effects on k_1 , which remains relatively insensitive to such changes of the reaction medium.

Inhibition of cytochrome *c* peroxidase by polycations. Cytochrome oxidase is inhibited by polycations (Smith & Minnaert, 1965) as well as by oxidized cytochrome *c* itself. This inhibition is essentially competitive towards cytochrome *c* (Kabel, 1969). Such competitive inhibition by a substrate analogue classically provides evidence for the existence of reversible enzyme-substrate complexes. As reported by Beetlestone (1960), and indicated in

Fig. 9, cytochrome *c* peroxidase is also inhibited by polycations such as salmine and polylysine, but remains quite unaffected by other proteins (e.g. serum albumin). Horseradish peroxidase, on the other hand, which oxidizes cytochrome *c* by a similar mechanism but without forming reversible complexes with the cytochrome, is unaffected by such polycations (Nicholls, 1964).

A striking feature of Fig. 9 is the apparent increase in the first-order velocity constant with time. Since this increase also occurs in the absence of enzyme and is not specifically induced by the polycation, it arises from an autoperoxidatic action of ferricytochrome *c* (Mochan & Degn, 1969), as discussed below.

DISCUSSION

Comparison of the two enzyme preparations. Enzyme preparations made by both procedures (Abrams *et al.* 1942; Yonetani & Ray, 1965) have similar kinetic properties. In phosphate buffer, both enzymes oxidize cytochrome *c* with a first-order time-course and have similar K_m values for ferrocytochrome *c*. The reaction is inhibited by ferricytochrome *c*, potassium phosphate and polycations. Ferricytochrome *c* inhibition for both enzymes is competitive with respect to ferrocytochrome *c*. The K_i for ferricytochrome *c* is approximately equal to the K_m for ferrocytochrome *c*, and therefore most likely explains the observed first-order kinetics. The kinetic behaviour is relatively independent of the purity of the preparation; for throughout the course of purification, employing both methods, all fractions behaved in a similar manner towards cytochrome *c*. The deviations from first-order kinetics in cytochrome *c* peroxidation reported by Yonetani & Ray (1966), which suggested possible mechanistic differences between the two preparations and prompted the present investigation, arise most noticeably in acetate buffer. Mochan & Degn (1969) have shown that ferricytochrome *c* acts as a catalyst for the peroxidation of ferrocytochrome *c*, thereby giving rise to an autocatalytic reaction. Although the rate of this reaction is too slow to interfere with the enzymic rate at low ferricytochrome *c* concentrations (i.e. the initial rates) it does become significant in the terminal part of the reaction, as the concentration of ferricytochrome *c* builds up. This peroxidative reaction is 40% faster in acetate buffer than is phosphate buffer at $\text{pH} 6$. We believe that this phenomenon accounts in large part for the deviations from first-order behaviour that are most noticeable in acetate buffers.

However, some enzyme properties may vary depending on the isolation procedure. One such difference may be reflected in the titration behaviour

Table 6. Rate of cytochrome *c* peroxidase reaction with H₂O₂

The half-time (*t*₁), the corresponding first-order rate constant (*k* = 0.693/*t*₁) and the bimolecular rate constant (*k*₁) for the interaction of H₂O₂ and peroxidase were obtained with the Gibson stopped-flow apparatus. The final concentration of peroxidase (prepared by method Y, as in Table 3) in the present study was 1.85 μM in potassium phosphate buffer or sodium acetate buffer at 20°C. Values of *t*₁ were obtained directly from the photographs of traces (when Δ*A* is small, Δ%*T* ∝ Δ*A*).

λ (nm)	Conditions phosphate buffer pH 7.0	H ₂ O ₂ (μM)	<i>t</i> ₁ (ms)	<i>k</i> (s ⁻¹)	<i>k</i> ₁ (M ⁻¹ s ⁻¹)	Reference
425	0.01 M	2.0	11	62	3.2 × 10 ⁷	Present study
425	0.01 M	4.0	5.5	126	3.2 × 10 ⁷	Present study
425	0.30 M	4.0	6.0	115	2.9 × 10 ⁷	Present study
380	0.01 M	2.0	11	63	3.2 × 10 ⁷	Present study
acetate buffer						
	pH 6.0					
425	0.05 M	4.0	6.5	10.7	2.7 × 10 ⁷	Present study
426	?	60	—	—	~10.0 × 10 ⁷	Yonetani, Schleyer, Chance & Ehrenberg (1966)
395	?	375	0.075	930	2.5 × 10 ⁷	Chance (1967)
424	?	375	0.112	620	1.7 × 10 ⁷	Chance (1967)

Fig. 9. Inhibition of cytochrome *c* peroxidase by poly-cations. The oxidation of 24 μM-ferrocyanide *c* by 170 μM-H₂O₂ in 0.01 M-potassium phosphate buffer, pH 7.0, at 25°C was carried out with 2.5 nM-peroxidase (prepared by method Y): alone (○); in the presence of 10 μM-albumin (■); 10 μM-polylysine (▲); or 10 μM-saline (●).

with ferrocyanide (Mochan & Nicholls, 1971). Another difference lies in the turnover numbers for cytochrome *c* peroxidation.

The enzyme prepared by method A has a relatively low turnover number. Although the reasons for this difference have not been clearly resolved, some suggestions can be made. The possibility of haem degradation seems unlikely since the concentration of enzyme, in this study, was based on peroxide compound formation, presumably a reflection of catalytically active species. Neither is the rate of the reaction with hydrogen peroxide sufficiently low to account for the low turnover, as

hydrogen peroxide concentration was not limiting. An inhibitory contaminant in the enzyme prepared by method A is also improbable in view of the low concentrations of enzyme employed in the kinetic studies and the observed linear dependency of rate on enzyme concentration. Nicholls (1966) suggested that variations in horseradish peroxidase activity towards cytochrome *c* were due to variations in activity and accessibility of an oxidized 'endogenous donor'. A similar explanation may be advanced to explain the lower activities of some enzyme preparations.

Kinetic analysis of the reaction. The kinetic study of Yonetani & Ray (1966) showed that the peroxidation of cytochrome *c* by peroxidase proceeds via a 'ternary-complex mechanism'. They interpreted this oxidation as involving a compulsory-order mechanism. In agreement with these findings, our kinetic study has also indicated the formation of a rate-limiting complex involving both cytochrome *c* and enzyme. However, in view of other results reported here as well as the equilibrium studies reported separately (Mochan & Nicholls, 1971) we propose a random-addition mechanism involving reversible formation of both ferricytochrome *c*- and ferrocyanide *c*-enzyme complexes. Among the kinetic findings favouring this idea are the following: (a) the first-order time-courses of oxidation in phosphate buffers (indicating that *K_m* for substrate is equal to *K_i* for product); (b) the parallelism between the effects of ionic strength on both *K_m* and *K_i* (indicating that *K_m* and *K_i* may involve similar binding reactions); (c) the similarity of *K_m* values for cytochrome *c* between different enzyme preparations of varying turnover (indicating that *K_m* is more likely to represent a binding constant than a

ratio of kinetic constants); and (d) the interpretation of zero-order kinetics in acetate buffers as a consequence of autocatalytic peroxidation of cytochrome *c* superimposed upon an otherwise first-order enzymic reaction.

Each of these points is in accord with the view that the observed kinetic constants associated with the ferrous and ferric forms of cytochrome *c* are of similar origin. As the binding of ferricytochrome *c* with the free enzyme is reversible and competitive towards ferrocyanochrome *c*, we conclude that the binding of the latter is also a reversible process that can occur with free enzyme as well as the peroxide complex(es). However, if this is the case, it is necessary to assume that the initial reaction of free enzyme with peroxide is not affected by the combination with either form of cytochrome *c*. The value of k_1 for the reaction of enzyme with hydrogen peroxide is the same whether calculated from initial rates at low peroxide and high cytochrome *c* concentrations with catalytic amounts of enzyme, or by direct measurement in the absence of cytochrome *c* in the stopped-flow system (above). Moreover, the presence of oxidized cytochrome *c* does not affect the titration of enzyme and hydrogen peroxide (Yonetani, 1965).

The overall reaction (eqn. 1) may be considered as brought about by the general mechanism of Scheme 1, where S = hydrogen peroxide and EP is a hypothetical one-equivalent oxidation state of the enzyme (Mochan & Nicholls, 1971).

This mechanism includes the following concepts: (a) only one molecule of cytochrome *c* can react with a molecule of enzyme at one time; (b) redox reactions are effectively irreversible; (c) there is at least one form of a one-equivalent oxidation state (and possibly two interconvertible forms, characterized by the electron spin resonance and optical spectra respectively); (d) bimolecular reactions with cytochrome *c* are binding reactions and not redox processes.

As the reaction with hydrogen peroxide is effectively irreversible (Yonetani & Ray, 1965), the binding of this substrate is independent of the

amount of cytochrome *c* present, both in this scheme and in the ordered mechanism. Scheme 1 and the ordered (irreversible) mechanism may thus be distinguished from other mechanisms in which this would not be the case. For example, if peroxide were a reversibly bound first substrate, the resulting ordered mechanism would require that increasing cytochrome *c* concentration decreased the apparent K_m indefinitely. Conversely, if peroxide were bound irreversibly as in Scheme 1, but reacted only with free enzyme, the experimental value of k_1 would be a function of the concentrations of ferro- and ferricytochrome *c*, decreasing as the binding of these species removed free enzyme from the system. At low peroxide concentrations, therefore, Scheme 1 indicates that the reaction rate is given simply by the expression $k_1 se$ (where *e* is the total enzyme present). As with the mechanism of Yonetani & Ray (1966), initial-rate measurements give us the value of k_1 directly.

Scheme 1 is, however, rather too complex for a practical steady-state expression to be derived describing the reactions with cytochrome *c*. In the general case, such mechanisms give non-linear reciprocal plots ($1/v$ versus $1/s$). Although such plots can sometimes be observed experimentally, they are a poor mechanistic test because such non-linearity can also arise for trivial reasons. It is possible to simplify the postulated reaction pathway (a) by setting certain rates and equilibria as equal (all K_m and K_1 values), and (b) by considering only the case where the reactions of E, Ec^{3+} and Ec^{2+} with hydrogen peroxide are fast. The kinetics then become formally very similar to those for the oxidase discussed by Minnaert (1961).

The 'ordered' and 'random' mechanisms are now indistinguishable, but this simplified form of Scheme 1 may be distinguished from the model of Yonetani & Ray (1966) by retaining the dissociation of Ec^{3+} (the complex of oxidized cytochrome *c* and free enzyme) as the final step (k_4) and by postulating a large finite value for k_{-2} (eqn. 2 below). The reaction mechanism is now that of eqn. (2):

Steady-state analysis of eqn. (2) gives the relationship of eqn. (3):

$$v = \frac{2k_3 k_4}{k_3 + k_4} \frac{e[c^{2+}]}{k_4(k_{-2} + k_3) + [c^{2+}]} \quad (3)$$

Here:

$$V_{\max.} = \frac{2k_3 k_4 e}{k_3 + k_4} \quad \text{and} \quad K_m = \frac{k_4(k_{-2} + k_3)}{k_2(k_4 + k_3)}$$

Scheme 1.

Although the intermediate ES compound (Scheme 1) oxidizes two molecules of cytochrome *c*, any obligatory formation of a complex with two ferrocytochrome *c* molecules is eliminated by the linear Lineweaver-Burk plots (Figs. 2 and 7). Eqn. (2) is only acceptable, however, if the two sets of rate constants for the two molecules of cytochrome *c* (Scheme 1) are equal. There is some evidence that this is not the case (Mochan & Nicholls, 1971). Eqn. (3) is nevertheless adequate for the initial description of the results, and can be used to describe the kinetics of both types of preparation.

The K_m (eqn. 3) is a function of four velocity constants, but it may approximate a true dissociation constant (k_{-2}/k_2) if either $k_{-2} = k_4$, implying that the dissociation constant is independent of the oxidation state of cytochrome *c*, or k_{-2} and k_4 are both much greater than k_3 . In the latter case V_{max} simplifies to $2k_3e$ (the rate of the intramolecular electron-transfer reaction), and eqn. (3) becomes eqn. (4):

$$v = \frac{2k_3e[c^{2+}]}{k_{-2}/k_2 + [c^{2+}]} \quad (4)$$

If the increase in K_m with increasing salt concentration (Tables 1 and 3) is due to mutual competition between small cations and positively charged cytochrome *c*, then the observed value of k_2 may be expected to decrease with increasing potassium concentration. K_m would then increase. Such a result is indicated in Fig. 4, where K_m is seen to be proportional to phosphate concentration. An additional effect of buffer concentration on turnover (k_3), such that k_3 is proportional to [phosphate]⁻¹ (Fig. 4 and Tables 1 and 3), gives rise to the relationship that k_3k_2/k_{-2} is proportional to [phosphate]⁻². This applies to the rate of cytochrome *c* oxidation at low cytochrome *c* concentration (Fig. 4). This pattern of buffer effects argues in favour of eqn. (4) and against an interpretation in terms of a largely steady-state origin of K_m , such as that implied by the model of Yonetani & Ray (1966). In Table I of that paper, k_2 is given as $5.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, k_3 as $2.6 \times 10^3 \text{ s}^{-1}$ and K_m as $4.5 \mu\text{M}$ for cytochrome *c*. Thus K_m does not appreciably exceed k_3/k_2 in their calculations.

Applying eqns. (3) and (4) to the experimental results of Figs. 2, 4 and 7 as summarized in Tables 1 and 3, we obtain the results listed in Table 5. For comparison, the values obtained by Yonetani & Ray (1966) with both yeast and horse cytochrome *c* are also listed. Since one molecule of cytochrome *c* peroxidase-hydrogen peroxide compound oxidizes two molecules of ferrocytochrome *c* the following relationships exist between the observed constants and the rate constants given by the simplified eqn. (3): $k_1 = 0.5 \times \text{apparent } k_1$; $k_2 = 0.5 \times \text{apparent } k_2$ when the two intrinsic k_2 values are very different

(eqn. 2); $k_2 = \text{apparent } k_2$ when intrinsic k_2 values are the same; $k_3 = 0.5 \times \text{apparent } k_3$ when the intrinsic k_3 values are very different (Scheme 1); $k_3 = \text{apparent } k_3$ when the intrinsic k_3 values are the same. The maximum rates (controlled by k_3 or k_4) are those given in Tables 1 and 3. The constant k_2 approaches the diffusion-controlled value of $8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$.

A value of $3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ is obtained for k_1 , the rate of formation of the enzyme-substrate 'complex', both in the direct measurement by stopped-flow technique and in the indirect measurement by overall kinetics. It is larger than the value observed for the horseradish enzyme, but lower than that of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ reported by Yonetani & Ray (1966). However, it appears to be a true parameter of the enzyme studied under the present conditions, and independent of the binding of free enzyme to cytochrome *c*, as indicated in the following paper (Mochan & Nicholls, 1971). It does not seem to represent a diffusion-controlled rate.

As suggested in Table 5, the major effects of increasing ionic strength are on k_2 (the formation of the enzyme-cytochrome *c* complex) and k_3 (the proposed intra-complex electron-transfer process). The peroxide reaction (k_1) and the dissociation steps (governed by k_2 and k_4) are relatively insensitive. Nevertheless, a comparison of the observations of Yonetani & Ray (1966) on the effect of ionic strength on the oxidation of yeast cytochrome *c* with those obtained here with horse heart cytochrome *c* [cf. Fig. 2 of Yonetani & Ray (1966) with Figs. 4 and 7 above] shows that the former system, unlike the latter, shows increasing activity with increasing ionic strength up to I 0.2 (0.2M-acetate or 0.12M-phosphate buffer). This may be explained if, for yeast cytochrome *c* at low ionic strength, k_3 is very large and k_4 therefore dominates the expression for V_{max} (eqn. 3 above), whereas for heart cytochrome *c* under these conditions both k_3 and k_4 contribute to that expression. When I is 0.005, both heart and yeast cytochromes show a k_{max} of approximately 1500 s^{-1} . Increasing I to 0.2, which diminishes k_3 , slows the rate with heart cytochrome to 500 s^{-1} or less, whereas the rate with yeast cytochrome increases to 14000 s^{-1} . The latter observation suggests that k_4 may be increasing as I increases. Above I 0.2, the rate with both types of cytochrome diminishes, indicating that the intramolecular rate constant (k_3) has now become rate limiting in the yeast system as well as the heterologous case (heart cytochrome *c* plus yeast enzyme). In other words, the difference between the oxidation of the two cytochromes may be explicable if electron transfer within the complex with yeast *c* is faster, but if that complex is also more vulnerable to dissociation at higher ionic strengths.

Since the maximum turnover observed here did

not exceed 500s^{-1} , $27\mu\text{M}$ -hydrogen peroxide was the minimum amount of substrate permitting the saturation of enzyme with peroxide ($27 \times 10^{-6} \times 3 \times 10^7 = 810\text{s}^{-1}$). The lower turnover of the enzyme prepared by method A should facilitate saturation with peroxide at lower concentrations; the experimental K_m values (Table 2), however, do not bear out this expectation. Lower values of k_1 , or other slow steps not giving rise to a species capable of reacting with hydrogen peroxide (e.g. in the formation of EP from ES, Scheme 1) must occur in this case.

An alternative kinetic simplification of Scheme 1 occurs if a 'quasi-equilibrium' assumption is made for the reactions of enzyme with cytochrome *c*. A Michaelis expression in S may be obtained for which $V_{\max}$ is given by

$$k_3[c^{2+}]e/(K_m + [c^{2+}] + \frac{K_m}{K_I}[c^{3+}])$$

and K_m (for S) by

$$k_3[c^{2+}]/k_1(K_m + [c^{2+}] + \frac{K_m}{K_I}[c^{3+}])$$

These expressions are also related to those obtained for cytochrome oxidase by Minnaert (1961). They are less helpful than the simplification based on eqn. (2) above, because as shown in the preceding discussion the assumption that $k_4 \gg k_3$, implicit in the 'quasi-equilibrium' treatment, is probably not valid.

Validity of kinetic evidence for an enzyme-cytochrome complex. Certain kinetic observations have been regarded as supporting the idea of complexes between oxidative enzymes and cytochrome *c*. Thus kinetic evidence (Nicholls, 1964) for an active cytochrome *c*-cytochrome oxidase complex included the facts that: (a) the rate of oxidation of cytochrome *c* does not become very small as the K_m becomes small with decreasing buffer concentration (as would be expected if the complex were inhibitory); (b) the binding of cytochrome *c* by the intact respiratory chain (reconstitution) shows the same stoichiometry and cation sensitivity as the binding by the oxidase alone; (c) the maximum turnover number of the isolated oxidase is the same in solution as in the respiratory chain containing cytochrome *c* bound quasi-irreversibly; (d) polycations and ferri-cytochrome *c* inhibit the isolated oxidase but not the succinoxidase activity of the respiratory chain containing 'endogenous' cytochrome *c*.

Although cytochrome *c* peroxidase is a mitochondrial enzyme (Yonetani & Ohnishi, 1966), there is no evidence that it is associated with the inner membrane in the same way as the oxidase, permitting the acceptance of electrons from dehydrogenases via bound cytochrome *c*. A tentative

picture (Nicholls, Mochan & Kimelberg, 1969) places the peroxidase in the intracristal space (the space between the two mitochondrial membranes) reacting with the excess of cytochrome *c* present in yeast mitochondria. The type of evidence in (b) and (c) above is therefore not available for cytochrome *c* peroxidase. However, as shown in the present paper, in other respects this enzyme behaves kinetically in a very similar way to the oxidase. Thus: (i) linear Lineweaver-Burk plots are observed indicating that only one cytochrome *c* molecule reacts with each enzyme molecule at any one time; (ii) both $1/K_m$ and $V_{\max}$ values increase with decreasing buffer concentration, down to 10mM -phosphate; no sign of a decrease in $V_{\max}$ at low buffer concentration is seen, as would be expected if any complexes were inhibitory; (iii) the time-course of ferrocyanochrome *c* oxidation in phosphate buffer follows first-order kinetics and is consistent with the inhibitory role of ferri-cytochrome *c* (Fig. 4).

The following paper (Mochan & Nicholls, 1971) presents direct evidence for the existence of complexes between enzyme and both ferri- and ferro-cytochrome *c*; similar evidence has been obtained for cytochrome oxidase (Nicholls, 1964). Comparison of the two types of complex-formation undergone by the enzyme, with peroxide (H_2O_2) and with hydrogen donor (cytochrome *c*), then permits a revised reaction pathway to be postulated that preserves at least some of the analogies with the other hydroperoxidases.

This work was supported by U.S. Public Health Service Grant GM11691. The technical assistance of Miss Mary Park with enzyme preparations is acknowledged.

REFERENCES

- Abrams, R., Altschul, A. M. & Hogness, T. R. (1942). *J. biol. Chem.* **142**, 303.
- Altschul, A. M., Abrams, R. & Hogness, T. R. (1940). *J. biol. Chem.* **136**, 777.
- Beetlestone, J. (1960). *Archs Biochem. Biophys.* **89**, 35.
- Chance, B. (1951). In *Enzymes and Enzyme Systems*, p. 95. Ed. by Edsall, J. T. Cambridge, Mass.: Harvard University Press.
- Chance, B. (1967). *Abstr. Pap. Am. chem. Soc.*, 154th Meet., Chicago, no. C77.
- Dalziel, K. (1957). *Acta chem. scand.* **11**, 1706.
- Dixon, M. (1953). *Biochem. J.* **55**, 170.
- Kabel, B. S. (1969). Ph.D. Dissertation: State University of New York at Buffalo.
- Keilin, D. & Nicholls, P. (1958). *Biochim. biophys. Acta*, **29**, 302.
- Lemberg, R. (1949). In *Hematin Compounds and Bile Pigments*, p. 397. Ed. by Lemberg, R. & Legge, J. W. New York: Interscience Publishers Inc.
- Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.
- Minnaert, K. (1961). *Biochim. biophys. Acta*, **50**, 23.

- Mochan, E. & Degn, H. (1969). *Biochim. biophys. Acta*, **189**, 354.
- Mochan, E. & Nicholls, P. (1971). *Biochem. J.* **121**, 69.
- Nicholls, P. (1964). *Archs Biochem. Biophys.* **106**, 25.
- Nicholls, P. (1966). In *Hemes and Hemoproteins*, p. 307. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Nicholls, P., Mochan, E. & Kimelberg, H. K. (1969). *FEBS Lett.* **3**, 242.
- Smith, L. & Conrad, H. (1956). *Archs Biochem. Biophys.* **63**, 403.
- Smith, L. & Minnaert, K. (1965). *Biochim. biophys. Acta*, **105**, 1.
- Yonetani, T. (1965). *J. biol. Chem.* **240**, 4509.
- Yonetani, T. (1966a). *J. biol. Chem.* **241**, 2562.
- Yonetani, T. (1966b). *Biochem. Prep.* **11**, 14.
- Yonetani, T. & Ohnishi, T. (1966). *J. biol. Chem.* **241**, 2983.
- Yonetani, T. & Ray, G. S. (1965). *J. biol. Chem.* **240**, 4503.
- Yonetani, T. & Ray, G. S. (1966). *J. biol. Chem.* **241**, 700.
- Yonetani, T., Schleyer, H., Chance, B. & Ehrenberg, A. (1966). In *Hemes and Hemoproteins*, p. 293. Ed. by Chance, B., Estabrook, R. W. & Yonetani, T. New York: Academic Press Inc.
- Yonetani, T., Schleyer, H. & Ehrenberg, A. (1966). *J. biol. Chem.* **241**, 3240.