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

EQUILIBRIUM AND TITRATION STUDIES

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1. Physical studies of complex-formation between cytochrome *c* and yeast peroxidase are consistent with kinetic predictions that these complexes participate in the catalytic activity of yeast peroxidase towards ferrocytochrome *c*. Enzyme–ferricytochrome *c* complexes have been detected both by the analytical ultracentrifuge and by column chromatography, whereas an enzyme–ferrocytochrome *c* complex was demonstrated by column chromatography. Estimated binding constants obtained from chromatographic experiments were similar to the measured kinetic values. 2. The physicochemical study of the enzyme–ferricytochrome *c* complex, and an analysis of its spectrum and reactivity, suggest that the conformation and reactivity of neither cytochrome *c* nor yeast peroxidase are grossly modified in the complex. 3. The peroxide compound of yeast cytochrome *c* peroxidase was found to have two oxidizing equivalents accessible to cytochrome *c* but only one readily accessible to ferrocyanide. Several types of peroxide compound, differing in available oxidizing equivalents and in reactivity with cytochrome *c*, seem to be formed by stoicheiometric amounts of hydrogen peroxide. 4. Fluoride combines not only with free yeast peroxidase but also with peroxidase–peroxide and accelerates the decomposition of the latter compound. The ligand-catalysed decomposition provides evidence for one-electron reduction pathways in yeast peroxidase, and the reversible binding of fluoride casts doubt upon the concept that the peroxidase–peroxide intermediate is any form of peroxide complex. 5. A mechanism for cytochrome *c* oxidation is proposed involving the successive reaction of two reversibly bound molecules of cytochrome *c* with oxidizing equivalents associated with the enzyme protein.

The preceding paper (Nicholls & Mochan, 1971) has described kinetic studies which were interpreted in terms of complex-formation between cytochrome *c* and cytochrome *c* peroxidase. Spectroscopic or other direct evidence for such combination is not, however, available, unlike the complexes formed by direct reaction with the peroxidase haem group, such as cyanide, fluoride and peroxide itself (Yonetani & Ray, 1965*b*). The nature of the link between the two kinds of complex formation is also unclear. Electrons (reducing equivalents) must be transferred from cytochrome *c* to the peroxide moiety associated with the haem iron or to the free radical species on the protein (Yonetani, Schleyer, Chance & Ehrenberg, 1966*a*). If the latter is far from the haem iron, the problem of its formation within 30 μs (Yonetani *et al.* 1966*a*) also arises. The one-electron oxidation of cytochrome *c* has to be adapted to the two-electron reduction of hydrogen peroxide or the enzyme–substrate complex, the structure of which appears to differ from that of the analogous compounds of metmyoglobin, catalase and horseradish peroxidase (Keilin & Nicholls, 1958).

The problem of electron transfer is of interest because of the related reaction between cytochrome *c* and cytochrome oxidase, in which a more complicated four-electron reduction of oxygen occurs (Lemberg, 1969). The nature of the peroxide compound has also been the subject of dispute (Wittenberg, Kampa, Wittenberg, Blumberg & Peisach, 1968; Yonetani *et al.* 1966*a*; Yonetani, Schleyer & Ehrenberg, 1966*b*); in particular the absence of a peroxide compound of type III in yeast peroxidase is an unexpected finding. For all the other haem proteins that react with peroxide engage in

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further reactions with hydrogen peroxide giving rise to oxyferrous species of various kinds (Keilin & Hartree, 1954; Yamazaki & Yokota, 1965; Wittenberg *et al.* 1967).

The present paper describes some experiments that examine the complexes or compounds formed between peroxidase and cytochrome *c*, and between peroxidase and hydrogen peroxide or other haem iron ligands. Peroxide compounds are themselves sometimes capable of binding further peroxide or inhibitor molecules (Nicholls, 1961). The relationships between the several kinds of binding reaction will be discussed in the context of the postulated involvement of a reversible cytochrome *c*–enzyme complex in the catalytic reaction (Nicholls & Mochan, 1971).

MATERIALS AND METHODS

Materials. 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 (Margoliash & Lustgarten, 1962). 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 (1966*a*) and Yonetani & Ray (1965*a*).

Cytochrome *c* peroxidase from yeast (Anheuser-Busch) was prepared by a slight modification of the procedure of Yonetani & Ray (1965*a*). The enzyme, the kinetics of which are described in the preceding paper (Nicholls & Mochan, 1971), had a purity ratio (E_{408}/E_{280}) of 1.0, was homogeneous in the analytical ultracentrifuge, and gave one band on disc electrophoresis. The spectrum of the isolated enzyme at pH 7 was of 'acid met' type. At pH 8.5 an alkaline form was produced resembling alkaline horseradish peroxidase. The complexes with fluoride (high spin) and cyanide (low spin) were as reported by Yonetani & Ray (1965*b*).

Horseradish peroxidase (type VI) and crystalline bovine serum albumin were obtained from the Sigma Chemical Co. and employed without further purification. Sephadex G-75 and G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Hydrogen peroxide was prepared by diluting a 30% solution immediately before use. Its concentration was determined spectrophotometrically (E_m at 230nm 62.7 M^{-1} cm^{-1}) and by using peroxidase (Yonetani, 1965). Other chemical and spectrophotometric methods were as described in the preceding paper (Nicholls & Mochan, 1971).

Ultracentrifuge methods. Analyses were carried out in a Spinco model E ultracentrifuge equipped with both Rayleigh interference and schlieren optical systems. The molecular weight was determined by sedimentation equilibrium according to the meniscus-depletion technique of Yphantis (1964) with Rayleigh interference optics. A 0.02% solution of cytochrome *c* peroxidase in 0.1 M-potassium phosphate buffer, pH 7.0, was centrifuged at 20°C for 24h after which photographic exposures were made. Calculation of the weight-average molecular weight (M_w) was carried out with the aid of a computer program devised by Dr Yphantis. The partial specific volume ($\bar{v}$) of peroxidase, estimated from the amino acid composition (Yonetani, 1967) as described by Cohn & Edsall (1943), was 0.73 cm^3/g, neglecting the haem contribution. This value agrees well with the measured value of 0.733 obtained by Ellfolk (1967*b*).

Sedimentation-velocity studies were carried out with schlieren optics. The rotor speed was 59780 rev./min and photographs were taken at appropriate intervals after reaching this speed. Because of the absorption of cytochrome *c*, red-sensitive Eastman Kodak IN plates and a Wratten no. 25 filter were employed. The position of the boundary was taken to be that of the peak and was measured with a Nikon micro-comparator.

Values of the sedimentation coefficients were approximated by employing cytochrome *c* as a 'standard' ($s_{20,w}$ = 1.83S; Margoliash & Lustgarten, 1962) according to eqn. (1):

$$s_{20,s}\text{(peroxidase or peroxidase–cytochrome } c) = s_{20,w}\text{(cytochrome } c) \times R \quad (1)$$

where

$$R = \frac{\text{distance travelled from meniscus by peroxidase or peroxidase–cytochrome } c}{\text{distance travelled from meniscus by cytochrome } c}$$

Since $s_{20,w}$ is a function of the partial specific volume of the protein, this assumes that $\bar{v}$ values for both enzyme and enzyme–cytochrome *c* complex are approximately equal to $\bar{v}$ for cytochrome *c*. Margoliash & Schejter (1966) have reported $\bar{v}$ 0.725ml/g for horse heart cytochrome *c*. Sedimentation coefficients ($s_{obs.}$) for the enzyme and enzyme–ferricytochrome *c* complex were then more accurately estimated as described by Schachman (1957) from a plot of the logarithm of the distance of the protein from the centre of rotation versus time. The sedimentation constants thus obtained were corrected for water at 20°C as described by Svedberg & Pedersen (1940). The resulting sedimentation coefficient at the indicated protein concentration ($s_{20,w}$) is related to the $s^0_{20,w}$ (sedimentation coefficient at infinite dilution) by eqn. (2):

$$s^0_{20,w} = s_{20,w}(1 + KC) \quad (2)$$

where C = the protein concentration and K = concentration-dependency of $s_{20,w}$.

Chromatographic methods. The molecular sieve resins Sephadex G-75 and G-100 were employed. The flow rate of the columns was approximately 3ml/h and 0.5ml fractions were collected at 4°C. Elution patterns were monitored by the extinction in the Soret region.

Cytochrome *c*/enzyme ratios in the fractions were determined as follows.

(I) Enzyme–ferricytochrome *c* complex. Ferricytochrome *c* and cytochrome *c* peroxidase have the same extinction at 404nm (E 92 mM^{-1} cm^{-1}); the difference extinction coefficients for the wavelength pair (412–380nm) for cytochrome *c* peroxidase and ferricytochrome *c* are 29 and 69 mM^{-1} cm^{-1}, respectively. This gives rise to the following relationships:

$$E_{(412-380)} = 29[\text{enzyme}] + 69[\text{cytochrome } c^{3+}]$$

$$E_{404} = 92[\text{enzyme}] + 92[\text{cytochrome } c^{3+}]$$

which may be solved for [cytochrome c^{3+}]/[enzyme]:

$$\frac{[\text{cytochrome } c^{3+}]}{[\text{enzyme}]} = \frac{E_{(412-380)} - 0.32E_{404}}{0.75E_{404} - E_{(412-380)}} \quad (3)$$

(II) Enzyme–ferrocytochrome *c* complex. The addition of sodium dithionite to the complex results in the production of ferrocytochrome *c* and reduced enzyme. The concentration of ferrocytochrome *c* can be determined from the change in extinction at 550–540nm, by employing 17mM^{-1}cm^{-1} as the difference extinction coefficient for the reduced enzyme–ferrocytochrome *c* system. In a similar manner the concentration of reduced enzyme can be determined at 430nm before and after the addition of dithionite, by employing a difference extinction coefficient of 90mM^{-1}cm^{-1}.

The ferricytochrome *c*/enzyme ratio could also be determined by this method. The two methods gave similar values for the ferricytochrome *c*/enzyme ratio.

The ferricytochrome *c*/albumin ratio was determined from eqn. (4). The respective millimolar extinction coefficient values employed for ferricytochrome *c* at 408nm and 278nm were 106 and 24. The millimolar extinction coefficient for albumin at 278nm determined under the present conditions was 35.

$$\frac{[\text{cytochrome } c^{3+}]}{[\text{albumin}]} = \frac{0.009E_{408}}{0.029E_{278} - 0.006E_{408}} \quad (4)$$

Extinction coefficients used for cytochrome *c* and cytochrome *c* peroxidase were those of Margoliash (1954) and Yonetani & Ray (1965*b*).

RESULTS

Combination between enzyme and cytochrome c

Molecular weight of cytochrome c *peroxidase.* Fig. 1 illustrates the point-average value of the molecular weight (M_w) of cytochrome *c* peroxidase at various concentrations of the enzyme across the cell. As can be seen there is some variation across the cell, indicating trace amounts of a higher-molecular-weight species. This species may be a high-molecular-weight contaminant or an aggregated form of the enzyme. The behaviour of the enzyme on disc electrophoresis, as well as its schlieren pattern, would seem to favour the latter. Extrapolating the point-average molecular weight of the major lighter component to zero gives an apparent weight-average molecular weight of 34400. Since a low initial protein concentration (0.02%) was employed, this value may be close to the true molecular weight; it may be compared with the value of 34100 obtained by sedimentation diffusion (Ellfolk, 1967*b*).

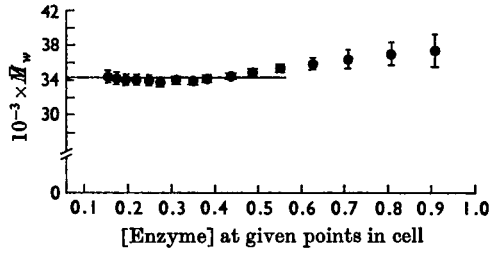

Fig. 1. Molecular-weight determination of cytochrome *c* peroxidase by sedimentation equilibrium. Point-average molecular weights of peroxidase (determined as described in the text) are indicated as a function of the concentration of peroxidase at given points in the cell. The initial concentration of enzyme was 0.02% in 0.1M-potassium phosphate buffer, pH7.0, at 20°C. The determination was performed at a rotor speed of 29500rev./min 24h after the start of the run. The indicated error limits represent the standard error of the five-point least-squares fit. The weight-average molecular weight was determined by extrapolating the point-average-molecular-weight values to zero concentration.

Ultracentrifugal studies in the presence of cytochrome c. Because of the intense absorption of cytochrome *c*, ultracentrifugal analysis could be accurately performed only with a limited set of concentrations of cytochrome *c* and peroxidase. A schlieren trace is represented diagrammatically in Fig. 2. The mixture in the top cell contained 0.6ml of a 3.5 molar excess of ferricytochrome *c* over

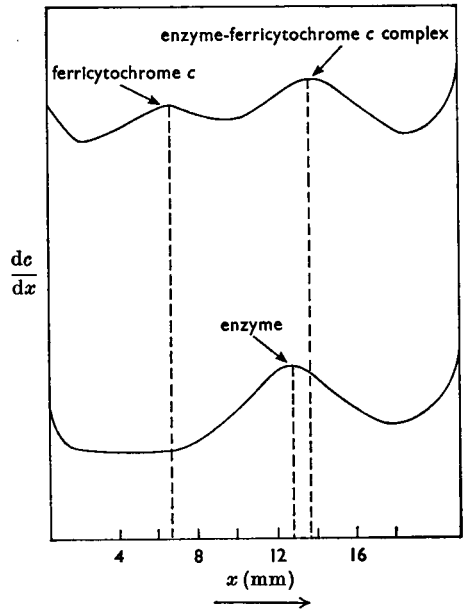

Fig. 2. Sedimentation-velocity schlieren pattern of cytochrome *c* peroxidase and its ferricytochrome *c* complex. The top cell contained 0.6ml of a mixture containing 550μM-ferricytochrome *c* and 160μM-enzyme in 0.05M-potassium phosphate buffer, pH6.25, at 20°C. The bottom cell contained only 160μM-free enzyme in the same buffer. This tracing was obtained 128min after a rotor speed of 59780rev./min was reached.

peroxidase (550μM; 160μM), and the bottom cell contained 160μM-free enzyme. The sedimentation velocity of the enzyme is increased in the presence of ferricytochrome *c*. Compared with the reported $s_{20,w}$ value for cytochrome *c*, the $s_{20,w}$ values for the enzyme and enzyme–ferricytochrome *c* complex (cf. eqn. 1) were about 3.5 and 3.7S, respectively. More accurate $s_{20,w}$ values for the enzyme and enzyme–cytochrome *c* complex, determined by Schachman's (1957) method, were 3.24 and 3.53S, respectively. By using the reported (Ellfolk, 1967*b*) concentration-dependency factor of 0.019 for peroxidase the $s^0_{20,w}$ (sedimentation coefficient at infinite dilution) was found to be 3.58S, in close agreement with the value of 3.55S obtained by Ellfolk (1967*b*). An estimate of the $s^0_{20,w}$ value for the enzyme–cytochrome *c* complex is difficult since neither the extent of complex-formation nor its concentration dependency could be accurately determined. If the faster sedimenting component corresponds to a 1:1 stoicheiometric complex between cytochrome *c* and peroxidase (as the chromatographic experiments described below indicate) and if its concentration dependency is similar to that for unbound enzyme (i.e. K of eqn. 2 = 0.019), then $s^0_{20,w}$ for the complex is about 4.0.

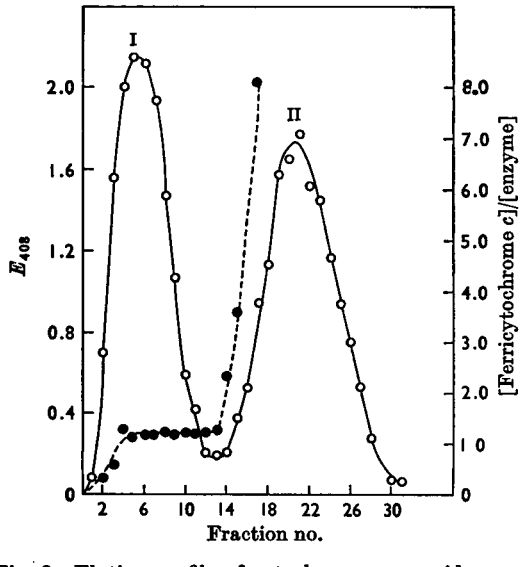

Fig. 3. Elution profile of cytochrome *c* peroxidase and peroxidase–ferricytochrome *c* complex on Sephadex. A 0.8ml mixture containing an excess of ferricytochrome *c* (550μM) over peroxidase (160μM) in 0.05M-potassium phosphate buffer, pH6.25, at 4°C, was placed on a column (1cm×60cm) of Sephadex G-75 that had been previously equilibrated with the same buffer. The flow rate of the column was 3ml/h and 0.5ml fractions were collected. The extinction at 408nm (——) was recorded for each fraction. The ferricytochrome *c*/enzyme ratio in the fractions (– – – –) was determined as described in the text. Peak I, enzyme–ferricytochrome *c* complex; peak II, ferricytochrome *c*.

Chromatographic evidence for an enzyme–ferricytochrome c *complex.* Interaction between cytochrome *c* and peroxidase was also examined by column chromatography. A concentrated solution, containing approximately 0.44μmol of ferricytochrome *c* and 0.13μmol of enzyme in 0.005M-potassium phosphate buffer, pH6.25, was placed on a column (1cm×60cm) of Sephadex G-75 that had been previously equilibrated with the same buffer. The elution profile of the 0.5ml fractions monitored at 408nm is shown in Fig. 3, which clearly indicates the separation of two distinct haem protein peaks. The ferricytochrome *c*/enzyme ratio in each fraction was obtained from the spectrum before and after addition of sodium dithionite as described in the Materials and Methods section.

The formation of a stoicheiometric complex between the enzyme and ferricytochrome *c*, corresponding to the first peak, is demonstrated by the constancy of a ratio close to one in the major portion of the peak. This is followed by a rise in the ratio corresponding to the second peak, which contains exclusively ferricytochrome *c*. The presence of free enzyme in the first peak is indicated by the low ferricytochrome *c*/enzyme ratio in the leading edge. The system appears to be a typical associating–dissociating equilibrium in which free enzyme, enzyme–ferricytochrome *c* complex, and free ferricytochrome *c* are all present (I in Table 1).

Since the system contains mixtures of the components it is not easy to determine the dissociation constant for the complex, which is also a function of the properties of the column. If it is assumed, however, that enzyme and complex are in rapid equilibrium and that the total cytochrome *c* in the leading peak is equal to that amount originally combined, then an estimate of the dissociation constant would be about 1μM. This value is similar to the observed kinetic K_i of 2.4μM under these conditions (cf. Table 4 in Nicholls & Mochan, 1971).

Fig. 4 shows the spectrum of the complex. The reduced form was obtained by the addition of sodium dithionite to the oxidized form.

To eliminate the possibility that the elution profile was due to non-specific 'trailing' of cytochrome *c*, a second Sephadex column was run. In this case, fractions containing the complex were pooled, concentrated, and placed on a column (1cm×35cm) of Sephadex G-75 previously equilibrated with 0.005M-potassium phosphate buffer, pH6.25. The elution profile, obtained by monitoring the extinction of the various fractions at 408nm indicated the

Table 1. *Complex-formation between cytochrome* c *and enzymes on Sephadex*

The results indicate the amount of cytochrome *c* and enzyme in the appropriate dilution of a fraction representative of the indicated peak. The concentrations of cytochrome *c* and enzyme were determined as described in the Materials and Methods section. Experiments were performed in 5mM-potassium phosphate buffer, pH 6.25, at 4°C.

		First peak			Second peak	
	Experimental conditions	Cytochrome *c* (μM)	Enzyme (μM)	Cytochrome *c*/ enzyme ratio	Cytochrome *c* (μM)	Enzyme (μM)
I	550μM-cytochrome c^{3+} + 160μM-peroxidase (1 cm × 60 cm Sephadex G-75)	0.53	0.56	0.93	5.4	<0.3
II	complex from I (1 cm × 34 cm Sephadex G-75)	0.37	0.9	0.41	<0.2	<0.3
III	500μM-cytochrome c^{3+} + 100μM-horseradish peroxidase (1 cm × 70 cm Sephadex G-100)	<0.2	6.59	—	4.5	<0.3
IV	320μM-cytochrome c^{2+} + 160μM-peroxidase + 50mM-ascorbate (1 cm × 70 cm Sephadex G-100)	0.29	0.84	0.34	6.1	<0.3
V	550μM-cytochrome c^{2+} + 160μM-peroxidase + 200μM-KCN (1 cm × 70 cm Sephadex G-75)	0.89	0.79	1.1	1.62	<0.3

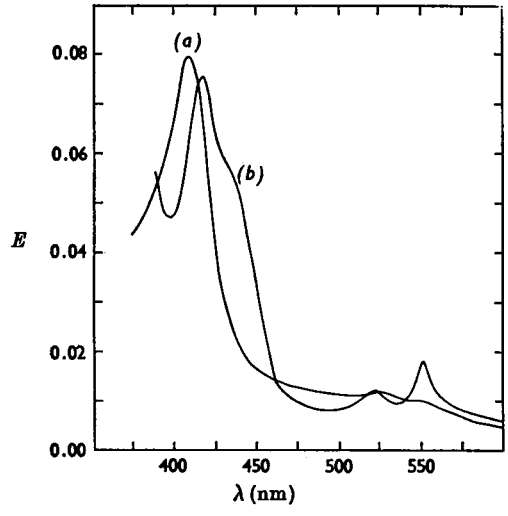

Fig. 4. Spectra of enzyme–cytochrome *c* complexes. The spectrum of the enzyme–ferricytochrome *c* complex (*a*) corresponds to the appropriate dilution of fraction 6 in Fig. 3. The reduced form (*b*) was obtained by the addition of dithionite to the oxidized form.

presence of one haem protein peak. The ferricytochrome *c*/enzyme ratio in the major portion of the peak, as in Fig. 3, remained constant. The value of the ratio, however, decreased to approx. 0.4 (II in Table 1) indicating that some ferricytochrome *c* had dissociated. Lower ratios in the leading fractions indicated that some free enzyme also existed. This system, as before, represents an equilibrium mixture, further evidence that ferricytochrome *c* can bind the enzyme reversibly.

Since cytochrome *c* is highly basic (isoelectric point 10.05; Margoliash & Schejter, 1966) and cytochrome *c* peroxidase is acidic (isoelectric point 5.25; Ellfolk, 1967*a*), a possible objection to both ultracentrifugal and column studies is that the observed interaction between the two proteins is non-specific. The interaction of cytochrome *c* with bovine serum albumin (isoelectric point 4.8; Mahler & Cordes, 1966) was therefore examined. The elution profile indicated some interaction between the two proteins, but the cytochrome *c*/albumin ratio in the leading peak was much lower than with peroxidase. Serum albumin, an acidic protein with a wide spectrum of binding properties, thus has a markedly lower affinity for cytochrome *c* than peroxidase, indicating a certain specificity in the latter reaction. The interaction of cytochrome *c* and horseradish peroxidase, which has similar physical properties to yeast peroxidase, and also catalyses the peroxidation of cytochrome *c*, was also examined. In this case, there appeared to be only a trailing of cytochrome *c* alone with the leading, predominantly horseradish peroxidase, peak (III in Table 1). No evidence of a constant cytochrome *c*/horseradish peroxidase ratio in the

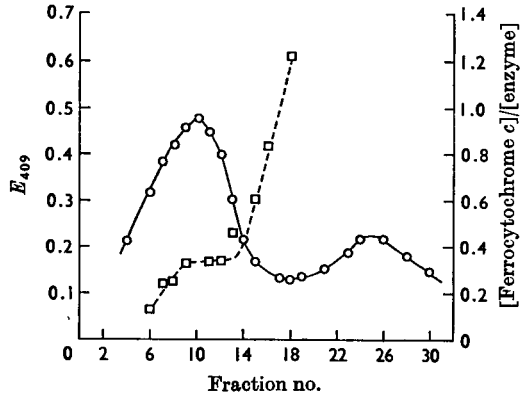

Fig. 5. Elution profile of a mixture of cytochrome *c* peroxidase and ferrocytochrome *c*. A mixture containing 160 μM-peroxidase and 320 μM-ferrocytochrome *c* (reduced with 50mM-ascorbate) was placed on a column (1cm × 70cm) of Sephadex G-100 that had been equilibrated with nitrogen-saturated 5mM-potassium phosphate buffer containing 5mM-ascorbate. The protein mixture was eluted with the same buffer at a flow rate of 3ml/h and 0.5ml fractions were collected. The extinction at 409nm (○) was recorded for the indicated fractions and the ferrocytochrome *c*/enzyme ratio (□) was calculated as described in the text.

leading peak was obtained. Therefore, if any complex is formed between horseradish peroxidase and cytochrome *c* it has a much higher dissociation constant than that of the yeast peroxidase–cytochrome *c* complex.

Chromatographic evidence for an enzyme–ferrocytochrome c complex. Preliminary investigations revealed that when ferrocytochrome *c* and peroxidase were placed on a Sephadex column the elution profile indicated that oxidation of ferrocytochrome *c* had taken place. This suggested that hydrogen peroxide was present or being formed, permitting peroxidase to act enzymically. The most satisfactory method of avoiding this was found to be the addition of ascorbate to a buffering system previously flushed with nitrogen. By using this procedure a mixture containing 320 μM-ferrocytochrome *c* (reduced with 50mM-sodium ascorbate) and 160 μM-peroxidase in 5mM-potassium phosphate buffer, pH6.25, was placed on a column (1cm × 70cm) of Sephadex G-100 previously equilibrated with the same buffer containing 50mM-sodium ascorbate and saturated with nitrogen. Due to the intense absorption spectrum of ferrocytochrome *c* it was helpful to use this lower initial concentration to determine the ferrocytochrome *c*/enzyme ratio in the trailing fractions of the leading peak. The sample was eluted with the same buffer saturated with nitrogen. The elution profile, monitored at 409nm (Fig. 5), shows the separation of two distinct haem protein peaks. The ferrocytochrome *c*/enzyme ratio (indicated by the broken line) suggests the formation of a complex corresponding to the first peak. The second peak contains exclusively ferrocytochrome *c*. Hence this system also represents an equilibrium containing free peroxidase (indicated by the low [ferrocytochrome *c*]/[enzyme] ratio in the first peak), enzyme–ferrocytochrome *c* complex, and free ferrocytochrome *c*. It should be noted, however, that the ferrocytochrome *c*/enzyme ratio was low (approx. 0.34, IV in Table 1). This value represents the ratio determined by the addition of sodium dithionite to the fractions. The ferrocytochrome *c*/enzyme ratio determined directly from the column was even lower since some oxidation of ferrocytochrome *c* by the enzyme had occurred. Thus ferrocytochrome *c* can also form a reversible complex with peroxidase. The lower cytochrome *c*/enzyme ratio is probably due to lower initial concentrations of ferrocytochrome *c* used and to the presence of 50mM-ascorbate to keep the cytochrome *c* reduced, raising the ionic strength. A dissociation constant of about 20 μM was estimated for this system, which may be compared with a kinetic K_m of about 10 μM under the same ionic conditions (Nicholls & Mochan, 1971).

Chemical behaviour of the enzyme–cytochrome c complex. The reaction between the cyanide complex of peroxidase and cytochrome *c* was also examined. A mixture containing 200 μM-potassium cyanide, 550 μM-ferrocytochrome *c* and 100 μM-peroxidase in 5mM-potassium phosphate buffer, pH6.25, was placed on a column (1cm × 60cm) of Sephadex G-75 previously equilibrated with the same buffer containing 200 μM-potassium cyanide. An elution profile was obtained similar to that for free enzyme and cytochrome *c*. Samples in the first peak showed extinction at 425nm indicating formation of enzyme–cyanide (Yonetani & Ray, 1965*b*).

The ferricytochrome *c*/enzyme ratio in this peak was 1:1 and the second peak contained only cytochrome *c* (V in Table 1). The cyanide derivative of peroxidase is thus also capable of forming a reversible complex with ferricytochrome *c* (most of the ferrocytochrome *c* added to the initial mixture was converted into ferricytochrome *c* even in the presence of CN^-).

The spectra of all the complexes, including that formed between peroxidase and ferricytochrome *c* (Fig. 4), can be obtained by optical addition of the spectra of the individual components. The reaction between ferricytochrome *c* and peroxidase (or peroxidase–CN^-) does not result in new absorption bands nor in any gross alteration of the spectral characteristics of the individual components.

The addition of 1mM-sodium ascorbate to a solution of 0.3μM-enzyme–ferricytochrome *c* complex in 5mM-potassium phosphate buffer, pH 6.25, did not appear to reduce the cytochrome *c* in the complex since no change in the 550–540nm extinction occurred. Upon the addition of 5mM-sodium fluoride to this mixture, however, several changes were noted. First, an increase in extinction at 407nm corresponding to the formation of 0.3μM-enzyme–fluoride complex was observed. Secondly, an increase in the 550–540nm extinction indicated that approximately 30% of the total cytochrome *c* was now reduced. The original inability of ascorbate to reduce the cytochrome *c* was probably due to the formation of hydrogen peroxide, enabling the enzyme rapidly to oxidize the cytochrome *c* reduced by the ascorbate. Fluoride, by inhibiting the enzyme, produces a steady state between complete oxidation and complete reduction of cytochrome *c*. The bound cytochrome *c* is completely reduced on the addition of sodium dithionite to the oxidized complex.

Combination between enzyme and peroxide

Formation and reduction of the peroxidase–hydrogen peroxide compound. The addition of 6μM-hydrogen peroxide to 5.25μM-peroxidase results in the formation of a compound with an absorption spectrum similar to horseradish peroxidase compound II, as reported by Yonetani & Ray (1965*b*). The presence of ferrocyanide did not increase the extent of peroxidase–hydrogen peroxide compound formation. No changes at the isosbestic point of 412nm were observed during this reaction, indicating the absence of any 'compound I'. This is consistent with kinetic observations. The reported 1:1 stoicheiometry of the enzyme–hydrogen peroxide reaction was confirmed with our enzyme preparation. According to Yonetani (1965), cytochrome *c* peroxidase forms only one such peroxide compound, a derivative spectroscopically analogous to horseradish peroxidase compound II but titrimetrically analogous to horseradish peroxidase compound I. Two oxidizing equivalents are associated with each molecule, one with the haem iron, the other apparently involving a nearby group oxidized to give a free radical (Yonetani *et al.* 1966*a*,*b*). This result conflicted with the result obtained by George (1953*a*), who detected only one oxidizing equivalent per haem iron by titration with ferrocyanide (see discussion by George, 1966). We have therefore repeated both kinds of titration of the enzyme–peroxide compound.

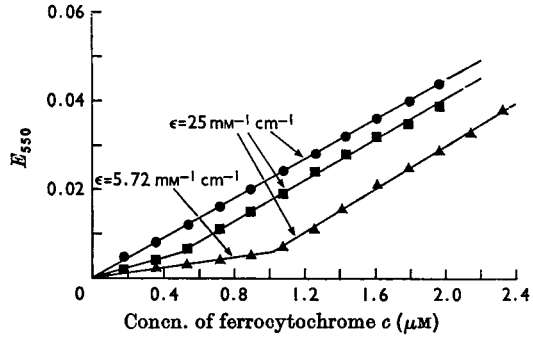

Fig. 6. Titration of enzyme–peroxide compound with ferrocytochrome *c*. The change in extinction at 550nm was recorded on the addition of ferrocytochrome *c* to: 0.66μM-peroxidase+0.65μM-H_2O_2 (▲); 0.66μM-peroxidase (■); 0.65μM-H_2O_2 (●), all in 0.01M-potassium phosphate buffer, pH 5.4, at 25°C. The indicated extinction coefficients correspond to the slopes of the appropriate lines.

The titration with cytochrome *c* was carried out indirectly (Yonetani, 1965) by observing extinction changes at the α-peak as follows. Cytochrome *c* peroxidase was initially converted into enzyme–peroxide by the addition of one equivalent of hydrogen peroxide. This enzyme–peroxide compound was titrated with ferrocytochrome *c* and the resulting change at 550nm recorded (Fig. 6). In the absence of enzyme or hydrogen peroxide no appreciable oxidation of ferrocytochrome *c* occurred. This was indicated by the steep increase in extinction, the slope of which corresponded closely to the reported extinction coefficient of 27.7 $mM^{-1}cm^{-1}$ for ferrocytochrome *c*. On the other hand, when enzyme–peroxide compound was present, the 550nm extinction increased much less steeply upon addition of ferrocytochrome *c*. The slope of 5.7 $mM^{-1}cm^{-1}$ corresponded to the composite extinction changes resulting from the formation of ferricytochrome *c* and the conversion of enzyme–peroxide compound to free enzyme. After all the oxidizing equivalents were exhausted a rapid increase in the extinction was observed, due to the appearance of ferrocytochrome *c*. At pH 5.4, the 'equivalence point' for 0.66μM-enzyme–peroxide occurred with 1.01μM-ferrocytochrome *c*, corresponding to an enzyme–peroxide compound/ferrocytochrome *c* ratio of 0.65±0.04. These results, then, indicate that up to two oxidizing equivalents are detectable by titration with ferrocytochrome *c*, in agreement with the observations of Yonetani (1965).

The reaction with ferrocyanide can be followed directly (George, 1953*a*) by measuring the change in extinction at 425nm ($\Delta E = 0.038\,\mu M^{-1}cm^{-1}$).

At pH 5.4 the reaction between enzyme–peroxide and ferrocyanide is fast ($k \simeq 10^6\,M^{-1}s^{-1}$). Fig. 7 illustrates a typical titration. The average equivalence point corresponded to an enzyme–peroxide

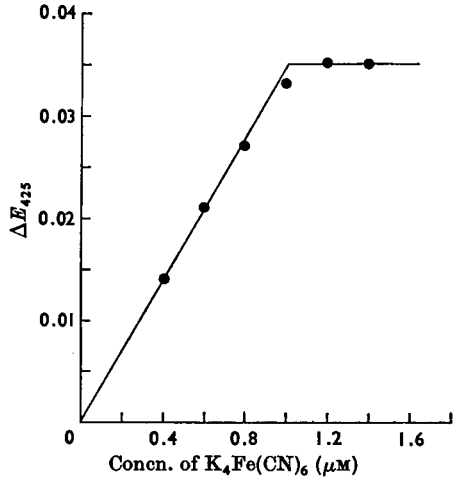

Fig. 7. Titration of enzyme–peroxide compound with ferrocyanide. The enzyme–peroxide compound was formed by the addition of 1 μM-H_2O_2 to 0.87 μM-peroxidase in 0.01 M-potassium phosphate buffer, pH 5.4, at 25°C. The titration was performed by measuring the decrease in extinction at 425nm on the addition of the indicated quantity of potassium ferrocyanide.

compound reduced/ferrocyanide equivalents added ratio of 0.94 ± 0.19. In this case little more than one reducing equivalent is needed to return the enzyme to its ferric form. This is essentially in agreement with the observations of George (1953*a*). Since it was possible that additional reducing equivalents are used up in the reaction associated with the formation of the enzyme–peroxide compound (as with horseradish peroxidase), the effects of adding the donor before the addition of hydrogen peroxide to enzyme were studied. A small amount of donor was added to enzyme, followed by the addition of a stoicheiometric quantity of hydrogen peroxide. The reaction was allowed to proceed to completion, the change in extinction at 425nm was recorded and the remaining enzyme–peroxide compound was titrated as before. The results with both ferrocyanide and cytochrome *c* are summarized in Table 2. (At pH 7.0 the reaction of ferrocyanide and enzyme–peroxide compound was too slow since in our hands enzyme–peroxide itself decomposed at a rate of $3.7 \times 10^{-4} s^{-1}$.)

As discussed below, these experiments suggest that of the two oxidizing equivalents retained in the peroxide compound, only one, the 'spectroscopically active' species, reacts readily with ferrocyanide at pH 5.4, whereas cytochrome *c* reacts with both equivalents. Titration of the product of the ferrocyanide reaction with cytochrome *c* could not be carried out because of the presence of ferricyanide in the final mixture.

Table 2. *Titrations of enzyme–peroxide compound with ferrocytochrome* c *and ferrocyanide*

The experimental conditions were as follows: 0.01 M-phosphate buffer, pH 5.4 or 7.0; approx. 1 μM-peroxidase plus 1 μM-H_2O_2. Reduction of peroxide compound by ferrocyanide was measured at 425nm; cytochrome *c* was measured at 550nm by the method of Yonetani (1965).

pH	Reductant	Ratio of peroxide compound reduced/reducing equivalents added: Reductant added last	Reductant added first
5.4	Ferrocyanide	0.94 ± 0.19	0.83 ± 0.07
5.4	Cytochrome *c*	0.65 ± 0.04	0.51 ± 0.02
7.0	Cytochrome *c*	0.78 ± 0.11	0.57

Evidence against the occurrence of ternary peroxide compounds of cytochrome c *peroxidase.* Fig. 8(*a*) shows the visible spectrum of an enzyme–peroxide compound obtained by adding a tenfold excess of hydrogen peroxide to 5.25 μM-peroxidase. A decrease occurred in the extinction at 560nm. At the same time we observed a shift in the position of the Soret band from 419 to 412nm (Fig. 8*b*). A plot of an extended titration of enzyme with peroxide, followed at 560nm as before, shows the progressive formation of the new derivative as the hydrogen peroxide/enzyme ratio increases above 1.0 (Fig. 9).

Such a compound might have been considered as the missing compound III. The following evidence suggests that it is not such a compound, but a mixture of at least two products of the reduction of the peroxide compound by the excess of peroxide. First, hydrogen donors such as ferrocyanide do not restore the spectrum of ferric peroxidase. A tenfold excess of hydrogen peroxide was added to a micromolar solution of peroxidase and the mixture was left for 30min until the transition was complete. Subsequent addition of 5 μM-ferrocyanide had no effect on the resulting compound. Secondly, ligands such as fluoride promote the formation of this derivative and also appear to combine with part of it as if it were free enzyme. Addition of 2mM-sodium fluoride to 5 μM-peroxidase containing 40 μM-hydrogen peroxide induced the immediate formation of the inert derivative, and fluoride combined with at least part of the product, indicating the presence of free ferric haem protein. Thirdly, full formation of the compound requires a finite time (up to 30min) for completion. And lastly, the transition from the regular peroxide compound involves no clear isosbestic points.

The peroxide compound is known to react with excess of peroxide 'catalatically' (Yonetani *et al.* 1966*b*), re-forming free enzyme while the peroxide is

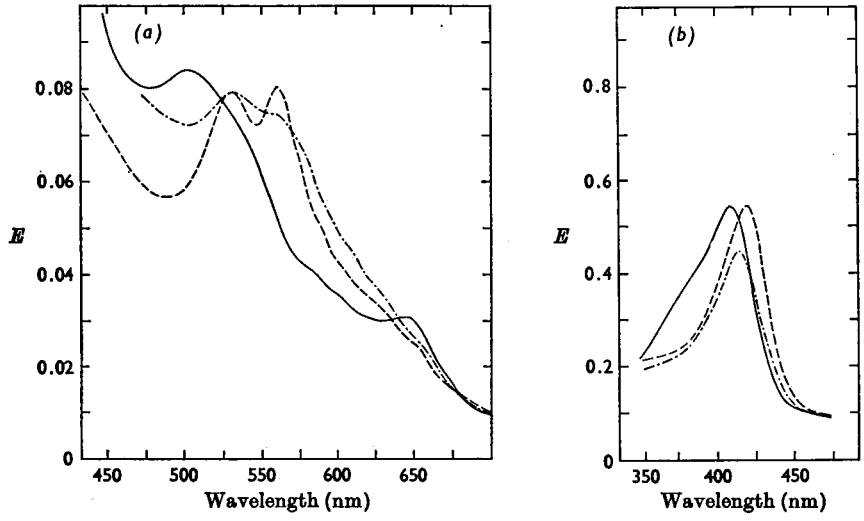

Fig. 8. Absorption spectra of cytochrome *c* peroxidase and peroxide derivatives. The following amounts of H_2O_2 were added to 5.25μM-peroxidase, in 0.01M-potassium phosphate buffer, pH7.0, at 25°C: ——, none; – – – –, 4.3μM-H_2O_2; –·–·–, 51μM-H_2O_2 (after approx. 30min). Both visible (*a*) and Soret regions (*b*) are shown.

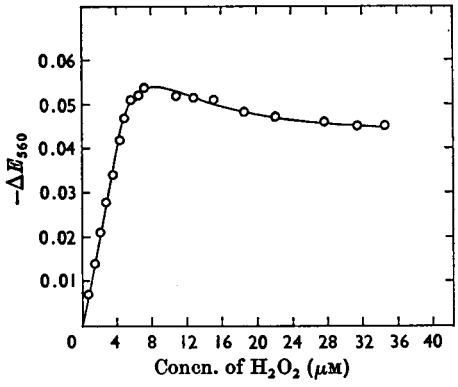

Fig. 9. Titration of cytochrome *c* peroxidase with H_2O_2. The changes in extinction at 560nm are recorded immediately after the addition of the given amounts of H_2O_2 to 4.76μM-peroxidase in 0.01M-potassium phosphate buffer, pH7.0.

converted into molecular oxygen. In addition to this 'catalatic' reaction other processes occur that involve some degradation of the haem moiety. The spectroscopic observations in excess of peroxide are in accord with the production of approximately equimolar amounts of a form of free peroxidase and of another derivative with a low Soret band ($\lambda_{max.}$ approx. 415–420nm, Σ_{max} approx. 45mM^{-1}cm^{-1}) and a weak extinction in the visible region. The latter product resembles the pseudo-peroxide compounds formed under acid conditions by metmyoglobin (George & Irvine, 1952). The former product resembles cytochrome *c* peroxidase itself, but is apparently unaffected by the hydrogen peroxide still present in solution.

Evidence for ligand binding by the enzyme–peroxide compound. Enzyme–peroxide in the absence of added donors spontaneously decomposes to free enzyme. As noted by George (1953*b*) this spontaneous decomposition of enzyme–peroxide is first-order with respect to peroxidase concentration (see Fig. 11), indicating that the reducing substance is associated with the same protein molecule as the haem. A rate of 3×10^{-4}s^{-1} was obtained at pH7 in 10mM-phosphate buffer, compared with rates of 10^{-3}s^{-1} reported by George (1953*b*) and 10^{-4}–10^{-5}s^{-1} by Yonetani *et al.* (1966*b*).

This decomposition is accelerated by certain anions, including fluoride. Cytochrome *c* peroxidase has an unusually high affinity for fluoride compared with other haemoproteins, although the spectrum is that of a typical high-spin derivative with extinction bands at 610 and 490nm. Titration of free peroxidase with sodium fluoride gave a response indicating the reaction of 1mol of ligand with 1mol of peroxidase, as in the titration with cyanide (Yonetani & Ray, 1965*b*). The dissociation constant (K_d) obtained from such titrations was 28μM at pH5.6 and 180μM at pH6.75 [a previous value (George, 1953*b*) of 240μM was obtained at pH7.0]. A dissociation constant of 0.15μM with respect to undissociated hydrogen fluoride may be calculated,

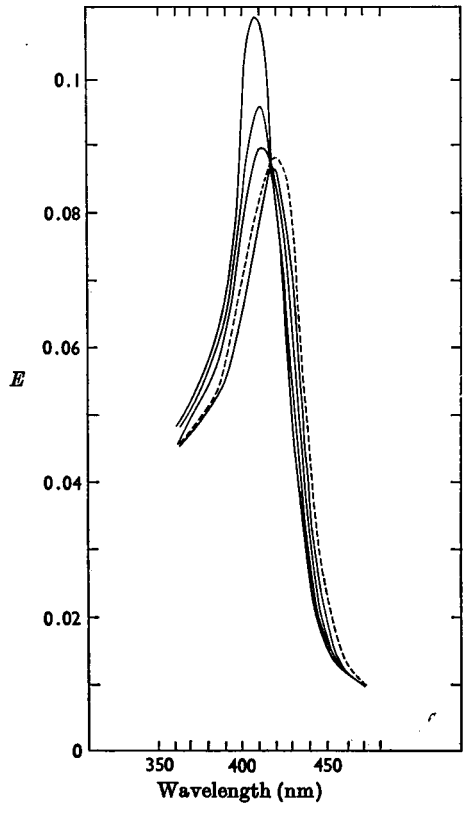

Fig. 10. Decay of the enzyme–peroxide compound on the addition of NaF. Enzyme–peroxide (0.87 μM) compound was formed by the addition of 1.15 μM-H_2O_2 to 0.87 μM-peroxidase, in 0.01 M-potassium phosphate buffer, pH 6.75. Progressive extinction spectra were obtained by rapidly scanning the solution (5 nm/s). ----, 0.87 μM-enzyme–peroxide compound; ——, 0.87 μM-enzyme–peroxide compound + 10 mM-NaF.

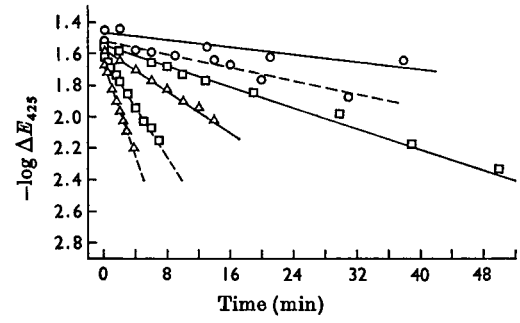

Fig. 11. Time-course for decomposition of enzyme–peroxide compound by fluoride. The enzyme–peroxide compound was formed by the addition of 1 μM-H_2O_2 to 0.94 μM-peroxidase in 0.01 M-potassium phosphate buffer, pH 5.6 and 6.75, at 25°C. The decomposition of the enzyme–peroxide compound was followed by recording the decrease in extinction at 425 nm in the absence (○) and presence of fluoride (□, 0.5 mM-NaF; △, 1 mM-NaF). The same reactions were observed at pH 5.6 (----) and pH 6.75 (——).

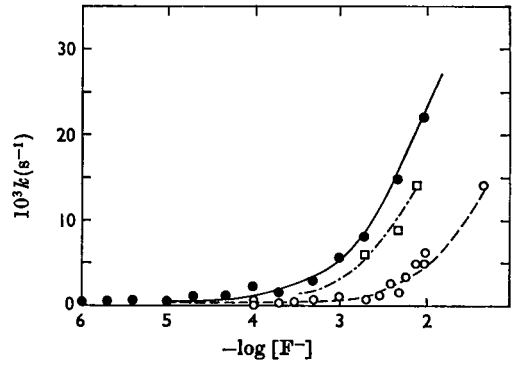

Fig. 12. Variation of the first-order rate constant for peroxide compound decomposition with NaF concentration. The addition of 1.15 μM-H_2O_2 to 1 μM-peroxidase formed 1 μM-enzyme–peroxide compound. The decrease in extinction at 425 nm was followed in the presence of various quantities of NaF. First-order rate constants (k') were obtained from a plot of log ΔE versus time. Experiments were performed at 0.01 M- and 0.1 M-potassium phosphate buffer, pH 7.0 (○) and 5.6 (●), respectively. The data from George (1953*b*) are also indicated (□). Theoretical curves: ——, K_d' 8 mM, k 0.4×10^{-3} s^{-1} and k' 4.0×10^{-2} s^{-1}; ----, K_d' 63 mM, k 0.3×10^{-3} s^{-1} and k' 3.0×10^{-2} s^{-1}; ·–·–·, K_d' 50 mM, k 1.0×10^{-3} s^{-1} and k' 9.0×10^{-2} s^{-1}.

compared with values of 19 μM for horseradish peroxidase and 45 μM for catalase (Nicholls, 1961).

Fig. 10 shows the effect of adding 10 mM-sodium fluoride to the enzyme–peroxide compound, formed with a stoicheiometric amount of peroxide. Isosbestic points at 415 and 470 nm are obtained, as the peroxide compound decomposes to give the enzyme–fluoride complex without detectable intermediates. Fig. 11 illustrates the kinetics of the reaction at 425 nm. The time-course is first order, although the velocity constant is a function of pH and fluoride concentration. If the first-order rate constants, however, are plotted against fluoride concentration (Fig. 12), the linear relationship found by George (1953*b*) is not obeyed at high fluoride concentrations. The addition of these large quantities of fluoride (approx. 10 mM) did not appreciably affect the pH of the system. Nor did they influence the decomposition of enzyme–peroxide compound by exerting a 'salt effect' since an equivalent amount of sodium chloride did not

Table 3. *Catalysis by fluoride of the decomposition of enzyme–peroxide compound*

The following symbols are used: K_d, dissociation constant for enzyme–fluoride complex; K'_d, concentration of F^- for half-maximal stimulation of peroxide compound decay; k, velocity constant (25°C) for decay in the absence of fluoride; k', velocity constant (25°C) for decay in the presence of excess of fluoride; k'', second-order velocity constant for reaction with low concentrations of F^-.

pH	K_d (mM)	K'_d (mM)	K'_d/K_d ratio	10^3k (s^{-1})	$10^3k'$ (s^{-1})	k'' ($M^{-1}s^{-1}$)
6.8	0.3	63.0	210	0.3	30	0.48
5.6	0.035	8.0	230	0.4	40	5.0

alter the spontaneous rate of enzyme–peroxide compound decomposition. This deviation from linearity indicates the approach to a maximum limiting rate, characteristic of complex formation. As quite high concentrations of fluoride (63mM) were needed to reach the estimated [by extrapolation to infinite fluoride concentration, assuming 1mol of fluoride reacts reversibly with 1mol of enzyme–peroxide complex (see Fig. 12)] half-maximal velocity at pH6.8, the affinity of the enzyme–peroxide compound for fluoride at this pH was very low. Fluoride apparently has a higher affinity for the enzyme–peroxide compound at lower pH. This is illustrated both in Fig. 11, where the decomposition of enzyme–peroxide compound by fluoride is greatly accelerated at pH5.6, and in Fig. 12, where the concentration of fluoride required for half maximal velocity at pH5.6 is lower than at pH7.0. This suggests that both free enzyme and enzyme–peroxide react with the undissociated acid. Table 3 summarizes the effects of fluoride on peroxidase and its peroxide compound and lists the minimum and maximum velocity constants obtained. The estimated half-maximal velocity at both pH values is obtained at a fluoride concentration some 200 times greater than that required to bind 50% of the free enzyme. The data of George (1953*b*) can be brought into agreement with the present findings if for his preparation the velocity constant without fluoride equalled $10^{-3}s^{-1}$ whereas the velocity with excess of fluoride equalled $0.09s^{-1}$, some three times the values with the present enzyme. Although Yonetani & Ray (1965*b*), treating the process as an equilibrium, claim that fluoride 'only partially' converts enzyme–substrate complex into the enzyme–inhibitor complex, it is probable that with their very stable enzyme–peroxide compound, complete conversion would take some time. Present results show that although complex-formation between enzyme–peroxide compound and fluoride cannot be detected spectrophotometrically, it is indicated by the curves in Fig. 12. These curves are drawn assuming the indicated constants and a value of $n = 1$ (1mol of anion/mol of enzyme–peroxide compound).

DISCUSSION

Complex formed with cytochrome c. The detection of peroxidase–cytochrome *c* complexes in the present study supports the idea that such complexes participate in the catalytic activity of the enzyme towards cytochrome *c*. These observations are therefore in accord with the kinetic mechanism presented previously (Nicholls & Mochan, 1971). Spin-label studies by Drott (1969) have provided additional evidence for cytochrome *c*–peroxidase complex-formation.

The present study also gives some clues as to the nature of complex-formation between peroxidase and cytochrome *c*. The interaction of peroxidase with cytochrome *c* is rapid (10^8–$10^9 M^{-1}s^{-1}$) and approaches the value for a diffusion-controlled process for the two proteins (approx. $10^9 M^{-1}s^{-1}$). This rapid rate indicates the 'correctness of fit' of cytochrome *c* to peroxidase, since deviations from diffusion-controlled rates are usually associated with rate-determining orientations of substrate, or to a requirement for conformational changes before binding is complete (Eigen & Hammes, 1963). This seems to be consistent with the limited ultracentrifugal observations. For some indication of the conformations of ferricytochrome *c* and peroxidase in the complex may be obtained from a comparison of the molecular frictional ratios (f/f_0) of the free components with that estimated for the complex. If the molecular weight and $\bar{v}$ for the cytochrome *c*–peroxidase complex are assumed to be 46800 and 0.73ml/g, respectively, then the frictional ratio for the enzyme is 1.02 compared with 1.12 for the complex. Since f/f_0 for cytochrome *c* is 1.09 (Margoliash & Schejter, 1966), it appears that the formation of a complex between cytochrome *c* and peroxidase does not markedly alter the symmetry of the components.

Inhibition of complex-formation at high ionic strengths and by polycations (Nicholls & Mochan, 1971) suggests that the predominant force between peroxidase and cytochrome *c* is electrostatic in nature. Studies by Mochan & Kabel (1969) with modified cytochromes *c* support this view. Other

forces may be involved in complex-formation, as neither with the albumin nor with horseradish peroxidase was the interaction as great as that observed with cytochrome *c* peroxidase. In addition, the lower affinity of cytochrome *c* for horseradish peroxidase is consistent with the kinetic differences between horseradish peroxidase and cytochrome *c* peroxidase discussed by Nicholls (1964).

Since complex-formation does not markedly alter the spectral characteristics of the components, the primary electrostatic forces are probably associated with the protein moiety rather than the haem. Thus the cyanide derivative of cytochrome *c* peroxidase is still capable of forming a physically detectable complex with ferricytochrome *c*, and ascorbate can still reduce cytochrome *c*, and fluoride can bind peroxidase, when the components are linked together in the complex. The importance of the cytochrome *c* protein groups in complex-formation might be expected; Dickerson *et al.* (1968) have reported that the ferricytochrome *c* haem is buried within a crevice of the protein, and Okunuki, Wada, Matsubara & Takemori (1965) have implicated lysine residue 72 (or 73) in complex-formation with cytochrome oxidase.

Compounds formed with peroxides and other ligands. The titrimetric results suggest that the oxidizing equivalents in the enzyme–peroxide complex may be distinguishable in reactivity towards hydrogen donors, that cytochrome *c* peroxidase is, however, unusually vulnerable to small excesses of hydrogen peroxide, and does not produce a compound of type III, and that the enzyme–peroxide compounds can decompose spontaneously in a reaction accelerated by fluoride, the kinetic characteristics of which suggest complex formation between fluoride and peroxide compound.

The oxidative reactions with both cytochrome *c* and with ferrocyanide may be tentatively summarized in the simplified eqns. (5*a*), (5*b*) and (5*c*) where YH_2 indicates the 'endogenous donor' (Nicholls, 1966) whose semiquinone state is responsible for the free radical signal, YH$^{\bullet}$ indicates the oxidized form of YH_2, and CcP represents cytochrome *c* peroxidase.

A slow secondary reaction such as that given in eqn. (5*c*) can explain the titration values (ferrocyanide–peroxide compound decomposed) intermediate between 1 and 2 sometimes obtained (Table 2). Such variability is a likely explanation of the differences between the present results and those of George (1953*a*), and those reported by Yonetani (1965) and by Wittenberg *et al.* (1968). The pathways indicated in eqns. (5*b*) and (5*c*) may be preferred rather than obligatory pathways for the two hydrogen donors. Electron-spin-resonance data indicate that cytochrome *c* may react preferentially with the free radical species, though Yonetani *et al.* (1966*a*) were reluctant to draw firm conclusions; a $g = 2$ signal disappears at low cytochrome *c* concentrations, and the $g = 2.2$, 2.6 and 6.0 signals, characteristic of the ferric enzyme, appear only at considerably higher cytochrome *c* concentrations. An electron-spin-resonance study of the ferrocyanide reaction has yet to be made. The alternative pathways are consistent with the minimal two-step model of cytochrome *c* oxidation required if the reaction intermediates are 1:1 complexes of enzyme and cytochrome *c*.

The reaction with fluoride may be written formally as in eqn. (6), where the product of reaction (6*a*) is an intermediate of unknown structure containing both the hydrogen fluoride ligand and the OH$^{\bullet}$ moiety of compound II.

The observed limiting velocity is given by k_7 (eqn. 6*b*) and the apparent K_m value, if $k_{-6} \gg k_7$, is equal to the dissociation constant for the enzyme–peroxide complex with fluoride, k_{-6}/k_6 (eqn. 6*a*).

The existence of this reaction, which in the other

$$\underset{\text{CcPFe}^{3+}}{\text{CcPFe}^{3+}}\ \underset{\text{YH}_2}{\text{YH}_2} + \text{H}_2\text{O}_2 \longrightarrow \underset{\text{CcPFeOH}^{3+}}{\text{CcPFeOH}^{3+}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} + \text{H}_2\text{O} \qquad (5a)$$

$$\underset{\text{CcPFeOH}^{3+}}{\text{CcPFeOH}^{3+}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} \xrightarrow{\text{cytochrome } c\text{Fe}^{2+}} \underset{\text{CcPFeOH}^{3+}}{\text{CcPFeOH}^{3+}}\ \underset{\text{YH}_2}{\text{YH}_2} \xrightarrow{\text{cytochrome } c\text{Fe}^{2+}} \underset{\text{CcPFe}^{3+}}{\text{CcPFe}^{3+}}\ \underset{\text{YH}_2}{\text{YH}_2} \qquad (5b)$$

$$\underset{\text{CcPFeOH}^{3+}}{\text{CcPFeOH}^{3+}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} \xrightarrow{\text{Fe(CN)}_6^{4-}} \underset{\text{CcPFe}^{3+}}{\text{CcPFe}^{3+}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} \xrightarrow{\text{Fe(CN)}_6^{-4}} \underset{\text{CcPFe}^{3+}}{\text{CcPFe}^{3+}}\ \underset{\text{YH}_2}{\text{YH}_2} \qquad (5c)$$

$$\underset{\text{CcPFeOH}^{3+}}{\text{CcPFeOH}^{3+}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} + \text{HF} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \underset{\text{CcPFeOH}^{3+}\text{HF}}{\text{CcPFeOH}^{3+}\text{HF}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} \qquad (6a)$$

$$\underset{\text{CcPFeOH}^{3+}\text{HF}}{\text{CcPFeOH}^{3+}\text{HF}}\ \underset{\text{YH}^{\bullet}}{\text{YH}^{\bullet}} \xrightarrow{k_7} \underset{\text{CcPFe}^{3+}\text{HF}}{\text{CcPFe}^{3+}\text{HF}}\ \underset{\text{Y}}{\text{Y}} + \text{H}_2\text{O} \quad \text{or} \quad \underset{\text{CcP}_{ox}\text{Fe}^{3+}\text{HF}}{\text{CcP}_{ox}\text{Fe}^{3+}\text{HF}}\ \underset{\text{YH}_2}{\text{YH}_2} \qquad (6b)$$

$$\text{Per Fe}^{2+} \overset{O_2}{\rightleftharpoons} \text{Compound III} \overset{H_2O_2}{\rightleftharpoons}$$

$$\text{Compound II} \overset{AH_2}{\rightleftharpoons} \text{Per Fe}^{3+} \quad (7)$$

$$H_2O + CcPFe^{2+} \quad YH_2 + O_2 \longrightarrow CcPFeOH^{3+} \quad YH_2 + HO_2^- \quad (8)$$

haem enzymes is always a one-electron process, is further evidence for independence of the two oxidizing equivalents in the peroxide compound. The exact fate of the free radical during this process is, however, unknown. The decomposition pathway may be compared with that postulated for horseradish peroxidase during the oxidation of cytochrome *c* by that enzyme (Nicholls, 1966). The maximum turnover of cytochrome *c* peroxidase (approx. $10^3 s^{-1}$), unlike horseradish peroxidase, greatly exceeds the maximum rate of 'endogenous donor'-catalysed decomposition of the peroxide compound (approx. $0.1 s^{-1}$). The second molecule of cytochrome *c* must therefore either react directly with the haemperoxide in the peroxidase–cytochrome *c* complex, or a pathway for electron transfer much faster than that given by fluoride must be available.

With horseradish peroxidase, compound III formation (eqn. 7) involves the reduction of compound II by hydrogen peroxide or the oxygenation of the ferroenzyme (Wittenberg *et al.* 1967).

Although no compound III has been found with cytochrome *c* peroxidase, Wittenberg *et al.* (1968) have shown that the addition of molecular oxygen to ferrous cytochrome *c* peroxidase results in the formation of a compound with the optical spectrum of the peroxide compound but without the electron-spin-resonance signal. This product would seem to be analogous not to the plant peroxidase compound III produced under similar conditions, nor to the inert products with excess of peroxide described above, but to the compound probably produced in one of the initial reactions with ferrocytochrome *c* (eqn. 5*b*). A stoicheiometric reaction such as that of eqn. (8) would produce this form of the compound directly.

An irreversible reaction in this direction would be the converse of the irreversible formation of oxyhaemoglobin from methaemoglobin peroxide (Keilin & Hartree, 1954); cytochrome *c* peroxidase and haemoglobin may represent extreme cases of the equilibrium of eqn. (7).

The titration data support the picture, elaborated previously (Nicholls, 1964, 1966), of a primary role for 'endogenous donor' groups in the oxidation of cytochrome *c* by peroxidases. The pathways indicated give priority to the reduction of the 'endogenous donor' radical by cytochrome *c*. Whether the second molecule of cytochrome *c* then reacts with the iron-peroxide species, or whether that species dismutes to give the form Fe^{3+} $YH^{\bullet}$ (otherwise generated by ferrocyanide) is still uncertain.

The previous kinetic (Nicholls & Mochan, 1971) and the present physicochemical studies indicate that only one molecule of cytochrome *c* is associated with a molecule of cytochrome *c* peroxidase at one time. The intermediate formation of a one-equivalent oxidation state during the time required for dissociation of oxidized cytochrome *c* and reaction with the second molecule of reduced cytochrome *c* is thus obligatory. We propose the following sequence of events: (*a*) formation of ternary enzyme–peroxide–cytochrome c^{2+} complex by random equilibrium processes, (*b*) intramolecular oxidation–reduction reactions involving 'endogenous donor' and cytochrome *c* to give an enzyme–cytochrome c^{3+} complex retaining one oxidizing equivalent from the peroxide, (*c*) dissociation of oxidized cytochrome *c* and combination with a second molecule of reduced cytochrome *c*, (*d*) a second intramolecular reaction to give ferric peroxidase–cytochrome c^{3+}, and (*e*) formation of the same complex as in (*a*) by random equilibrium, except that dissociation of cytochrome c^{3+} precedes binding of cytochrome c^{2+}.

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