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# Studies on the Peroxidase Effect of Cytochrome c

## IV. The Influence of pH and Certain Anions on the Over-all Reaction

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The peroxidation of pyrogallol to purpurogallin, catalyzed by ferricytochrome c, was studied in the presence of different anions in the pH range 2.3 to 6.8, and the following results were obtained:

(1) The pH-activity curves in citrate, acetate, chloride, and sulphate were of very similar form, differing from each other primarily in the position of and the height at the apparent pH optimum. The pH optima with the different anions were obtained within 0.2 pH unit; the highest value (approx. 3.5) was found in citrate buffer.

(2) In a double logarithmic plot the pH dependence of the catalytic activity revealed the dissociation of two functional groups with $pK_1' = 3.13$ and $pK_2' = 3.78$ (25°C, 40 mM citrate buffer). Evidence is presented in support of the view that these groups are the hemochromogen-forming groups of the cytochrome c molecule, and their nature is discussed.

(3) The peroxidase activity of cytochrome c is greatly influenced by the presence of anions, which seems to be essential in order to obtain measurable rates. The change in activity with the anion concentration follows ordinary, simple dissociation curves, indicating that ferricytochrome c is dissociably combined with the anions. The degree of activation increased in the following order: chloride, citrate, and acetate. The mechanism of the anion activation is discussed.

(4) The influence of pH and anions on the peroxidase activity of ferricytochrome c is paralleled by changes in the light absorption of the pure hemoprotein, indicating a common mechanism for both these effects.

In a previous paper of this series [1] the kinetics of the peroxidation of pyrogallol to purpurogallin, catalyzed by ferricytochrome c,* were studied. A definite pH "optimum" was observed at about 3.5 in 40 mM citrate buffer at 25°C, and the higher the citrate concentration the greater was the catalytic activity at this pH optimum. In the present communication are described

* The following abbreviation will be used: Cyt. c = cytochrome c.

further experiments undertaken for the interpretation of the pH-activity curve and in order to get a clearer idea of the anion effect, *i.e.* the specificity and the essential role of anions for the peroxidase activity of cyt. c.

## MATERIALS AND METHODS

The following materials and methods were used in addition to those previously described: [1]

*Ferricytochrome c* was obtained by autoxidation following acidification to pH 3.0 with 1 N acid; [2] the acid used corresponded to the species of the anion to be studied. When complete oxidation was ensured, the solution was neutralized by 0.1 N NaOH. The acid and the base were added by means of an Agla micrometer syringe.

*Electrolyte and buffer solutions.* Buffers of increasing hydrogen-ion concentration, but fairly constant anion concentration, were prepared by mixing NaOH (of suitable, but constant normality) and acetic or citric acid (of different molarities) (1:1, v/v). The molar concentration of anion ($A^-$) was calculated from the equation,

$$pH = pK_a + \log ([A^-]/[HA]) \quad (1)$$

where $[HA] = [HA_{added}] - [A^-]$.

For acetic and citric acid the $pK_a$ values used were 4.76 and 3.13, respectively (25°C); [3] from a practical point of view the concentration of citrate will be expressed only as univalent anions.

Buffers of increasing anion concentration, but constant pH, were prepared from a series of stock solutions of the acid and the salt (sodium form). 50 ml of the acid was titrated with the salt, of the same molar concentration, to the wanted pH value; the salt was added by means of a 10 ml burette. The molar concentration of anion ($A^-$) was calculated from eqn. (1) where $[HA] = [A_{tot}] - [A^-]$. Solutions of increasing chloride (sulphate) ion concentration, but constant pH, were prepared from dilutions of NaCl ($Na_2SO_4$) by adding the corresponding acid by means of an Agla micrometer syringe.

*Assay of peroxidase activity.* The rate of peroxidation of pyrogallol was assayed as described previously.[1] The composition of the medium was: pyrogallol, 4 mM; hydrogen peroxide, 80 mM; ferri-cyt. c, $2.3 \times 10^{-1}$ μM; pH and the anion concentration of the buffer solutions will be reported for the different experiments. Except where otherwise stated, the activities, will be expressed as "initial" reaction rates, $v = \Delta A_{430} \times \text{min}^{-1}$.[1] The maximal rate ($V$) and the apparent Michaelis constant ($K_m$) were determined graphically according to Lineweaver and Burk; [4] the straight lines fitting the experimental values were calculated by the least-square method.

*Difference spectrophotometry.* The effect of anions on the spectrum of ferri-cyt. c in acid solutions was studied by using a difference spectral technique. Spectra were obtained by a Beckman DK-2A recording spectrophotometer, but for the spectrophotometric titrations a Beckman DU spectrophotometer was used. Both instruments were supplied with equipment for temperature regulation, and the cell compartments were maintained at 25°C by water circulation. Two solutions with identical concentration of ferri-cyt. c, but different anion concentration, were compared. The solution containing the low anion concentration was placed in the reference beam, the solution containing the high anion concentration in the sample beam. In order to test the effect on the difference spectrum of increasing anion concentrations at constant pH, buffers (solutions) were prepared as described above.

In order to study the effect of pH on the difference spectrum obtained by comparing a solution of low (10 mM) with a solution of high (2.7 M) concentration of chloride, stock solutions of 0.01 N HCl and 2.8 M NaCl were prepared and pH was adjusted in both solutions to the desired value by means of 1 N NaOH and 1 N HCl, respectively; acid and base were added by means of an Agla micrometer syringe. After the addition of ferri-cyt. c (in 10 mM NaCl), the pH of the reference and sample solution always agreed within 0.1 pH unit. The final anion concentration was calculated by making corrections for the dilutions.

*pH-measurements.* A glass electrode pH-meter — Radiometer, Copenhagen, model 25 SE — was used. It was standardized against a Beckman standard buffer (pH = 4.01 at 25°C). The pH values of reaction solutions were determined at the temperatures reported in results.

## RESULTS

### Assay of peroxidase activity

The peroxidation of pyrogallol to purpurogallin, catalyzed by cyt. c, was studied as a function of pH and the anion concentration by using the ferric form of the purified hemoprotein.

*a) Effect of pH on V and* $K_m^{AH_2}$. At each pH value $v$ was determined at a series of pyrogallol concentrations and a linear relationship was observed between $v$ and the pyrogallol concentration within the whole pH-range 2.5—4.8, when plotted in a double reciprocal manner.[4] The pH curve for the maximum rate ($V'$) (Fig. 1, A) fits in well with that found by plotting $v$ (as obtained at

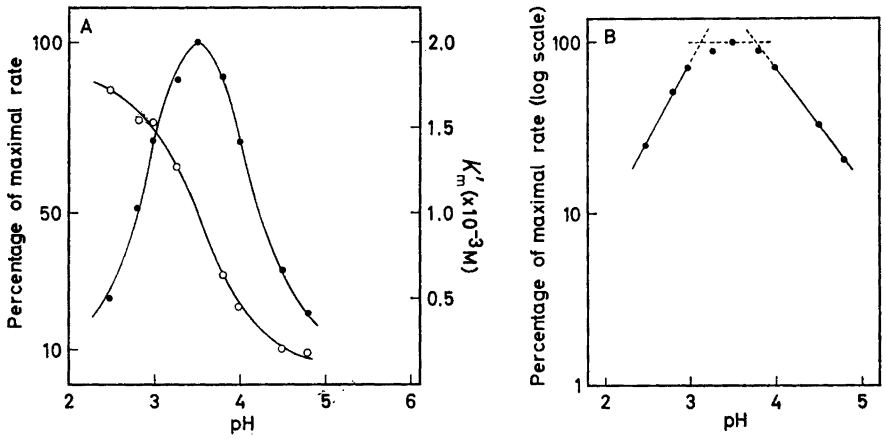


*Fig. 1. A.* Effect of pH on $V'$ (●) and $K_m^{'pyrogallol}$ (○). Standard assay system; $H_2O_2$, 80 mM; cyt. c, $2.3 \times 10^{-1}$ μM; citrate, approx. 40 mM; 25°C. $V' = 100\ \% \sim \Delta A_{430} \times \text{min}^{-1} = 0.361$. For details see text.
*B.* Effect of pH on log $V'$. The data were obtained from Fig. 1, *A*, and the equation of the straight line fitting the three lowest values of the acid and the alkaline branch was calculated by the least-square method. The left-hand intersection ($pK_1$) and the right-hand intersection ($pK_2$) were calculated to be 3.13 and 3.78, respectively.

a pyrogallol concentration of 4 mM) against pH (Fig. 2), and when the pH curve was plotted as devised by Dixon and Webb (Fig. 1, B),[5] the dissociation of functional groups with $pK_1' = 3.13$ and $pK_2' = 3.78$ were revealed; this result is in good agreement with that found by plotting log $v$ against pH ($pK_1' = 3.25$ and $pK_2' = 3.74$). Thus, since it is far more complicated to study the influence of pH on $V'$ than on $v$, the latter procedure will be used in what follows.

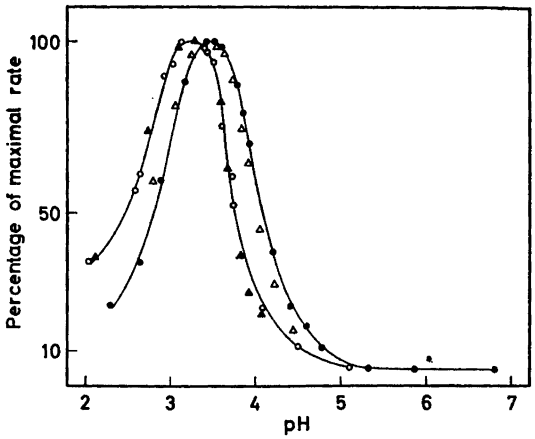


*Fig. 2.* The influence of some anions on the pH-activity curve. Standard assay procedure, 25°C. ●, citrate, approx. 40 mM; ○, acetate, approx. 5 mM; ▲, chloride, approx. 40 mM; △, sulphate, approx. 40 mM.
Only the curves fitting the experimental points in citrate and acetate buffer have been drawn. The maximal rates (100 %) were $\Delta A_{430} \times \text{min}^{-1} = 0.265$, 0.276, 0.119, and 0.113 for citrate, acetate, chloride, and sulphate, respectively. The curves have all been reduced to the same vertical scale, the better to illustrate the small change in shape and position of the maximum.

When $K_m{}'^{AH_2}$ was plotted *vs.* pH an S-shaped curve with inflection point at about pH 3.5, *i.e.* at the optimum of peroxidase activity, was found (Fig. 1, A).

*b) Effect of anions on the pH-activity curve.* The pH-activity curves obtained in the presence of citrate, acetate, chloride, and sulphate are shown in Fig. 2.

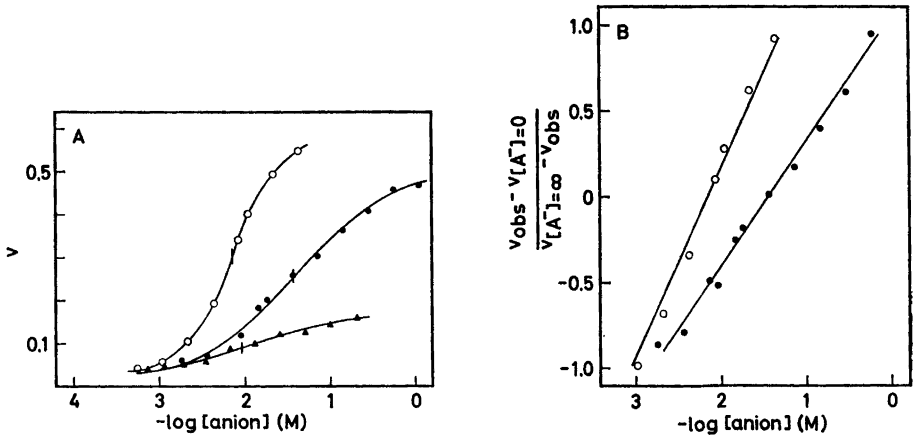


*Fig. 3. A.* The influence of the concentration of some anions on $v$ ($\Delta A_{430} \times \text{min}^{-1}$). Standard assay procedure; pH 3.5; 25°C. ○, acetate; ●, citrate; and ▲, chloride.
*B.* Graphical determination of the number $n$ of activating anions which combine with one molecule ferri-cyt. c at pH 3.5. Symbols as in Fig. 3, A.

The curves were of very similar form, and differed from each other primarily in the position of and the height at the apparent pH optimum, depending on the species of the anion present; the pH-optima were obtained within 0.2 pH-unit, and the highest value (3.5) was found in citrate.

c) *The activating effect of anions.* The reaction rate was greatly influenced by the concentration of buffer anions. If $v$ was determined at increasing concentrations of anions at the pH optimum (3.5) in citrate, there were obtained S-shaped activation curves (Fig. 3, A) which approach asymptotically to zero at low anion concentrations. This effect indicates that anions are essential if the reaction is to proceed at a measurable rate.

Assuming the anion-activity curves to be completely symmetrical even at extreme anion concentrations, the half maximal activity ($v_{0.5}$) was obtained from the inflection points. If the negative logarithm of the anion concentration required to obtain this activity (*i.e.* $pK_A{}^-{}'$) was used as an expression of the apparent affinity of ferri-cyt. c for the anion, the affinities increased in the order: citrate, chloride, and acetate (Table 1). Furthermore, the rate at $[A^-] = \infty$ can be calculated, and by plotting

$$\log \frac{v_{obs} - v_{[A^-]=0}}{v_{[A^-]=\infty} - v_{obs}}$$

*vs.* $-\log [A^-]$, the number ($n$) of anions ($A^-$) combining with one molecule of ferri-cyt. c at pH 3.5 can be calculated (Fig. 3, B). The values thus obtained were $n_{Ac^-} = 1.1$ and $n_{Cit^-} = 0.7$; the effect of chloride ions was, however, too small to allow accurate calculation of $n$.

*Table 1.* The apparent affinity of ferri-cyt. c for some anions and their activating effect in the peroxidase reaction.[a]

| Anion | $pK_A{}^-{}' = -\log[A^-]_{0.5}$[b] (M) | $[A^-]_{0.5}$ (mM) | $v_{0.5}$ ($\Delta A_{430} \cdot min^{-1}$) |
|---|---|---|---|
| Acetate | 2.15 | 7.1 | 0.306 |
| Chloride | 2.04 | 9.1 | 0.109 |
| Citrate[c] | 1.44 | 36.3 | 0.254 |

[a] Calc. from Fig. 3 (for details see text).
[b] $[A^-]_{0.5}$ = molar concentration of anion at which half maximal activity ($v_{0.5}$) is obtained.
[c] Calc. as univalent anion.

On the other hand, the activating effect increased in the order: chloride, citrate, and acetate (Table 1). Complete anion-activity curves were made only at pH 3.5, but the same order of activating effect was observed within the whole pH range 2.2—5.0.

d) *Effect of temperature on the pH-activity curve.* Complete pH-activity curves determined at 17.2°C and 36.1°C, are given in Fig. 4. A marked displacement of both $pK_1'$ and $pK_2'$ to more alkaline values was obtained at the

*Table 2.* Effect of temperature on the apparent pH optimum, the apparent p$K$ values of catalytically active groups and the concentration of catalytically active hemoprotein.[a]

| Tp (°C) | Apparent pH optimum | p$K_1'$ | p$K_2'$ | (p$K_2'$−p$K_1'$) | [Cyt−H$^+$][b] (%) | $v_{max}$[c] ($\Delta A_{430}$·min$^{-1}$) |
|---|---|---|---|---|---|---|
| 17.2 | 3.46 | 3.19 | 3.73 | 0.54 | 32 | 0.190 |
| 36.1 | 3.88 | 3.53 | 4.21 | 0.68 | 38 | 0.435 |

[a] Calc. from Fig. 4.
[b] Percentage of total cyt. c concentration calc. as shown in Fig. 8 by using the p$K'$ values given in this table (for details see text).
[c] $v_{max}$ = $v$ at the apparent pH optimum.

highest temperature, and $\Delta pK_2' > \Delta pK_1'$ (Table 2). The apparent heats of dissociation were calculated from the modified van't Hoff equation,[6]

$$\Delta H' = -4.5787 \frac{d(\log K)}{d(1/T)} \quad (2)$$

and found to be −7.4 kcal mole$^{-1}$ (p$K_1'$) and −10.4 kcal mole$^{-1}$ (p$K_2'$). Furthermore, there was an increase of $v$ at the apparent pH optimum when the temperature was increased from 17.2°C to 36.1°C (Table 2).

*e) Effect of pH on the stability of cytochrome c.* The enzyme was exposed to the range of pH values of 2.0—3.5 for 30 min by means of 40 mM citrate buffer. Following this procedure, no significant difference in "specific" activity ($\Delta A_{430}$· min$^{-1}$ · $\mu$M$^{-1}$) could be demonstrated at any pH when tested afterwards at the pH optimum (3.5) in 40 mM citrate buffer.

## Difference spectrophotometry

In order to get a clearer idea of the mechanism by which cyt. c is activated as a peroxidase, it was of essential interest to see whether or not the changes in peroxidase activity, determined as a function of pH and anion concentration, could be correlated with changes in other physico-chemical properties of the ferri-cyt. c molecule. Generally, determination of absorption spectra is perhaps the easiest and most accurate analytical method which can be used in the study of hemoproteins and their reactions with hydrogen ions and anions.

*a) Effect of anions.* In 1941, Theorell and Åkeson[2] reported that ferri-cyt. c gives a spectrophotometrically measurable fluoride compound around pH 3 that is similar to ferrihemoglobin fluoride. Later, Boeri *et al.*[7] found that other anions too ($Cl^-$, $Br^-$, and $SO_4^{2-}$) form spectrophotometrically and magnetometrically measurable compounds with ferri-cyt. c in strong acid solutions. At pH values higher than 2.0 the changes in light absorption produced by chloride were found to be too small to allow accurate calculations, and anions of the more weak carboxylic acids were not tested. In the present study, therefore, the more sensitive difference-spectral technique was used,

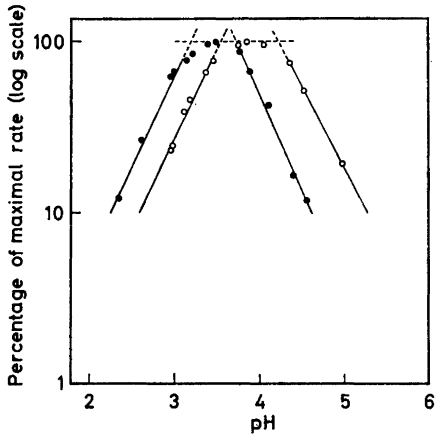


*Fig. 4.* The influence of temperature on the pH-activity curve. Standard assay procedure; citrate, approx. 120 mM at pH 3.5 (calc. as univalent anion by using $pK_a = 3.13$ at 25°C); ●, 17.2°C and ○, 36.1°C. The maximal rates (100 %) were $\Delta A_{430} \times min^{-1} = 0.190$ and 0.435 at 17.2°C and 36.1°C, respectively. $pK_1'$ was calculated to be 3.19 (17.2°C) and 3.53 (36.1°C), and $pK_2'$ 3.73 (17.2°C) and 4.21 (36.1°C). Symbols as in Fig. 1, *B*.

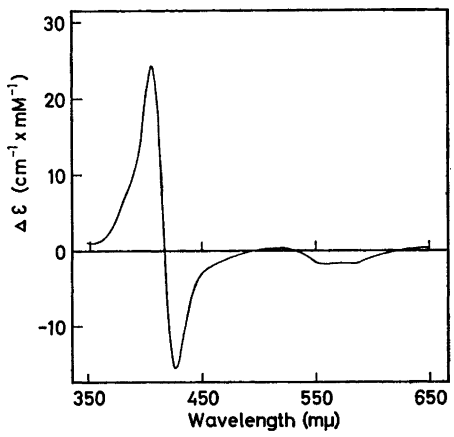


*Fig. 5.* Difference spectrum of ferri-cyt. c in 2.7 M chloride *vs.* a reference solution containing the same concentration of ferri-cyt. c in 10 mM chloride; pH 3.3 and 25°C. For details see text.

and from among the anions tested above chloride and acetate were selected because they are univalent anions of an inorganic and an organic (carboxylic) acid and exhibit great differences in the anion-activity curve (Fig. 3, A).

Fig. 5 shows the difference spectrum of ferri-cyt. c obtained by comparing a solution of low (10 mM) with a solution of high (2.7 M) concentration of chloride at the apparent pH optimum (3.3) for the peroxidase activity with this anion. In the Soret region the maximal change of the absorption spectrum was observed at 398 mμ. Very similar spectral changes were observed in acetate, but the maximal change of the absorption spectrum was observed at 396 mμ. Furthermore, the value of $\Delta\varepsilon$ ($cm^{-1} \times mM^{-1}$) was higher in acetate than in chloride at equal anion concentrations (Fig. 6). The relative effect of acetate and chloride ions on the Soret band spectrum of ferri-cyt. c was of the same order of magnitude as that obtained by measurement of peroxidase activity (Fig. 3, A). However, as was to be expected, no symmetrical, S-shaped titration curves were obtained, since the reference solution also contained an appreciable concentration of anions. Thus, no p$K$ values can be calculated from these curves.

*b) Effect of pH.* Fig. 7 shows the effect of pH on the difference in absorbancy at 298 mμ of ferri-cyt. c obtained by comparing a solution of low (10 mM) and a solution of high (2.7 M) concentration of chloride. The curve revealed

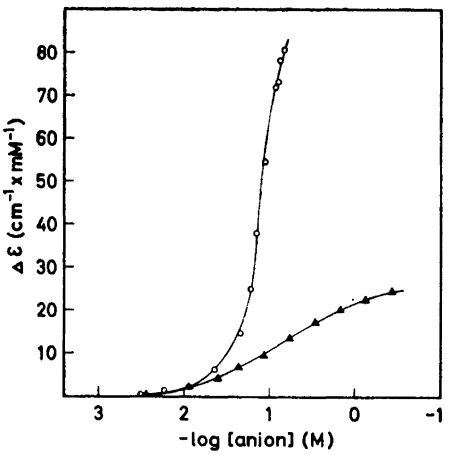


*Fig. 6.* Spectrophotometric titration of ferri-cyt. c with anions at pH 3.3 and 25°C. A solution containing anions at a concentration of 10 mM was used as a reference; for details see text. O, acetate at 396 mμ; ▲, chloride at 398 mμ.

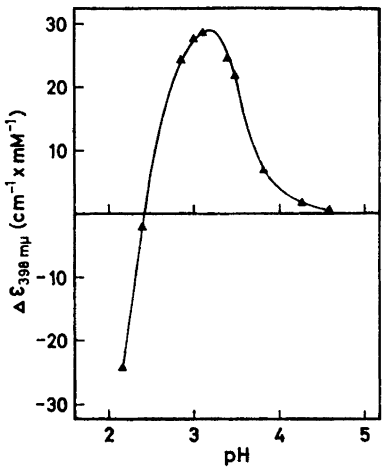


*Fig. 7.* Effect of pH on the difference spectrum of ferri-cyt. c in 2.7 M chloride *vs.* a reference solution containing 10 mM chloride. 25°C.

an apparent positive optimum value at pH about 3.2. The alkaline branch of the curve approaches asymptotically to zero over approximately pH 5.0, and the acid branch becomes negative below pH 2.45.

## DISCUSSION

### The interpretation of the pH-activity curve

It was not possible to obtain pH-activity curves showing a pure hydrogen-ion effect, since no activity could be measured in the absence of added anions (Fig. 3, A). Thus, each of the pH-activity curves are composite curves representing a combined effect of hydrogen ions and anions, but since the concentration of anions was held at a fairly constant level throughout the whole pH region, the curves can be interpreted primarily on the basis of a pH effect.

Except for the common peroxidases, such as horse radish peroxidase, native hemoproteins do not show any (*e.g.* methemoglobin and catalase [8]) or only negligible (*e.g.* cyt. c [1]) peroxidase activity at physiological pH values. However, an activation of the peroxidase activity of, *e.g.*, methemoglobin and catalase has been obtained by different modifications of their native structure, *e.g.* by denaturation with acid or by alkali.[8] Such a mechanism may also explain the activation of ferri-cyt. c as a peroxidase. Thus, it has recently been shown by ultraviolet difference spectra that the conformation of the native hemoprotein is changed in acid solution;[9] the pH dependence revealed a typical (S-shaped) titration curve with an apparent p$K$ of 2.80

(23.2°C, $\mu = 0.1$) for the single protonated group involved in the spectral transition. However, an irreversible denaturation or destruction of the cyt. c molecule *per se* as a prerequisite for the apparent pH optimum of its peroxidase activity can be ruled out, since the conformational change is completely reversible at least down to pH 1.[9]

In a previous study [1] it was found that the apparent optimum concentration of pyrogallol depends on the concentration of hydrogen peroxide, and *vice versa*. When this donor-substrate relationship was studied at different pH values, $K_m'$ and consequently also the apparent optimum concentration of pyrogallol (at a given, constant concentration of $H_2O_2$) decreased the higher the pH (Fig. 1). This resulted, however, only in an insignificant effect on the pH-activity curve; almost the same form was obtained whether the rates were determined as $V_{max}'$ (Fig. 1) or as $v$ (Fig. 2). Therefore, the donor-substrate relationship has only a minimum effect on the pH-activity curve as determined in the standard assay procedure (Fig. 2).

The peroxidation of pyrogallol, catalyzed, *e.g.*, by horse radish peroxidase, proceeds through the formation of intermediary compounds.[10–13] Nilsson [13] has studied the kinetics of two consecutive reactions, *i.e.* (1) the formation of a blue, intermediary compound, and (2) the elimination of this compound, accompanied by chemiluminiscence, by which the final products are formed. The former reaction was stated to be almost independent of pH, but the latter reaction, studied in the interval of pH 4.0—9.0, revealed an "optimum" between pH 6.0 and 6.5. Furthermore, the elimination of the blue compound, which is peroxide-dependent, will not be rate-limiting in the over-all reaction at the high peroxide concentration (80 mM) used in the present study. Thus, these intermediary reactions do not explain the pH-activity curve obtained in the present study. On the other hand, the pH-activity curve may reflect the enzymic formation of a primary product, *i.e.* the free radical, which has been shown to occur [11] and which, it has been suggested,[13] precedes the formation of the blue compound.

The pH-activity curve, when plotted according to Dixon and Webb [5] (Fig. 1, B), revealed the dissociation of two functional groups; $pK_1' = 3.13$ and $pK_2' = 3.78$. Inasmuch as the reactants, $H_2O_2$[14] and pyrogallol,[15] contain no titratable groups in the pH range studied, the dissociation of groups in the ferri-cyt. c molecule seems very likely. The following facts all support the view that these are the hemochromogen-forming groups:

(1) The iron-protein bonds in ferri-cyt. c are broken in acid solution.[2,7] On the basis of the changes in absorption spectrum and magnetic properties, Boeri *et al.*[7] showed that two protons entered the ferri-cyt. c molecule and formed a new compound (Cyt-$2H^+$), and that the two dissociation steps were very closely linked. At low chloride concentrations, a plot of $\Delta A_{395}$ *vs.* pH had the form of a part of a dissociation curve which reached an asymptotic level of pH 5.5. This effect is close to that obtained in the present study for the activation of cyt. c as a peroxidase (*cf.* the alkaline branch of the pH-activity curve in Fig. 2).

(2) George *et al.*[16] studied the equilibrium constants for the formation of the azide and cyanide complexes of ferri-cyt. c over the pH-range 4—8, and showed that there is a change in the $[H^+]$ dependence for both reactions at

about pH 5, which was considered to be consistent with the onset of protonation of one of the hemochromogen-forming groups (and splitting of the iron-protein bond) at this pH.

(3) In his titration studies, Paul [17] found that the protein moiety of cyt. c consumed two equivalents of base per mole more than did the intact hemoprotein in the pH-range 4—6. These equivalents were attributed to the titration of amino acid groups, constituting the hemochromogen-forming groups in the intact hemoprotein, closer to their normal p$K$ values. In the intact hemoprotein, however, these groups were in the main titrated below pH 4.

(4) Ehrenberg and Szczepkowski [18] found that one histidine imidazole, which is released when ferri-cyt. c reacts with NO, gave by titration a p$K$ somewhere between 3 and 5.

Thus, the pH dependence for the activation of cyt. c to a peroxidase appears to agree well with the breaking of one of its iron-protein bonds by protons. On the other hand, the decrease in peroxidase activity at pH's below the optimum can be explained by breaking of the second bond in analogy with that observed for horse radish peroxidase. Thus, the activity of this peroxidase, as determined by the classical purpurogallin method, disappeared in parallel with the splitting of its single iron-protein linkage in acid solution.[19] Thus, the proposed mechanism assumes protonation of the hemochromogen-forming groups (and splitting of the iron-protein bonds) of cyt. c in two stages according to the following equations,

$$\text{Cyt} + \text{H}^+ \rightleftharpoons \text{Cyt}-\text{H}^+ \quad (3)$$

$$\text{Cyt}-\text{H}^+ + \text{H}^+ \rightleftharpoons \text{Cyt}-2\text{H}^+ \quad (4)$$

and that the intermediary form, Cyt—$H^+$, in the presence of anions, is the catalytically active form in the peroxidase reaction. In Fig. 8 the difference curve, representing the intermediary form, Cyt—$H^+$, has been calculated from the two ionization curves representing eqns. (3) and (4). The concentration of Cyt—$H^+$ has evidently a maximum value at about pH 3.5, *i.e.* at the pH optimum of peroxidase activity, and the curve is of the same form as the pH-activity curve. The good agreement between the theoretical curve of

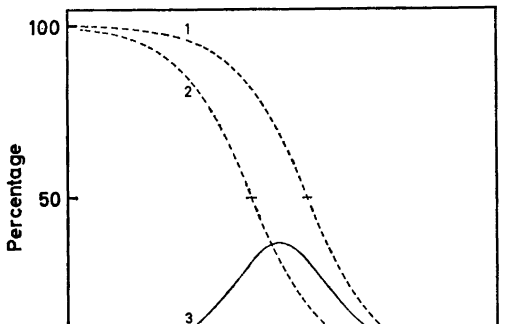


*Fig. 8.* Theoretical curve for the concentration of the intermediary form Cyt-$H^+$ (curve 3) based on the assumption that the protonation of the hemochromogen-forming groups in ferri-cyt. c proceeds in two stages according to eqns. (3) and (4) with p$K$'s at 3.78 (curve 1) and 3.13 (curve 2) for the first and the second ionization, respectively, (data from Fig. 1, B). Curve 3 was calculated as the difference between curves 1 and 2. The maximum concentration of Cyt-$H^+$ amounts to 36.5 % of the total.

Cyt—$H^+$ and the experimentally obtained pH-activity curve is regarded as strong evidence for the validity of eqns. (3) and (4); the slight asymmetry in the latter curve may be attributed to the small variation of the anion concentration with pH.

Thus, the general theory advanced by Theorell [20] that the catalytic activity of common peroxidases is determined by heme-linked groups may also serve to explain the peroxidatic activity of cyt. c.

In the foregoing the two p$K'$ values have been treated as corresponding to dissociations of the "free" hemoprotein. It may well be, however, that a possible combination of the "enzyme" with its substrates produces a change in its p$K$ values. But, as shown by Dixon and Webb,[5] the p$K$ of a group in an enzyme, which may affect the rate and appear in the log $V$ curve, will be cancelled out of the p$K_m$ curve if its ionization is not affected by combination with the substrate. This was actually the case in the present study (Fig.1) which indicates that the p$K$'s obtained corresponded to those of the "free enzyme" (*i.e.* ferri-cyt. c).

Generally, the concentration of hydrogen ions affects the stability of metal-ligand complexes by competition with the metal ion for the ligand. Thus, for ferri-cyt. c in acid solution we have the dissociation equilibria,

$$L_2 + H^+ \rightleftharpoons L_2H^+ \quad (5)$$

$$L_1 + H^+ \rightleftharpoons L_1H^+ \quad (6)$$

for the protonation of the hemochromogen-forming groups ($L_1$ and $L_2$), and the dissociation equilibria of the hematin iron is represented by the equations

$$L_1\text{-Fe-}L_2 \rightleftharpoons L_1\text{-Fe} + L_2 \quad (7)$$

$$L_1\text{-Fe} \rightleftharpoons Fe + L_1 \quad (8)$$

The effect of anions and the change in protein conformation on this equilibrium system will not be considered at present.

The dissociation constants for reactions (7) and (5) are given by the expressions,

$$K_{L_2} = \frac{[L_1\text{-Fe}]\,[L_2]}{[L_1\text{-Fe-}L_2]} \quad (9)$$

$$K_{H_2}{}^+ = \frac{[L_2]\,[H^+]}{[L_2H^+]} \quad (10)$$

and the combination of eqns. (9) and 10) gives

$$K_{L_2} = K_{H_2}{}^+ \frac{[L_1\text{-Fe}]\,[L_2H^+]}{[L_1\text{-Fe-}L_2]\,[H^+]} \quad (11)$$

By similar calculation there is obtained from eqns. (8) and (6)

$$K_{L_1} = K_{H_1}{}^+ \frac{[Fe]\,[L_1H^+]}{[L_1\text{-Fe}]\,[H^+]} \quad (12)$$

At 50 % ionization of $L_2$ and $L_1$, at $pH_2$ and $pH_1$, respectively, $[L_1\text{-Fe-}L_2] = [L_1\text{-Fe}] = [Fe] = [L_2H^+] = [L_1H^+] = \frac{1}{2}$ [cyt. $c_{tot}$] which, by combination of eqns. (11) and (12), gives

$$\frac{K_{L_2}}{K_{L_1}} = \frac{K_{H_2}^+}{K_{H_1}^+} \times 10^{(pH_2 - pH_1)} \quad (13)$$

or, in terms of association constants ($k$),

$$\frac{k_{L_1}}{k_{L_2}} = \frac{k_{H_1}^+}{k_{H_2}^+} \times 10^{(pH_2 - pH_1)} \quad (14)$$

Strong evidence has been presented in support of the view that both iron-linked groups of cyt. c are histidine imidazoles.[7,21-24] If it is assumed that these imidazole groups have the same $pK_H^+$ value and that $pH_1 = 3.13$ and $pH_2 = 3.78$ (see Fig. 1, B), we obtain $k_{L_1}/k_{L_2} = 4.1$. This difference in association constants shows the trend characteristic of metal ion-ligand complexes.[25] Provided that such influences as asymmetry and the effect of one metal-ligand bond on the strength of the other may be ignored, the ratio of successive association constants of identical ligands should be given by the expression [26]

$$\frac{k_n}{k_{n+1}} = \frac{(N - n + 1)\ (n + 1)}{(N - n)\ n} \quad (15)$$

where $N$ is the maximum number of ligands bond by the metal ion; this gives $k_1/k_2 = 4$ when $N = 2$. Thus, the experimental value (4.1) is close to that expected on a pure statistical consideration, which further supports the view that $L_1$ and $L_2$ are identical, but not equally firmly bound to the iron. The experimental procedure in this study, however, does not allow the calculation of the absolute value of the equilibrium constants.

The pH-activity curves obtained in acetate, citrate, chloride, and sulphate exhibited slightly different optima. This is not an unique effect; from the studies of other enzymic reactions it is known that pH-activity curves are shifted by buffer anions.[27-30] Thus, the $pK_a$'s for the ES complexes of fumarase are generally about 0.5 unit lower in acetate than in phosphate buffer,[29] and a difference of the same order of magnitude was found for the same anions in the oxidation of ferro-cyt. c by cytochrome *c* oxidase.[30]

The effect of the temperature calls for some comment. As seen from Fig. 4 and Table 2 the $pK'$ values of the catalytically active groups, and consequently also the pH optimum, are shifted towards the alkaline side when the reaction temperature rises. The large negative values of the apparent heat of dissociation ($\Delta H_1' = -7.4$ kcal mole$^{-1}$ and $\Delta H_2' = -10.4$ kcal mole$^{-1}$) thus obtained is of the same order of magnitude as that observed for the spectrophotometric titration of an abnormal ("buried") carboxyl group in ferri-cyt. c.[9] It is therefore probable that the $\Delta H'$ values obtained in the present study mainly reflect the thermodynamic parameters for the conformational change which takes place in acid solution.[9] Furthermore, it is noteworthy that the difference in the displacements of $pK_1'$ and $pK_2'$ will increase the concentration of the catalytically active form of the hemoprotein (Table 2) at the higher temperatures. This increase, as well as the displacement of the apparent pH optimum (Table 2), will both affect the temperature coefficient ($Q_{10}$) and the energy of activation ($E'$) as calculated in a previous paper.[1]

## Activation by anions

Anions were found to play an essential role in the peroxidation of pyrogallol catalyzed by cyt. c (Fig. 3) as the rate approaches zero at low anion concentrations. Since the anions themselves do not take part in the reaction catalyzed by the enzyme, they fulfil the criteria of enzyme activators.[31] The activating effect was shown to be fairly unspecific since all anions so far tested were found to have an effect. However, the extent of activation was greatly influenced by the nature of the anion and its concentration.

Generally, the effects of ions on enzymic reactions can be produced in many different ways, and in most cases the underlying physico-chemical factors are still not understood.[31] Regarding the mechanism of the effect of anions on the perioxidase activity of cyt. c, this will be discussed in the light of four different observations reported in the literature, *i.e.* (i) the effect of anions in the peroxidase reaction catalyzed by horse radish peroxidase, (ii) the anion compounds formed with ferri-cyt. c in acid solution, (iii) the activation of the peroxidatic activity of hematin upon the addition of heterogeneous ligands, and (iv) the effect of anions on protein titration curves.

(i) The increasing degree of activation with increasing anion concentration is in agreement with that observed by Ettori,[32] studying the effect of phosphate on the peroxidation of pyrogallol catalyzed by horse radish peroxidase (HRPO). He found that the activity of the enzyme increased markedly with the concentration of phosphate. The mechanism by which phosphate ions promoted this effect was not explored; it was only suggested that the harmful effect of $H_2O_2$ on the enzyme seemed to be greatly reduced in phosphate buffer. However, in his kinetic studies on the enzyme substrate compounds of HRPO and peroxide, Chance[33] observed that acetate, in comparison with phosphate, increased the rate constant for the decomposition of HRPO-$H_2O_2$ complex II, and that the higher the concentration of acetate the higher was the rate constant. To explain this effect he postulated that the enzyme contained an "acceptor" substance (later referred to as an endogenous donor or a reducing substance,[34–37]) which was present in a higher concentration in acetate than in phosphate buffer. Chance did not, however, arrive at any definite conclusion and left the problem open for further investigations.

(ii) It was shown by Boeri *et al.*[7] that anions form spectrophotometrically measurable compounds with ferri-cyt. c in acid solution, and a theory of the structure of the Cl-compound was advanced by them. Thus, two chloride ions were found to enter into Cyt-$2H^+$ through the formation of a chain, $\diagdown N^+H\ Cl^-\ Fe^+\ Cl^-\ H^+N\diagup$; the value of $n_{Cl^-}$ was calculated only at pH 1 and 2 since the changes in light-absorption were too small to allow accurate calculations at higher pH values. In the present study, however, the more sensitive difference-spectral technique revealed (Fig. 7) that, even in the pH range 2.5—5.0, chloride and acetate ions have a marked effect on the spectrum of ferri-cyt. c in the Soret region, but it did not allow the calculation of the $n$ value (see below). From the activity measurements, however, $n_{Ac^-,\ pH=3.5}$ was calculated to be 1.1. Acetate was more potent than chloride in promoting the spectral change, as was the case in the peroxidase reaction. The interpretation of the changes of the difference spectrum was, however, complicated

by the presence of the relatively high concentration of anions (10 mM) in the reference solution. When corrections were made for this concentration, good agreement was found between the effect of anions on the peroxidase activity and on the changes of the absorption spectrum of ferri-cyt. c, which suggests that in each case a common chemical effect lies behind.

Using the nomenclature of Boeri *et al.*,[7] it appears that the protonated forms of ferri-cyt. c (Cyt-$H^+$ and Cyt-$2H^+$), as defined in eqns. (3) and (4), are completely ineffective in catalyzing the peroxidation of pyrogallol; catalytic activity can be demonstrated only when anions enter into Cyt-$H^+$,

$$\text{Cyt-H}^+ + \text{A}^- \rightleftharpoons \text{Cyt-H}^+ \cdots \text{A}^- \qquad (16)$$

where $A^-$ can be the anion of an univalent or a polyvalent inorganic or organic (carboxylic) acid. The chemical nature of this anion complex is not clear, but the anions are probably bound to the iron atom of the cyt. c molecule, replacing one of the original hemochromogen-forming groups. On the other hand, the exact mechanism by which the anions promote the activating effect is still unknown. Perhaps anions may play the same role as previously described for the oxidation of one metal ion complex by another;[38] in such oxidation-reduction systems inorganic anions serve to transfer electrons from the reducing to the oxidizing agent, and greatly increase the rate of the over-all reaction. A similar anion effect has also been observed in the so-called Hill reaction.[39]

(iii) Nakamura *et al.*[40] have studied the non-protein hematin complexes with various nitrogenous substances as model enzymes of peroxidase. They found that complexes carrying different ligands (*e.g.* histidine-guanidine and pilocarpine-guanidine) in the fifth and sixth coordination position of the iron atom exhibited a much higher activity than complexes carrying two identical ligands.

The proposed mechanism of activation of cyt. c as a peroxidase is in good agreement with these observations, since the catalytically active form of ferri-cyt. c appears to be a hematin compound with heterogeneous ligands. Thus, cyt. c has only a minimal peroxidatic activity within the pH range where both iron-protein bonds are either intact (pH $> 5.5$) or completely broken (pH $< 1.0$). On the other hand, within the pH range where one of the iron-protein bonds is probably intact and an anion is positioned (as a ligand) on the other side of the heme disc, replacing the other hemochromogen-forming group, a high activity can be measured. Though no extensive studies have been performed on the relation between the effect on the peroxidase activity and the size, structure, and basicity of the anions, the activation of ferri-cyt. c by anions evidently becomes greater the lower the dissociation constant of the corresponding acid (Fig.3, A). In this connection it is noteworthy that the dissociation constant for chloride $K_{Cl^-,\ pH=3.5} = 10^{-2.04}$, based on activity measurements, is in good agreement with the value $K_{Cl^-,\ pH=1} = 10^{-2.12}$ previously found by spectrophotometric titration.[7]

(iv) In his acid-base titration studies on egg albumin, Steinhardt[41] found that the curves obtained with different anions were very similar in their general form; they differed from each other primarily by parallel displacements along the pH axis. This effect was attributed to a combination of the protein and

the anions, and the greater the affinity of the protein for the anion, the more the midpoint of the curve was displaced towards the alkaline side. In the present study, however, no such correlation was observed, which also indicates a somewhat more specific effect of the anions in this case.

In conclusion, from the results given in the present study two equilibria seem to be of predominant importance for the peroxidase activity of cyt. c, namely the protonation of ferri-cyt. c and the combination of anions with this protonated form. Several of the commonly used buffer anions are able to combine with ferri-cyt. c in acid solution, resulting in an activation of its peroxidase activity. However, the affinity for these anions and the degree of activation obtained differ.

*Note added in proof.* As far as the iron-protein bonds in beef heart cyt. c is concerned, the conclusion of the present work is in accordance with a recent paper by Horinishi *et al.* (*Arch. Biochem Biophys.* **111** (1965) 520) where they used a new coupling reagent for histidine residues.

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