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Studies on the Peroxidase Effect of Cytochrome c

III. A Kinetic Study of the Over-all Reaction

T. FLATMARK

*Institute for Thrombosis Research, University Hospital (Rikshospitalet), Oslo, Norway and Department of Medical Chemistry, University of Umeå, Sweden**

(1) The peroxidation of pyrogallol to purpurogallin, catalyzed by ferricytochrome c, satisfied the criteria of an enzymic reaction, which fitted the properties of a "true" peroxidase; *i.e.* the catalytic activity exhibited a definite pH "optimum" of about 3.5 (40 mM citrate buffer; 25°C), and was a linear function of the concentration of the hemoprotein.

(2) The apparent optimum concentration of either reactant (H_2O_2 and pyrogallol) was found to be higher, the higher the concentration of the other; no definite optimum concentrations can therefore be given. In a double reciprocal plot, the "initial" reaction rate was proportional to the concentration of pyrogallol and to the square of the concentration of H_2O_2. A high concentration of this reactant was always required to obtain a measurable reaction rate.

(3) Cytochrome c was progressively destroyed during the reaction, and this destruction proceeded more rapidly, the higher the concentration of H_2O_2 and the temperature. The reaction was further characterized by a nonlinear Arrhenius plot and a high apparent activation energy ($E' \approx 22\,200$ cal mole^{-1}) within the temperature range 15–25°C.

(4) Ferrocytochrome c was just as effective as the ferric form, inasmuch as it is rapidly oxidized to the trivalent condition.

(5) The peroxidase reaction is a sensitive and accurate method for the assay of cytochrome c. In the standard procedure of the present study, it was calculated to be approximately 40 and 130 times more sensitive than the spectrophotometric determination of the absorbancy in the α-band of ferrocytochrome c when the reaction product was measured after 1 and 10 min, respectively. The sensitivity could, however, be further increased by the use of higher concentration of the substrates and/or of buffer anions.

In a previous paper,[1] it was shown that cytochrome *c*** can be identified and assayed at very low concentrations in the soluble fraction of rat kidney homogenates after its separation by paper electrophoresis using a

* Present address: Institute for Thrombosis Research, University Hospital (Rikshospitalet), Oslo, Norway.

** The following abbreviations will be used: Cyt. c = cytochrome c; HRPO = horse radish peroxidase; MPO = myeloperoxidase; LPO = lactoperoxidase.

benzidine-peroxide-nitroprusside solution as location reagent. Since this peroxidase reaction represents a new approach in the assay as well as in the study of cyt. c, it was thought desirable to investigate the reaction mechanism in further detail by using a spectrophotometric method and a highly purified preparation of cyt. c. In the present experimental work it is demonstrated that cyt. c behaves like a "true" peroxidase at low pH values, and that the over-all kinetics mostly agree with those earlier reported on the peroxidase reaction catalyzed by horse radish peroxidase. A sensitive spectrophotometric method for the rapid and accurate assay of cyt. c based on this kinetic study is described. A preliminary report of certain aspects of this work has already appeared,[2] and an application of the method to the assaying of cyt. c in biological materials will be adduced in a forthcoming paper.[3]

MATERIALS AND METHODS

Materials

Cytochrome c was purified from beef heart muscle by gel filtration on Sephadex G-75,[4] and it was isolated in the ferric form by chromatography on Duolite CS-101.[5] The preparation, representing the native, monomeric form of cyt. c, displayed an iron content of 0.43(8) % and a ratio $A_{550\ \mathrm{red}}/A_{280\ \mathrm{ox}} = 1.26$.[4] Before use, the preparation was dialyzed against 2 mM ammonia. Ferro-cyt. c was obtained by reduction with Pd-H_2[6] in such a way that no H_2O_2 was generated during the procedure.[7]

Chemicals. A.g. chemicals (from E. Merck AG, Germany, and Hopkin & Williams Ltd., England) and water redistilled in quartz vessels, were used throughout.

Glassware. All glassware, including the cuvettes, were cleaned with bichromate-sulphuric acid.

pH-meter. A glass electrode pH-meter, Radiometer, Copenhagen, Denmark, Model 25 SE, was used.

Methods

The concentration of cytochrome c was determined spectrophotometrically after reduction with sodium dithionite in 65 mM sodium phosphate buffer, pH 6.8, using the extinction coefficients $E^{1\ \%}_{1\ \mathrm{cm}}$ at 550 mμ red = 23.94 and ε ($\mathrm{cm^{-1}} \times \mathrm{mM^{-1}}$) at 550 mμ red = 30.53.[4]

Assay of hydrogen peroxide. The concentration of H_2O_2 was assayed by ultraviolet spectrophotometry [8] using the molar extinction coefficient ε ($\mathrm{cm^{-1}} \times \mathrm{M^{-1}}$) at 301.5 mμ = 1.0 and ε ($\mathrm{cm^{-1}} \times \mathrm{M^{-1}}$) at 265.5 mμ = 10.0. The stock solutions, used for the determination of the extinction coefficients, were assayed by volumetric titration with potassium permanganate.[9] Though there is no peak in the absorption spectrum of H_2O_2,[8] reproducible measurements were obtained due to the exactness of setting the wavelength scale in this region (the same calibrated Beckman DU spectrophotometer was used throughout). Beer's law was found to be strictly obeyed at least up to 0.8 M at 301.5 mμ and 0.08 M at 265.5 mμ. The measurements were all made in the region of minimum relative error, *i.e.* at absorbancies between 0.2 and 0.7.[10]

Assay of peroxidase activity. Peroxidase activities were measured using H_2O_2 as the primary substrate and pyrogallol as chromogen hydrogen donor. The formation of strongly coloured reaction products (presumably mostly purpurogallin) was followed spectrophotometrically at 430 mμ which is the wavelength usually employed [11] for the registration of end products. The reagents used in the standard procedure were: (1) A 4.88 mM solution of pyrogallol in 50 mM citrate buffer,[12] pH 3.5. Pyrogallol is very stable at this pH; no autoxidation could be demonstrated spectrophotometrically when this solution was stored at 25°C for 6 h. (2) A 488 mM aqueous solution of H_2O_2 was

prepared immediately before use. Unless otherwise stated, the assay was carried out as follows: 2.5 ml pyrogallol solution and 0.5 ml H_2O_2 solution (previously warmed to the desired reaction temperature) were mixed in a standard cuvette (1 cm). The reaction was then started by the rapid addition ($t = 0$) of 0.05 ml of the cyt. c solution (14 μM) by means of a glass rod. The absorbancy at 430 mμ was measured by means of a Beckman DU spectrophotometer exactly 1 min after the reaction was started. The instrument was set at zero with a calibrated cuvette containing the same reagents as mentioned above, but distilled water instead of the peroxide solution. The assays were performed at least in duplicate. When the progress curves were determined, the reactions were followed continuously in a Beckman DK-2A recording spectrophotometer. Both spectrophotometers were supplied with equipment for temperature regulation. Except where otherwise stated, the temperature of the cell compartment was maintained at 25°C with water circulating from a constant temperature bath; the temperatures reported were measured directly in the cuvette. The reaction conditions for the standard procedure were chosen on the basis of the kinetic study presented in the results.

The kinetics of the over-all reaction were studied by varying the factors influencing the "initial" reaction rate; *i.e.* pH, the temperature and the concentration of reactants and enzyme.

Heat treatment of cytochrome c. Sealed glass tubes, containing 2 ml samples of the cyt. c solution, diluted in 40 mM citrate buffer of the desired pH, were heated over a water-glycerine bath for 10 min at temperatures ranging from 30 to 90°C; the temperatures were held constant within ± 0.5°C. After heating, the tubes were rapidly cooled in ice water and the peroxidase activity was assayed.

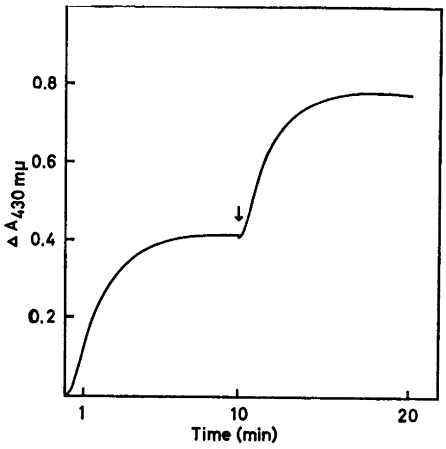

Fig. 1. Typical progress curve of the peroxidation of pyrogallol to purpurogallin catalyzed by ferricytochrome c. Standard assay procedure; H_2O_2, 80 mM; pyrogallol, 4 mM; cyt. c, 2.3×10^{-1} μM; 40 mM citrate buffer; 25°C. The change in absorbancy at 430 mμ was followed in a Beckman DK-2A recording spectrophotometer. At the time indicated by the arrow a quantity of cytochrome c, equal to that present initially, was added.

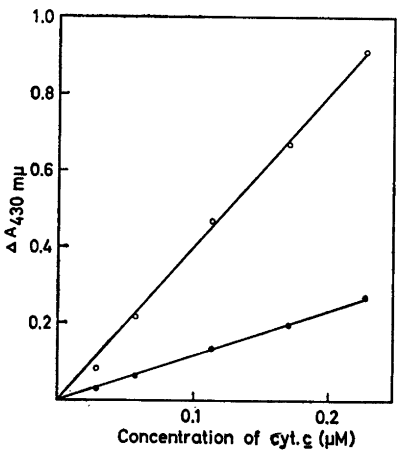

Fig. 2. Reaction rates as a function of the cytochrome c concentration. ●, standard assay procedure where the reaction product was measured after 1 min (*i.e.* "initial" rate). O, standard assay procedure except that the concentration of pyrogallol was 130 mM, and the reaction product was measured after 10 min.

RESULTS

Unless otherwise stated, the peroxidation of pyrogallol catalyzed by cyt. c was studied by using the ferric form of the purified hemoprotein.

a) The type reaction. The progress curve in the standard procedure (Fig. 1) displayed initially a short "lag" period which was followed by a period of an almost constant rate for approximately 1 min. Later, the rate fell off gradually, and the reaction proceeded slowly to its completion (>10 min). The cessation of colour development is presumably due to destruction of cyt. c *per se* or of its peroxidase activity, for the addition of an equal amount of the hemoprotein to the reaction mixture after the peak absorbancy had been reached allowed the reaction to proceed again for a while (Fig. 1); the increment was only slightly lower than after the first addition of cyt. c. The amount of product formed during the first minute of the reaction ($\Delta A_{430} \cdot min^{-1}$) will be used as a measure of the "initial" rate throughout the following study.

b) Effect of the concentration of cytochrome c. A straight-line relationship was obtained when the "initial" rate was plotted *vs.* the enzyme concentration (Fig. 2). This relationship was found even when measuring the reaction product after 10 min.

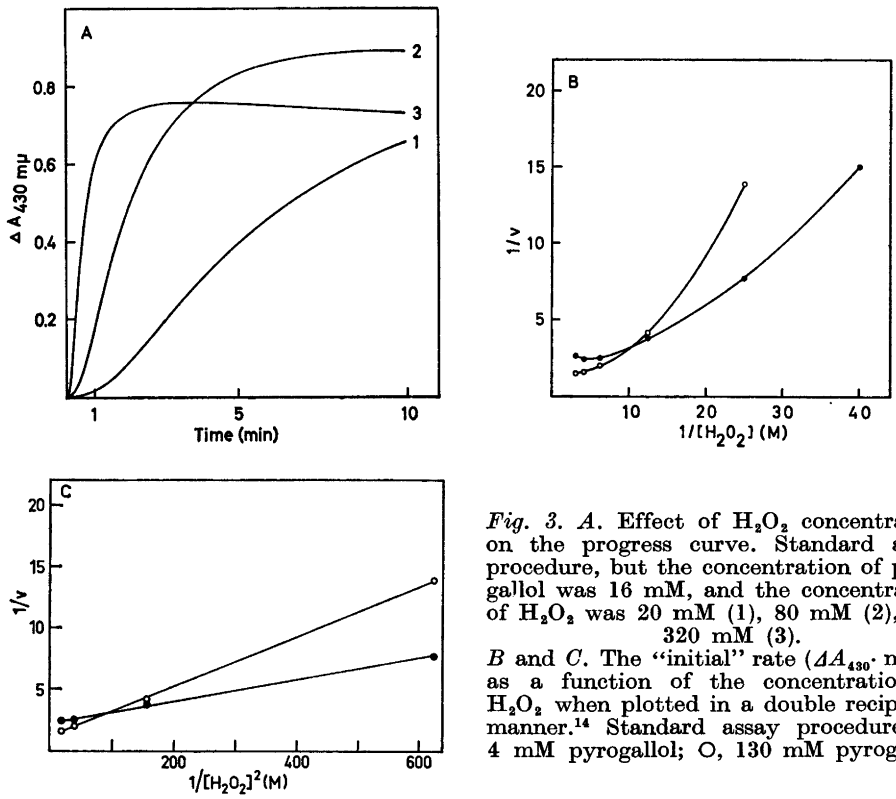

Fig. 3. A. Effect of H_2O_2 concentration on the progress curve. Standard assay procedure, but the concentration of pyrogallol was 16 mM, and the concentration of H_2O_2 was 20 mM (1), 80 mM (2), and 320 mM (3).
B and *C.* The "initial" rate ($\Delta A_{430} \cdot min^{-1}$) as a function of the concentration of H_2O_2 when plotted in a double reciprocal manner.[14] Standard assay procedure; ●, 4 mM pyrogallol; ○, 130 mM pyrogallol.

c) Effect of the concentration of reactants. At a low concentration of H_2O_2 (*e.g.* 20 mM) the progress curve (Fig. 3,A) showed a marked "lag" period, and the reaction proceeded slowly to its completion (>20 min). At a high concentration (*e.g.* 320 mM), however, there was no measurable "lag" period, and the reaction proceeded rapidly to its completion (within $3\frac{1}{2}$ min). The effect of the hydrogen donor concentration was less marked than the effect of the H_2O_2 concentration. Thus, when the latter reactant was kept constant at *e.g.* 80 mM, the reaction proceeded slowly to its completion irrespective of the donor concentration (Fig. 4,A).

The "initial" rate, when measured at a series of substrate concentrations, was found to increase up to a point, and then fall off gradually (Fig. 3,B); and the higher the donor concentration, the higher was the apparent optimum concentration of H_2O_2. Furthermore, it is noteworthy that the curves at the high and the low concentration of pyrogallol intersect, and that no linear relationship was obtained between the reciprocal value of the rate and the

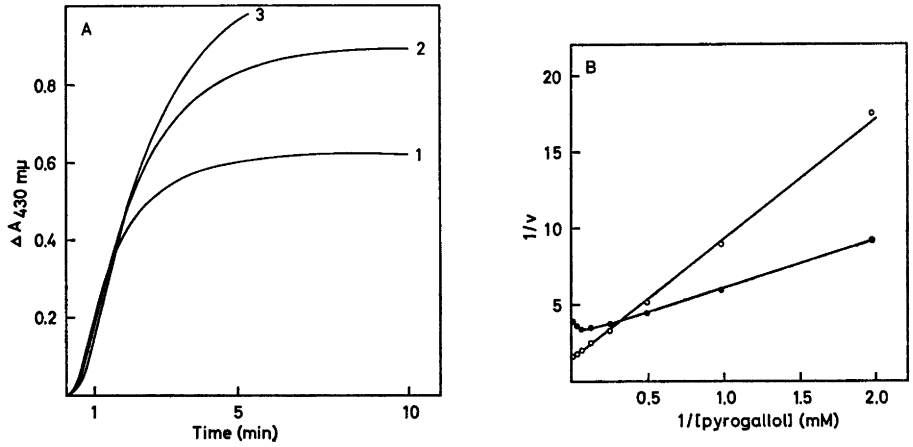

Fig. 4. A. Effect of pyrogallol concentration of the progress curve. Standard assay procedure; the concentrations of pyrogallol were 4 mM (1), 16 mM (2), and 130 mM (3). *B.* The "initial" rate ($\Delta A_{430} \cdot \text{min}^{-1}$) as a function of the concentration of pyrogallol when plotted in a double reciprocal manner.[14] Standard assay procedure; ●, 80 mM H_2O_2; O, 240 mM H_2O_2. At very high donor concentrations, an inhibition was obtained even at 240 mM H_2O_2.

concentration of H_2O_2. Straight lines were found, however, by plotting the rate *vs.* the square of the peroxide concentration (Fig. 3,C). The apparent optimum concentration of pyrogallol varied with the H_2O_2 concentration in a similar manner, except that a linear rate-donor relationship was obtained when the experimental data were plotted in a double reciprocal manner (Fig. 4,B).

d) Effect of pH. In Fig. 5 a plot of the "initial" rate *vs.* pH is shown; the range over which the influence of pH can be studied, is limited by the high

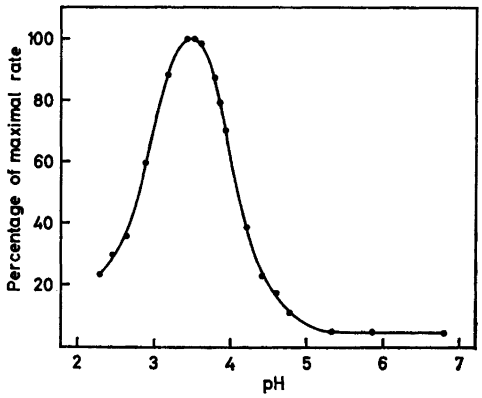

Fig. 5. Effect of pH on the "initial" rate (ΔA_{430}·min^{-1}). Standard assay procedure; the different pH values were obtained by citrate buffers (final concentration approximately 40 mM). Since no change in pH was observed during the reaction, the pH of the reaction mixtures was measured at 25°C immediately after each experiment. An apparent pH optimum of approximately 3.5 was obtained.

autoxidizability of pyrogallol in solutions of neutral and alkaline pH. An apparent pH optimum was obtained at approximately 3.5. Over the pH-range tested, the coloured reaction product exhibited the same absorption curve in the near ultraviolet region. No formation of colour could be measured when cyt. c was omitted from the reaction mixture. The pH of the reaction mixtures was checked before the addition of cyt. c and during the course of the reaction. However, no change in pH was observed.

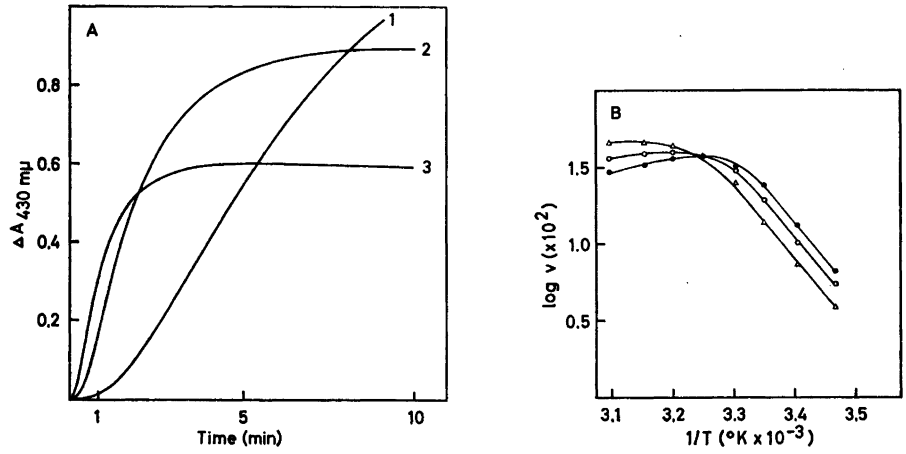

Fig. 6. Effect of temperature. *A.* Effect on the progress curve. Standard assay procedure, but the pyrogallol concentration was 16 mM, and the temperature was 10.7°C (1), 25.5°C, and 34.6°C (3).
B. Arrhenius plot of data on the "initial" rate as obtained in the standard assay procedure. The reaction product was measured after 1/2 min (△), 3/4 min (○), and 1 min (●), but in each case the rate was calculated as ΔA_{430}· min^{-1}. Note the gradual downward bend of the curves, corresponding to a decrease in activation energy, at the higher temperatures. The highest apparent value of the activation energy ($E' \approx 22\,200$ cal mole^{-1}) was calculated from the initial slopes (15.4—25.5°C).

The pH-activity curves in 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 effect of buffer anions will be studied in detail in a forthcoming paper.[13] Since the buffer capacity of citrate is maximal at about pH 3.6, *i.e.* near the pH "optimum", this buffer was preferred in the standard assay procedure.

e) Effect of temperature. A series of progress curves (Fig. 6,A) demonstrated that the higher the reaction temperature, the shorter was the initial "lag" period, and the more rapidly the reaction proceeded to its completion. After a certain period of time the progress curves cross due to a more rapid decrease of the reaction rate at higher temperatures, and the apparent optimum temperature will depend on the time at which the reaction product is measured (Fig. 6,B); it was found to be approximately 50, 40 and 35°C when the reaction product was measured after 1/2, 3/4, and 1 min, respectively.

From measurements of the temperature dependence of the "initial" rate in the range 15.4 to 25.5°C, a temperature coefficient (Q_{10}) of approximately 1.7 and an apparent activation energy (E') of 22 200 cal mole^{-1} was obtained whether the reaction product was assayed after 1/2, 3/4, or 1 min (Fig. 6,B). In each case, a pronounced deviation from the Arrhenius straight-line relationship was observed at higher temperatures.

To determine the effect of temperature *per se* on the stability of cyt. c, the hemoprotein was exposed to high temperatures for 10 min, before measuring the peroxidase activity. Fig. 7 shows that cyt. c was resistant to

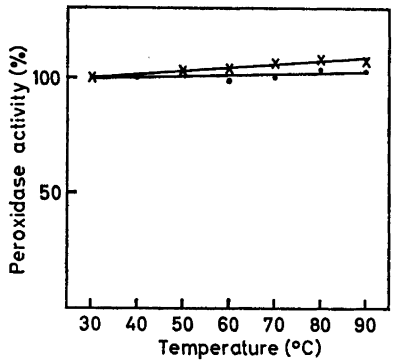

Fig. 7. Effect of heat treatment of ferricytochrome c on its peroxidase activity. The hemoprotein solution (14 μM in 40 mM citrate buffer) was heated for 10 min at different temperatures. Standard assay procedure; the rate at 30°C = 100 %. The straight lines, corresponding to the experimental values, were calculated by the least square method. For details see text. ●, pH 6.7; ×, pH 3.5.

heating at 90°C for 10 min at pH 3.5 and 6.7, and the decrease of the reaction rate observed during the peroxidase reaction can not be explained by instability of the enzyme itself towards heat. On the contrary, a slight increase in the peroxidase activity was observed following this treatment, most clearly seen at pH 3.5.

f) The sensitivity of the peroxidase reaction in the assay of cytochrome c. From data given in Fig. 2, it can be calculated that the peroxidase reaction is approximately 40 and 130 times more sensitive than the spectrophotometric determination of the absorbancy in the α-band (λ = 550 mμ) of ferro-cyt. c when measuring the reaction product after 1 and 10 min, respectively.

When the product was measured after 10 min, 1.25×10^{-2} μM cyt. c gave an increase in absorbancy at 430 mμ of 0.05, and this concentration may be used as the lower limit for exact assay of peroxidase activity in this test system. The sensitivity could be increased further by increasing the concentration of the substrates and/or of citrate anions (or by addition of acetate ions [13]).

g) Reproducibility. The reproducibility of the method was examined by assaying replicate samples, using the same reagent solution in all experiments. The standard procedure was used except that the concentration of pyrogallol was 130 mM when the reaction product was measured after 10 min. The results for the mean $\pm$ standard deviation and coefficient of variation, respectively were : $\Delta A_{430} \cdot \text{min}^{-1}$ ($n = 10$): 0.262 ± 0.008, 3.04 %; $\Delta A_{430} \cdot 10\ \text{min}^{-1}$ ($n = 10$): 0.918 ± 0.025, 2.75 %.

h) Comparison of the peroxidase effect of ferri- and ferrocytochrome c. For the purpose of comparison the peroxidase activity of ferro-cyt. c was investigated by the standard procedure. No significant difference was observed, however, in the "specific activity", *i.e.* $\Delta A_{430} \cdot \text{min}^{-1} \cdot \mu\text{M}^{-1}$, whether cyt. c was in the ferrous or in the ferric form. This result was expected inasmuch as ferro-cyt. c is rapidly oxidized to the ferric form under the experimental conditions used.[15]

DISCUSSION

Generally, peroxidase activity can be determined in many different ways, depending on the choice of hydrogen donor.[16] Guaiacol, extensively used in the peroxidase assays, was avoided because the product (tetraguaiacol) fades rapidly in acid solution which prevents accurate measurements of the rate,[17] and because this product, which is present in various amounts in nearly all stock solutions, increases the rate of the peroxidation of guaiacol.[18] Pyrogallol, one of the most commonly used donors, was introduced by Bach and Chodat [19] and extensively studied by Willstätter and Stoll.[20] As cyt. c did not display any absolute donor specificity, pyrogallol was preferred since this compound can be obtained with a high degree of purity. Furthermore, pyrogallol is very soluble in water, and non-autoxidizable at pH 3.5. Finally, in acid solution there is no oxidation of pyrogallol by H_2O_2 or by cyt. c *per se*, and the end products formed by the peroxidase reaction satisfy the criteria of a colorimetric analysis given, *e.g.*, by Vogel.[21] Unlike most chromogen hydrogen donors, the end products (presumably mostly purpurogallin) fade only very slowly, and the "true" progress curves of the reaction can be followed a wide period of time.

The oxidation of pyrogallol to purpurogallin by H_2O_2 and ferrous sulphate is accompanied by chemiluminiscence,[22] and the possibilities of utilizing this light emission for the assay of peroxidase activity has been studied by Ahnström *et al.*[23] The luminiscence intensity at the "steady state" was found to be dependent on the enzyme (HRPO) concentration, but the relationship was linear only within a very limited concentration range of hydrogen peroxide.[23] Nilsson [24] has recently demonstrated an intermediary, blue compound in the peroxidation of pyrogallol, preceding the luminiscence reaction, and proposed

that this compound gives a more reliable index of peroxidatic activity than does the conventional purpurogallin method. The formation of the strongly coloured oxidation products (presumably mostly purpurogallin) from pyrogallol, however, makes this assay inconvenient when using the usual spectrophotometric methods. Thus, neither of the proposed methods [23,24] seem to be useful for the present purpose, and a modification of the conventional method has therefore been used.

Unsubstituted hydrogen peroxide was preferred as the primary substrate since peroxidases generally show higher activity towards hydrogen peroxide than towards alkyl hydrogen peroxides.[25]

The progress curves showed a "lag" period, followed by a period with an almost constant rate and a final period where the rate gradually fell off. The higher the concentration of H_2O_2 and the reaction temperature, the shorter was the "lag" period, and the more rapidly the reaction proceeded to its completion. The cessation of colour development depends on a destruction of cyt. c *per se*, or of its peroxidase activity (Fig. 1), produced by the high concentration of H_2O_2 (Fig. 3,A); and the rate of destruction increases as the temperature rises (Fig. 6, A). Thus, the total concentration of enzyme is not constant during reaction, and hence the reaction becomes more complicated than is represented by the common simple reaction orders. This is in accordance with other peroxidase reactions where inactivation of the enzyme by H_2O_2 is well recognized *e.g.* for HRPO,[26-29] MPO,[30] LPO,[31] and thyroid peroxidase.[32]

A gradual downward bend of an Arrhenius plot, as shown in Fig. 6,B, usually means that a lower activation energy is obtained at the higher temperatures.[33] In the present peroxidase reaction, however, this effect is probably in the main part due to an increased rate of destruction of cyt. c by H_2O_2 since the deviation from linearity is greater when the reaction product is measured after 1 min than after $\frac{1}{2}$ min (Fig. 6,B). Furthermore, it may also indicate that the over-all activity involves more consecutive reactions with different activation energies.[33] This fits in well with recent studies on the reaction mechanism of pyrogallol oxidation. Thus, Nilsson [24] found the chemiluminiscence reaction, resulting from the elimination of an intermediary, blue compound, to have a much lower energy of activation than the enzymic formation of this compound. Finally, the slight displacement in the apparent pH optimum to more alkaline values with an increase of the reaction temperature,[13] may also attribute to this deviation from the Arrhenius straight-line relationship.

The effect of the initial concentrations of H_2O_2 and pyrogallol on the "initial" reaction rate is of primary interest. At a given concentration of one reactant the other demonstrates an apparent optimum concentration, which increases continuously with the increase in concentration of the other reactant, but without reaching any definite optimum concentration. Consequently there is little sense in calculating the Michaelis constant (K_m). One main difference is found, however, between the two reactants. When the initial concentration of H_2O_2 is kept constant and that of pyrogallol varied, the rate is proportional to the initial concentration of the latter (below the apparent optimum concentration) in a double reciprocal plot. On the other hand,

when the initial concentration of pyrogallol is kept constant and that of H_2O_2 varied, the rate appears to be proportional to the square of the latter. This fits in well with a reaction mechanism which involves an inhibition of the enzymic reaction by removal of substrate (H_2O_2), since such a mechanism leads to a rate equation in which the rate is, in general, a quadratic function of concentration terms.[34] This explanation seems reasonable in view of the recent studies by Nilsson [24] on the peroxidation of pyrogallol catalyzed by HRPO. He found two consecutive peroxide-dependent reactions: (1) the enzymic formation of a blue, intermediary compound, and (2) its non-enzymic elimination accompanied by chemiluminiscence. In this connection it may be noteworthy to mention that the same rate-substrate relationship was also found in the peroxidation of benzidine catalyzed by cyt. c.[35]

When pH was varied over a wide range in the acid region, at a constant concentration of reactants as well as electrolyte components of the buffer solution and reaction temperature, the peroxidase activity exhibited a definite pH optimum (about 3.5 in 40 mM citrate buffer at 25°C). This optimum is consistent with earlier observations made by Nakamura *et al.*;[36] in their comparative studies on the peroxidase activity of hemoproteins they observed that cyt. c exhibited a pH "optimum" at 3.4 for k_1, *i.e.* the rate constant for the reaction of cyt. c with H_2O_2 (in phosphate-HCl buffer and with leucomalachite green as hydrogen donor). Finally, the reaction rate is a linear function of the concentration of cyt. c, and this hemoprotein thus satisfies the criteria of a "true" peroxidase according to the nomenclature of Jayle.[37]

In principle, the over-all kinetics of the peroxidase reaction catalyzed by cyt. c agree with the results obtained with HRPO.[27,28,38] The former reaction differs, however, from the latter in that it requires a relatively high concentration of H_2O_2 to obtain a measurable reaction rate. Furthermore, the temperature coefficient of the peroxidase reaction catalyzed by cyt. c ($Q_{10} \approx 1.7$ at 15—25°C) is smaller than found for HRPO ($Q_{10} \approx 2.0$ at 15—25°C), [28,38] and the apparent activation energy ($E' \approx 22\,200$ cal mole^{-1} at 15—25°C) is higher than found for HRPO ($E' \approx 12\,700$ cal mole^{-1} at 15—25°C).[38]

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