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THE KINETICS OF LACTOPEROXIDASE REACTIONS

by

© ROBERT JAMES MAGUIRE

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

"THE KINETICS OF LACTOPEROXIDASE REACTIONS"

submitted by Robert James Maguire in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The optical rotatory dispersion of lactoperoxidase and its cyanide and fluoride complexes was studied over the spectral region 210-500 nm, and that of the azide complex from 350-450 nm. Results of the measurements of the reduced mean residue rotation at 233 nm lead to the conclusion that there are no significant changes in protein conformation upon the binding of these ligands to lactoperoxidase. Lactoperoxidase was estimated to have 17% α-helical character at pH 7.0. Results of studies in the Soret region indicate that lactoperoxidase, unlike horseradish peroxidase, hemoglobin, and myoglobin, exhibits a negative Cotton effect as do its cyanide, azide, and fluoride complexes. The binding of these ligands apparently causes an alteration of the geometry of the heme group with respect to the protein moiety.

The kinetics of the formation of the primary lactoperoxidase-hydrogen peroxide compound (compound I) at 25° have been studied by steady state methods. The second order rate constant k_1 is pH-independent over the pH region investigated, having a value of $(9.2 \pm 0.9) \times 10^6$ $M^{-1}sec^{-1}$. An anomalous effect of formate buffer on the kinetics of the formation of compound I is demonstrated.

The kinetics of the oxidation of iodide ion by lactoperoxidase compound II have been studied as a function of pH at 25° and an ionic strength of 0.05. Both a first order and a second order dependence on the concentration of iodide

ion were detected. The second order rate constant for the reaction of lactoperoxidase compound II with iodide ion decreases from 4.2×10^{6} to 2.6×10^{-1} $M^{-1}sec^{-1}$ and the third order rate constant decreases from 2.7×10^{10} to 1.4×10^{3} $M^{-2}sec^{-1}$ with increasing pH over the pH range 2.9 - 10.1. The pH dependence of the reaction is explained in terms of an acid dissociation outside the pH range of this study.

The kinetics of the oxidation of p-cresol by compound II of lactoperoxidase have been studied over the pH range 2.1 - 11.2 by the stopped-flow technique. The reaction is kinetically first order in p-cresol over the entire pH range. Use is made of the diffusion controlled limit to show that p-cresol reacts in the unionized form over the pH region of the study. The complexity of the pH-rate profile is discussed in terms of acid dissociation constants of groups in the enzyme, and the ionization of the substrate.

The rate of exchange of water with the iron atom of the heme group of horseradish peroxidase was studied at several pH values using a line broadening technique with O^{17} nuclear magnetic resonance. The results, while complicated by experimental error, indicate that the true values for the rate of exchange are not obtainable in the experimental temperature region 5° - 50° by the nuclear magnetic resonance line broadening technique.

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I would like to thank my research director, Dr. H. Brian Dunford, for the encouragement and advice he has given me throughout the course of my research. I would also like to thank my fellow graduate students and the post doctorate fellows for many helpful discussions.

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Chapter 1

Introduction

The hemoproteins are a distinct class of enzymes and proteins which include the hemoglobins and myoglobins concerned in oxygen transport and storage, the cytochromes, which are electron transfer agents, and several enzymes, the peroxidases and catalase. While the physiological role of the oxygen transport, storage, and utilization pigments are fairly well defined, the importance of peroxidases and catalase is not clear. The peroxidases can be classified as enzymes that catalyze the oxidation of certain substrates by such peroxides as hydrogen peroxide, methyl hydrogen peroxide, and ethyl hydrogen peroxide. Peroxidases are widespread in plant materials and have been implicated in the control of plant growth (Galston, 1956); they are also found in certain animal tissues. Catalase is generally present in aerobic cells, frequently in sizeable amounts. The interest in these enzymes is largely centered about their mechanisms of action, but many reasons for the presence of these vigorous catalysts remain unsolved.

Catalase is an enzyme, the primary function of which appears to be the destruction of hydrogen peroxide according to the equation

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalases from different sources are alike, while peroxidases are generally different from each other and from catalase. Catalase from all sources has a molecular weight of

250,000; the molecule is composed of four subunits, each of which has as prosthetic group the heme group known as ferriprotoporphyrin IX, shown in Fig. 1-1a. Under certain conditions catalase can act as a peroxidase by using hydrogen peroxide to oxidize certain compounds; however, the more generally observed reaction is the destruction of hydrogen peroxide in the so-called "catalatic" reaction. Peroxidases possess some catalatic ability in the absence of reducing agents, but they are only 10^{-4} as effective as catalase.

Peroxidases are widely distributed among plants; the richest sources are the sap of the fig tree and the root of the horseradish. Saunders et al. (1964) give an extensive list of sources of peroxidase activity in both plants and animals. Some of the peroxidases which have had a considerable amount of work performed on them in recent years are horseradish peroxidase, lactoperoxidase, thyroid peroxidase, cytochrome c peroxidase, and chloroperoxidase. In addition, there is another peroxidase which occurs in leucocytes called myeloperoxidase (verdoperoxidase); however, relatively little work has been done on this enzyme.

Horseradish peroxidase (EC 1.11.1.7; donor H_2O_2 oxidoreductase) is the peroxidase which has been most extensively investigated. This enzyme, which is brown, has a molecular weight of about 40,000 (Keilin and Hartree, 1951) and contains about 18% carbohydrate material by weight (Shannon et al., 1966). It contains as prosthetic group ferriproto-

porphyrin IX (Fig. 1-1a). Horseradish peroxidase was first observed to form spectroscopically distinct compounds with hydrogen peroxide over 30 years ago (Keilin and Mann, 1937). Much work has been done concerning the nature of these horseradish peroxidase-hydrogen peroxide compounds by studying their fast reaction kinetics, electron spin resonance spectroscopy, and magnetic susceptibilities, and the conclusions will be discussed in some detail later in connection with the enzyme lactoperoxidase. Saunders et al. (1964), and, more recently, Hasinoff (1970) provide good reviews of horseradish peroxidase.

Cytochrome c peroxidase (EC 1.11.1.5; cytochrome c : H_2O_2 oxidoreductase), which catalyzes the oxidation of ferrocytochrome c to ferricytochrome c in the presence of hydrogen peroxide,

$$H_2O_2 + 2 \text{ ferrocytochrome c} \longrightarrow 2 \text{ ferricytochrome c} + 2\,OH^-$$

was discovered in baker's yeast by Altschul et al. (1940). The enzyme is found exclusively in aerobically grown yeasts. It has a number of convenient properties that are difficult to find in other heme-containing enzymes. Large quantities of cytochrome c peroxidase can be prepared from commercially available yeasts by simple chromatographic techniques (Yonetani and Ray, 1965; Ellfolk, 1967; Yonetani, 1968). It is readily crystallized from dilute salt solutions (Yonetani et al., 1966; Ellfolk, 1967; Yonetani, 1968). The crystalline preparation of this enzyme is free of isozymes and has a consistent purity. The molecular weight was

determined to be 34,100 (Ellfolk, 1967a) and the prosthetic group to be ferriprotoporphyrin IX, shown in Fig. 1-1a (Altschul et al., 1940; Abrams et al., 1942; Yonetani and Ray, 1965). Unlike other peroxidases, it has been found to contain no carbohydrate material (Ellfolk, 1967). As reported originally by Altschul et al. (1940) and Abrams et al. (1942), the brown coloured cytochrome c peroxidase reacts with a stoichiometric amount of hydroperoxide to form a "red peroxide compound", which has two more oxidizing equivalents than the native enzyme. This red peroxide compound is highly stable in the absence of reducing agents, in contrast to the peroxide compounds of other peroxidases. The half-life of its decomposition is of the order of several hours at room temperature (Yonetani et al., 1966). This stability has lent itself to electron spin resonance studies of the peroxide compound and comparisons with the native enzyme (Yonetani et al., 1966a). In addition, Yonetani (1970) reports that X-ray studies of cytochrome c peroxidase are planned; this should give a clear picture of the heme environment of this enzyme, and will be indispensable for the understanding of its mode of action.

In the thyroid gland iodide ion is oxidized and iodinates tyrosine residues of the protein thyroglobulin. Two iodinated tyrosine residues presumably couple to form the hormone thyroxine which is secreted into the circulatory system. It is then carried to other tissues upon which the hormone exerts its influence. It has long been thought

that a peroxidase in the thyroid gland was involved in the oxidation of iodide ion. Klebanoff et al. (1962) showed that beef thyroid preparations were capable of the iodination of tyrosine. Yip (1966) studied the properties of a peroxidase purified from beef thyroid tissues. The purification was largely effected by the use of ion-exchange chromatogrphy. The molecular weight was determined to be 50,000. The prosthetic group was identified as ferriprotoporphyrin IX. Hosoya and Morrison (1967a) have described the isolation and purification of hog thyroid peroxidase. Gel filtration experiments have shown it to have a molecular weight of 104,000. The difference between this figure and that reported for beef thyroid peroxidase may be due to species difference or aggregation. Hosoya and Morrison (1967a) also found that hog thyroid peroxidase reacts with hydrogen peroxide about as quickly as horseradish peroxidase and lactoperoxidase ($k = 1.1 \times 10^7\ M^{-1}sec^{-1}$ at pH 7.4) and that this enzyme can iodinate tyrosine and thyroglobulin. Taurog et al. (1970) have described an improved procedure for the solubilization and purification of hog thyroid peroxidase; they obtained 29 milligrams of purified enzyme from 23 kg of frozen hog thyroid glands. Their spectral data suggest that the heme in thyroid peroxidase may not be ferriprotoporphyrin IX. Taurog (1970) has shown that the thyroid peroxidase-catalyzed iodination of thyroglobulin is inhibited by excess iodide. These findings offer a possible explanation for inhibitory effects of large doses

of iodide ion on thyroid hormone formation in humans (iodide-induced myxedema). Most of the problems involved in work with thyroid peroxidase may be the result of poor yields of impure preparations, and much work remains to be done on this important enzyme.

Morris and Hager (1966) have described the isolation and properties of chloroperoxidase. This enzyme is present in the mold Caldariomyces fumago, which synthesizes the chlorine-containing fungal metabolite called caldariomycin. They determined that the prosthetic group of chloroperoxidase is ferriprotoporphyrin IX, and that the molecular weight is 42,000. They also determined the amino acid composition of the enzyme and noted that 25-30% of the molecule is composed of carbohydrate material. Chloroperoxidase, unlike horseradish peroxidase, can oxidize chloride and bromide ions; Hager et al. (1966) have followed the chloroperoxidase-catalyzed halogenation of tyrosine and have shown that the relative activity among the halogen anions for the halogenation of tyrosine is approximately 5.1 : 4.8 : 1 for iodide, bromide, and chloride ions, respectively. Thomas and Hager (1968) presented evidence that chloroperoxidase can catalyze the oxidation of iodide ion beyond the molecular halogen level to iodate, i.e., to an oxidation state of +5. More recently, Thomas et al. (1970) have reported on the formation of peroxide and halide complexes of chloroperoxidase, and their relation to the mechanism of the halogenation. In addition, Thomas et al.

(1970a) have shown that chloroperoxidase can release oxygen from peroxy-acids, a finding which has some bearing on the nature of peroxidase-peroxide compounds. This point will be discussed later in connection with lactoperoxidase.

The subject of most of this thesis is lactoperoxidase, isolated from bovine milk, and which has also been detected in bovine salivary glands (Morrison et al., 1965) and bovine harderian and lacrimal glands (Morrison and Allen, 1966).

Historical Introduction of Lactoperoxidase

While searching for a method by which fresh milk could be distinguished from cooked milk, Arnold (1881) discovered that fresh bovine milk contained a substance which rapidly oxidized guaiacol when hydrogen peroxide was added. Previous studies had shown that oxidation of dyes could be carried out by various plant materials if hydrogen peroxide were added to the reaction mixture (Schonbein, 1863). Linnosier (1881) demonstrated the presence of a substance with similar activity in leucocytes and gave the name "peroxidase" to all of those materials which catalyzed the oxidation of dyes in the presence of hydrogen peroxide.

For some time the peroxidase of milk was assumed to be identical to the leucocyte peroxidase; its occurence in milk was believed to result from the disintegration of leucocytes. However, Theorell and Akeson (1943) demonstrated that the two peroxidases had different properties.

These authors suggested that the two green coloured animal peroxidases be classified as "verdoperoxidase" (or "myeloperoxidase") for the leucocyte peroxidase, and that the milk enzyme be named "lactoperoxidase".

Isolation and Purification

The first procedure for the isolation of lactoperoxidase was developed by Thurlow (1925). The peroxidase activity was obtained in a crude fraction by the use of ammonium sulfate fractionation. Elliot (1932) elaborated on this procedure and obtained the activity in a brown coloured fraction. Yakushiji (1939) further purified the peroxidase by subjection of the fraction which had been obtained by ammonium sulfate precipitation to precipitation by acetone.

Lactoperoxidase was finally obtained in pure form and crystallized by Theorell and co-workers (1943, 1944). Starting with skim milk these workers obtained a crude fraction of lactoperoxidase by the ammonium sulfate fractionation procedure of Elliot (1932). Inert proteins were removed from this crude fraction first by heat denaturation at 70°, followed by precipitation with basic lead acetate, and then by acetone precipitation. Repeated electrophoresis at pH 5.9 was required to remove a contaminating red protein. The lactoperoxidase, thus purified, was crystallized at its isoelectric point from ammonium sulfate solution. The crystals were in the form of thin leaves.

The isolation and purification procedures of early workers were never fully detailed, however, and the reported yields were low, although they did obtain crystals of the enzyme. Polis and Shmukler (1953) developed a more reproducible procedure in which the final purification was achieved by chromatography on calcium phosphate columns. Morrison et al. (1957) developed a method employing ion exchange resins for the isolation of the enzyme. Morrison and Hultquist (1963) later simplified and improved their previous procedure, so that yields of a more highly purified preparation were obtained. This improved procedure is described in Appendix 1, along with criteria used for the purity and activity of samples of lactoperoxidase. Although several criteria of purity are used, the ratio of the absorbance at 412 nm to that at 280 nm (called P.N., the "purity number") has been the most common criterion for comparing the purity of various preparations of lactoperoxidase. A P.N. value of 0.9 denotes a quite pure preparation of lactoperoxidase.

Rombauts et al. (1967) have determined the composition of lactoperoxidase. The molecular weight is about 77,500 and there is one iron atom per enzyme molecule. A quantitative determination showed that all the common amino acids were present. Carbohydrate material constituted 8% by weight of the enzyme molecule. Table 1-1 shows the amino acid and carbohydrate composition of lactoperoxidase.

Table 1-1

Amino Acid Composition of Lactoperoxidase[a,1]

	3 N HCl, 100°		6 N HCl, 110°			Av Values[b] (mol wt 77,500)
	4.5 hr	8 hr	24 hr	48 hr	106 hr	
Lys			32.1	33.2	32.9	33
His			13.8	14.1	14.0	14
Arg			38.5	38.9	39.0	39
$CySO_3H$						16
Asp			70.5	70.6	71.3	71
$MetSO_2$						12
Thr			27.3	27.0	24.2	28[c]
Ser			28.4	26.2	21.7	30[c]
Glu			59.4	60.3	60.9	60
Pro			40.1	42.3	42.6	42
Gly			40.1	41.4	41.0	41
Ala			38.9	40.7	39.6	40
Val			27.0	28.7	29.4	29[d]
Ile			24.5	27.1	28.0	28[d]
Leu			66.3	68.1	68.4	68[d]
Tyr			14.6	15.0	15.1	15
Phe			30.7	30.8	31.5	31
Trp				15.8[e]	15.8[e]	16[e]
Glucosamine	15.9	16.1	9.9	7.1	3.9	16[f]

continued

Table 1-1 continued

	3 N HCl, 100°		6 N HCl, 110°			Av Values[b] (mol wt 77,500)
	4.5 hr	8 hr	24 hr	48 hr	106 hr	
Galactosamine	9.7	9.5	5.5	3.6	1.0	10[f]
Neutral carbohydrates (%)						1.5
Acetylneuraminic acid						Negative
Acetyl groups						10 ± 2[g]

[a]Data are expressed as residues per mole of protein.

[b]Average values of the determinations unless noted otherwise.

[c]Corrected to zero time.

[d]The maximum value determined.

[e]Alkaline hydrolysis for 50 and 70 hr.

[f]Based on the data from hydrolysis in 3 N HCl.

[g]Average of two determinations.

[l]From Rombauts *et al.* (1967).

Carlstrom (1965,1966,1969) has claimed that lactoperoxidase exists as a number of closely related isozymes; however, Rombauts et al. (1967) have argued that Carlstrom's findings are the result of enzyme degredation during his extensive purification and separation procedures.

The nature of the prosthetic group (heme group) of lactoperoxidase has not been established. Hultquist and Morrison (1963) have indicated that the heme prosthetic group is probably a derivative of mesoheme 9, in which two double bonds are in conjunction with the tetrapyrrol nucleus and one or more hydroxyl groups are attached to the side chains. The linkage between hemin and protein was identified as an ester bond. Figure 1-1a shows the heme prosthetic group of horseradish peroxidase, hemoglobin, and myoglobin; ferriprotoporphyrin IX, which is closely related to mesoheme 9 (Falk, 1964). Figure 1-1b shows the structure of the heme prosthetic group of lactoperoxidase suggested by Hultquist (1962). The difference between the two heme groups is manifested by different absorbance characteristics in the native enzymes and the complexes they form.

Reaction of Lactoperoxidase with Oxidizing Agents

Spectrophotometric studies have demonstrated the existence of enzyme-substrate compounds between lactoperoxidase and peroxides, similar to the complexes which had been previously observed for horseradish peroxidase (Keilin and

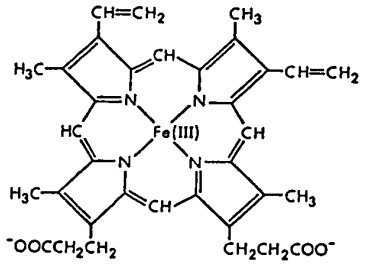

Fig. 1-1a. Ferriprotoporphyrin IX.

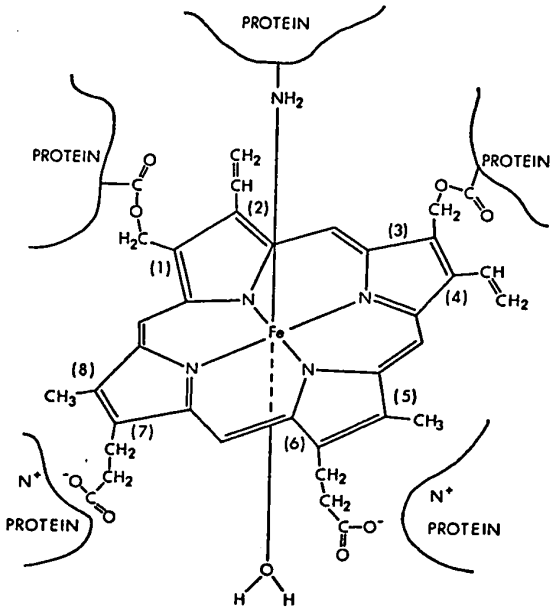

Fig. 1-1b. A proposed structure for the prosthetic group of lactoperoxidase. (Hultquist, 1962).

Mann, 1937; Theorell, 1942). The designations compound I and compound II refer to those spectroscopically distinct species that form, in order of appearance, after the addition of hydrogen peroxide to peroxidases. Using a rapid flow spectrophotometer, Chance (1949, 1950) was able to demonstrate the presence of the labile compound I of lactoperoxidase. He also demonstrated that compounds I and II of lactoperoxidase were formed with methyl hydrogen peroxide and ethyl hydrogen peroxide. The spectra of these compounds could not be distinguished from those of the hydrogen peroxide compounds. Figure 1-2 shows the spectra in the 400 nm region (the Soret region) of the primary (I) and the secondary (II) lactoperoxidase-hydrogen peroxide compounds, and the Soret band of lactoperoxidase (A). Chance (1950) also titrated lactoperoxidase with hydrogen peroxide, and this experiment revealed that one mole of peroxide had combined with one mole of enzyme.

Kinetic studies with the rapid flow spectrophotometer demonstrated that compounds I and II were involved in enzyme action (Chance, 1949,1950). From the results of these studies Chance concluded that the reaction mechanism for lactoperoxidase was identical to that which had been postulated for horseradish peroxidase (Chance, 1949a), and which may be represented by the following scheme:

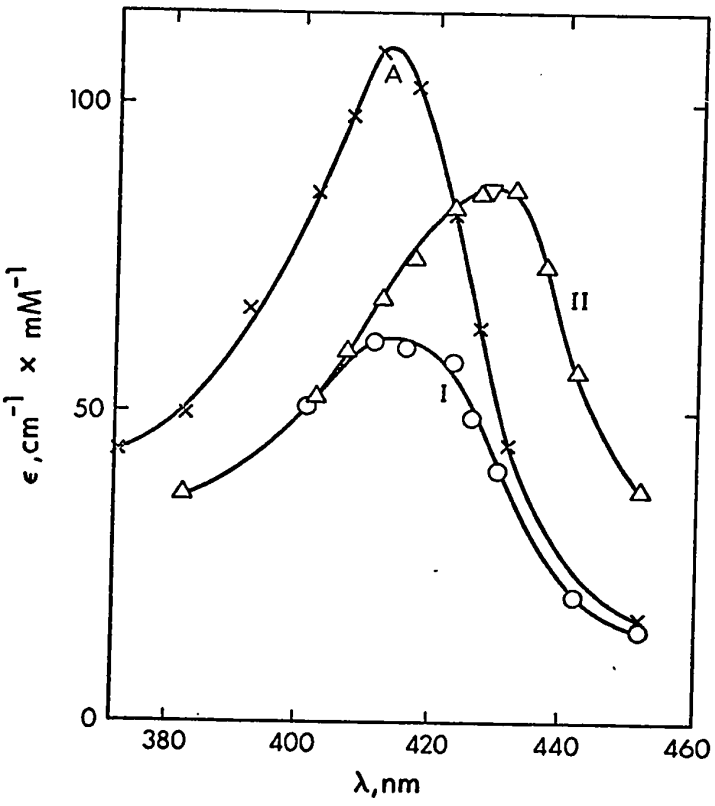

Fig. 1-2. The Soret bands of Lactoperoxidase (A), compound I, and compound II.[a]

[a]from Chance (1950).

$$LP + H_2O_2 \xrightarrow{k_1} LP\text{-}I \quad (1)$$

$$LP\text{-}I + AH_2 \xrightarrow{k_2} LP\text{-}II + AH\cdot \quad (2)$$

$$LP\text{-}II + AH_2 \xrightarrow{k_3} LP + AH\cdot \quad (3)$$

$$2AH\cdot \longrightarrow A + AH_2 \text{ (or AH - AH)} \quad (4)$$

where LP represents the native enzyme, LP-I and LP-II are the oxidized forms of the enzyme referred to as compounds I and II, respectively, and AH_2 is an oxidizable substrate, with A the product of oxidation. Various kinds of substrates have been used in lactoperoxidase-catalyzed oxidations, among them, phenols, amines, and inorganic ions such as iodide ion and ferrocyanide ion.

The reaction of lactoperoxidase with hydrogen peroxide to give compound I was shown to be very rapid. The somewhat slower rate of reaction with methyl hydrogen peroxide and the still slower rate of reaction with ethyl hydrogen peroxide suggested that there was a correlation between the size of the substrate molecule and the rate of reaction. Thus it appeared that the iron of lactoperoxidase was not completely accessible to substrate.

The nature of the intermediates involved in oxidations by horseradish peroxidase and lactoperoxidase is still a subject of some uncertainty. Virtually no work has been performed with lactoperoxidase in this regard, but since there is believed to be a general mechanism for the action of peroxidases, it may be considered valid to extend the conclusions obtained from horseradish peroxidase to lacto-

peroxidase.

Compound I of horseradish peroxidase (HRP-I) retains the two oxidizing equivalents of the hydroperoxide used in its formation, and is in an oxidation state of V (Chance, 1952). Compound II of horseradish peroxidase (HRP-II) can be formed from compound I and is in an oxidation state of IV (George, 1953). A number of structures have been proposed for HRP-I and HRP-II. For HRP-II these include the Fe(IV) and the Fe(IV)O (the ferryl ion) structures proposed by George (1953a). Brill and Williams (1961) agreed that the ferryl structure describes HRP-II, but proposed for HRP-I the existence of two components (1) a simple complex with hydroperoxide in the sixth coordination position and (2) an oxidized porphyrin ring compound. Brill and Sandberg (1968) later put forward proposals for the structure of HRP-I including (1) iron in a quadrivalent state plus a free radical or (2) iron in the ferric state plus a biradical on the porphyrin or protein. Moss et al. (1969) stated that the results of their Mössbauer study on HRP-I and HRP-II were compatible with a Fe(IV) type structure for HRP-II, but they also concluded that there was no difference in the configuration of the iron between HRP-I and HRP-II, indicating that the second equivalent on HRP-I is not stored on the iron. Peisach et al. (1968) and Blumberg et al. (1968) proposed structures of HRP-I and HRP-II consistent with low temperature optical spectra, electron paramagnetic resonance spectra, and previously

reported magnetic susceptibility data. For HRP-II they postulate that two oxygen atoms reside in the sixth ligand position of the iron atom:

$$[\text{HRP}(\text{heme } d_0^6)^-\text{OOH}]^{IV}$$

For the structure of HRP-I it was suggested that the Z axial ligands are not present or that they provide only a weak component to the ligand field consistent with:

$$[\text{HRP}(\text{heme}_{3/2}^5)]^{III},[X]^{II}$$

The two oxidizing equivalents are stored not on a ligand but either on the protein or the porphyrin represented as group X.

Dolphin et al. (1971) contend that the optical spectrum of HRP-I characterizes it as a porphyrin π-cation radical because of its similarity to the optical spectrum of a cobalt porphyrin π-cation radical. Thus one of the two oxidizing equivalents of HRP-I is accounted for by the loss of an electron from the porphyrin π-orbitals, and the second oxidation may then occur from the porphyrin ring, the protein, or the metal. Thomas et al. (1970a) have shown that a unique reaction catalyzed by a related enzyme, chloroperoxidase, is the evolution of oxygen from substituted peroxides such as ethyl hydrogen peroxide and peroxy acids. Although horseradish peroxidase reacts with the latter two compounds to form compound I, it can not complete a cycle of oxygen formation and regeneration of the native enzyme

(Chance, 1948,1949b). Provided that this oxygen evolution reaction proceeds through a compound I intermediate, definite conclusions regarding the chemical nature of compound I might be drawn once the origin of the oxygen atoms are known. Along this line of approach, Hager et al. (1971) have shown that the oxygen atoms in molecular oxygen evolved from peroxy acids arise from different substrate molecules. This leads to the conclusion that compound I must retain at least one oxygen atom after reaction with the first peroxy acid molecule. In a subsequent step, compound I reacts with a second peroxy acid molecule to form molecular oxygen.

Further work is necessary to define the chemical nature of peroxidase-peroxide compounds; however, it should be emphasized that any conclusions regarding the nature of HRP-I and HRP-II and compound I of chloroperoxidase may not necessarily apply to the structures of LP-I and LP-II.

The characteristic property and function of enzymes is the catalysis of chemical reactions. Any fundamental study of this catalytic function must be based on quantitative measurements of the rate of the catalyzed reaction. From the effect of varying the reaction conditions on the rate, inferences may be made about the mechanism of enzyme action. Ideally such kinetic studies should be brought into relation with chemical and structural studies on the enzyme in order to obtain a definite picture of the process, but this is only possible if the enzyme has been obtained

in a high degree of purity. Many enzymes have not been so purified and kinetic studies are the only approach possible at present. It is well known that biological systems, particularly living cells, are more sensitive to changes of temperature and pH than most non-biological chemical reactions, and this is due largely to the properties of the enzymes on which these systems depend. A knowledge of enzyme kinetics thus helps in the understanding of biological phenomena. A particularly important tool in the investigation of the kinetics of enzyme catalyzed reactions is the variation of the pH of the reaction medium, and it is this approach that is used in the kinetic investigations described in this thesis.

Chapter 2

The Effect of Ligand Binding on the Optical Rotatory Dispersion of Lactoperoxidase

Introduction

A beam of plane polarized light may be considered to be composed of two circularly polarized rays, one whose vector rotates clockwise and one counterclockwise as the beam advances. A medium containing asymmetric molecules transmits the two components with unequal velocity, that is, it has different refractive indices for the two circularly polarized rays. If neither ray is absorbed, the two rays have a resultant which is a plane-polarized ray whose vibration is in a plane which has been rotated with respect to that of the incident ray. The plane of polarization moves through a steadily increasing angle as the beam passes through the medium. The amount of rotation is measured as the angle between the incident and emergent beams, and is normally expressed as specific rotation, $[\alpha]_{\lambda}^{t}$, which is given by

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{cl}$$

where λ and t specify the wavelength at which measurements are made, and the temperature, respectively, α is the observed rotation in degrees, l is the path length of the sample in decimeters, and c is the number of grams of solute in one ml of solution. Since the indices of refraction are dependent on wavelength, different wave-

lengths of light are rotated by different amounts, giving rise to the phenomenon known as optical rotatory dispersion.[1]

If the medium also absorbs the two circularly polarized components unequally, the emergent light beam is elliptically polarized and the medium exhibits circular dichroism. The combination of unequal absorption (circular dichroism) and unequal velocity of transmission (optical rotation) of left and right circularly polarized light in the region in which optically active absorption bands are observed is a phenomenon called the Cotton effect. The C.R.D. curve of a Cotton effect is a symmetrical curve shaped like a derivative of an absorption peak and is centered at a wavelength corresponding to the maximum of the absorption peak. The maximum of a Cotton effect curve is known as the peak, and the minimum as the trough. When the peak occurs at a wavelength longer than that of the trough, the Cotton effect is termed "positive"; in the reverse

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[1]Abbreviations used in this chapter: O.R.D., optical rotatory dispersion: LP, LP-CN, LP-N_3, and LP-F, ferric lactoperoxidase and its cyanide, azide, and fluoride complexes, respectively; PGA, poly-α-L-glutamic acid; P.N., (purity number), ratio of the absorbance of a solution of LP at 412 nm to the absorbance at 280 nm.

situation, the Cotton effect is "negative".

Cotton effects have been classified into two groups. Using the terminology suggested by Blout (1964), "intrinsic Cotton effects" reflect the optical rotatory power of the protein itself, and "extrinsic Cotton effects" are generated by a chromophoric site on the protein or by a chromophoric molecule interacting with an asymmetric site on the molecule.

Optical rotation, generated by a metal-protein complex, when manifested as a specific Cotton effect, may reflect simultaneously the chemical composition of the metal-protein ligand site, its configuration, and also the spatial disposition of the chromophoric site with respect to neighboring groups. Hence, studies of optical rotatory dispersion of metal-protein systems disclose features in addition to those discernible from absorption spectra. The heme proteins serve to illustrate this point.

The conformation-dependent Cotton effects have provided a means for characterizing secondary structures. The depth of the 233 nm trough has been suggested as a measure of the helical content in proteins (Simmons et al., 1961). However, its quantitative significance is still not certain because of the lack of agreement in reference scales.

The 233 nm trough method has been used to study the effect of ligand binding on the conformation of heme

proteins. It has been found that while myoglobin shows no change in helical content upon the binding of ligands (Samejima and Yang, 1964; Breslow et al., 1965), and while hemoglobin shows no change upon the binding of most ligands (Beychok, 1964), hemoglobin seems to undergo an 8.5% decrease in helicity upon the binding of oxygen (Brunori et al., 1967). A similar study on horseradish peroxidase (Ellis and Dunford, 1968) showed no change in helical content upon the binding of the ligands fluoride, cyanide, and hydroxide ions. The present study was undertaken with a view to determining whether a gross conformational change takes place upon the binding of ligands to lactoperoxidase. This chapter describes in greater detail the work already published (Maguire and Dunford, 1971).

Experimental methods

Lactoperoxidase was isolated by the method of Morrison and Hultquist (1963). Further purifications were carried out by column chromatography with Sephadex G-100 and G-200 gels. Activity tests were performed on lactoperoxidase using the guaiacol method of Maehly and Chance (1954). LP solutions were dialyzed against the appropriate buffer and passed through a Millipore filter before use. The P.N. of all solutions was at least 0.82. The maximum P.N. attained was 0.93. All buffers were of an ionic strength of 0.05. Buffers used in the order of

increasing pH were: glycine-HCl, formate, acetate, phosphate, tris-HCl, glycinate, sodium hydroxide-disodium hydrogen phosphate, and sodium hydroxide-potassium chloride. Overlapping buffer systems were used to check for possible buffer effects. Inorganic chemicals were of reagent grade and were used without further purification.

Lactoperoxidase concentrations were determined from absorbance measurements at 412 nm using a Cary 14 spectrophotometer. The molar absorptivity used was $\varepsilon_{412} = 1.14 \times 10^5\ M^{-1}cm^{-1}$ (Morrison et al., 1957). Typical concentrations of lactoperoxidase used were 7×10^{-6} M for O.R.D. measurements in the ultraviolet region and 5×10^{-5} M for measurements in the visible region. All pH measurements were made with an Orion model 801 digital pH meter equipped with a Fisher combination electrode.

Optical rotatory dispersion spectra were obtained using a Jasco model O.R.D./UV5 recording spectrometer. Both 1 mm and 5 mm cells were used. At least five O.R.D. determinations were carried out on the LP samples at each pH value, except for pH 7.0 where 14 determinations were made. Multiple determinations were carried out to determine the reproducibility of the O.R.D. spectrum for a given sample (maximum observed deviation of 1%) and to check the variation in spectrum with change in concentration of lactoperoxidase.

The LP-F complex was studied at pH 4.5 in a solution which was 0.025 M with respect to NaF, LP-CN at pH 7.0 with 0.01 M KCN, and LP-N_3 at pH 3.9 with 0.5 M NaN_3. All spectra of the complexes were taken six times and were reproducible to within 2%.

For all solutions used in this study, the maximum absorption was less than two in the wavelength regions of interest. This procedure was followed to help ensure that the O.R.D. spectra obtained were not caused by rotatory artifacts produced by regions of high absorbance (Urnes and Doty, 1961). The values of the specific rotation [α] were calculated using the molar concentrations determined spectrophotometrically and a value of 78,000 for the molecular weight of lactoperoxidase. All measurements were made at 25°.

Results

Figure 2-1 shows the O.R.D. curve for lactoperoxidase over the wavelength range 210-350 nm, plotted as corrected specific rotation, [α'], vs. wavelength. Within experimental error, the O.R.D. curves of the fluoride and cyanide complexes of LP are the same as that of pure LP. The O.R.D. spectrum of LP-N_3 could not be obtained in this region because of the high absorbance of the azide ion. The curve in Fig. 2-1 shows a minimum at 233 nm which is characteristic of the conformation-dependent trough of the negative Cotton effect centered at 224 nm, which is

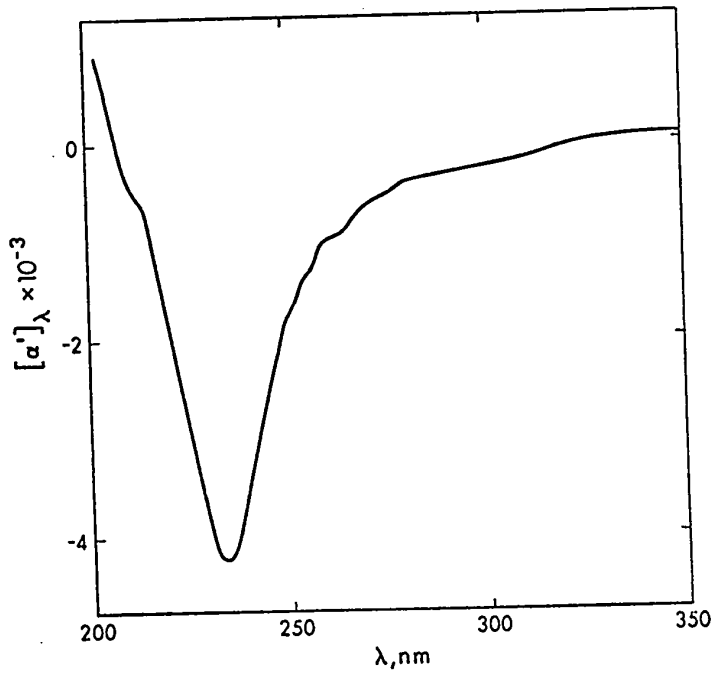

Fig. 2-1. Optical rotatory dispersion of lactoperoxidase in the ultraviolet region. Within experimental error, the corresponding curves for the fluoride and cyanide complexes of LP are the same as those for pure LP.

exhibited by poly-α-amino acids in the α-helical conformation. For a quantitative measure of the amplitude of this trough, we have used the reduced mean residue rotation $[R']_{233}$, as suggested by Simmons et al. (1961). The values of $[R']_{233}$ for LP and its cyanide and fluoride complexes were calculated from the relation

$$[R']_{233} = \frac{M_R}{100} \cdot \frac{3}{n_{233}^2 + 2} [\alpha']_{233} \qquad (2)$$

where the refractive index of water, n_{233}, was taken as 1.39, and the mean residue molecular weight, M_R, as 115. Lactoperoxidase contains about 8% carbohydrate material by weight (Rombauts et al., 1967), and thus a corrected specific rotation $[\alpha']_{233}$ was used based on an effective protein molecular weight of 72,000. Table 2-1 contains the values obtained for $[R']_{233}$ and percent helix content for pure LP over the pH range 3.4-10.3. Table 2-2 contains similar O.R.D. results for LP-F and LP-CN at 233 nm.

The values of percent helix show no trend with pH. In general, an LP solution with a higher P.N. exhibited a slightly lower helix content (about 2% in going from P.N. 0.83 to 0.88). This may be due to degradation as a result of extensive purification procedures. The use of $[R']_{233}$ to calculate the helical content of proteins seems to result in an underestimate in many cases (Urnes and Doty, 1961), which may be due to the fact that poly-α-L-glutamic acid has been used as the standard for 100% helicity. Although there seems to be agreement that $[R']_{233}$ is equal

Table 2-1. $[R']_{233}$ and percent helix for LP solutions as a function of pH.

pH	$-[R']_{233}$	% helix
3.4	4480	19
4.0	3490	11
4.6	4190	17
5.0	3930	15
5.6	5100	24
6.1	3940	15
7.0	4270	17
7.6	4360	18
8.0	4040	16
8.2	4490	19
8.8	3710	13
9.1	3360	10
9.5	4650	20
9.9	4030	16
10.3	4510	19

Table 2-2. Reduced mean residue rotation at 233 nm and helix content for lactoperoxidase and its fluoride and cyanide complexes.

Species	pH	$-[R']_{233}$	% helix
LP	7.0	4270	17
LP-CN	7.0	4190	17
LP	4.5	4080	16
LP-F	4.5	4220	17

to -2000° for random PGA, there is a wide disparity in the reported values for completely helical PGA (Simmons et al., 1961; Jirgensons, 1965; Yang and Samejima , 1963). After Yang (1967), we will assume that $[R']_{233}$ (helix) = -15,000° and $[R']_{233}$ (coil) = -2000° and obtain a rough estimate of the α-helical content by simple interpolation:

$$\text{fraction of } \alpha\text{-helix} = -\frac{([R']_{233} + 2{,}000)}{13{,}000} \qquad (3)$$

The randomness of the $[R']_{233}$ results would probably have obscured any concentration or buffer effect, if these were present. At either extreme of pH, denaturation occurred. O.R.D. determinations were not carried out below pH 3.4 or above 10.4. Figure 2-2 shows the extrinsic Cotton effects associated with the heme Soret bands of lactoperoxidase and its fluoride, cyanide, and azide complexes.

The activity of lactoperoxidase was determined by the oxidation of guaiacol (Maehly and Chance, 1954), which was monitored spectrophotometrically at 470 nm. It was observed that the activity of lactoperoxidase was independent of the P.N. exhibited (from P.N. 0.50 to 0.90), indicating that protein or peptide contaminants do not interfere with the oxidation reaction.

Discussion

The values of $[R']_{233}$ for the LP-ligand complexes were about the same as for pure lactoperoxidase, the

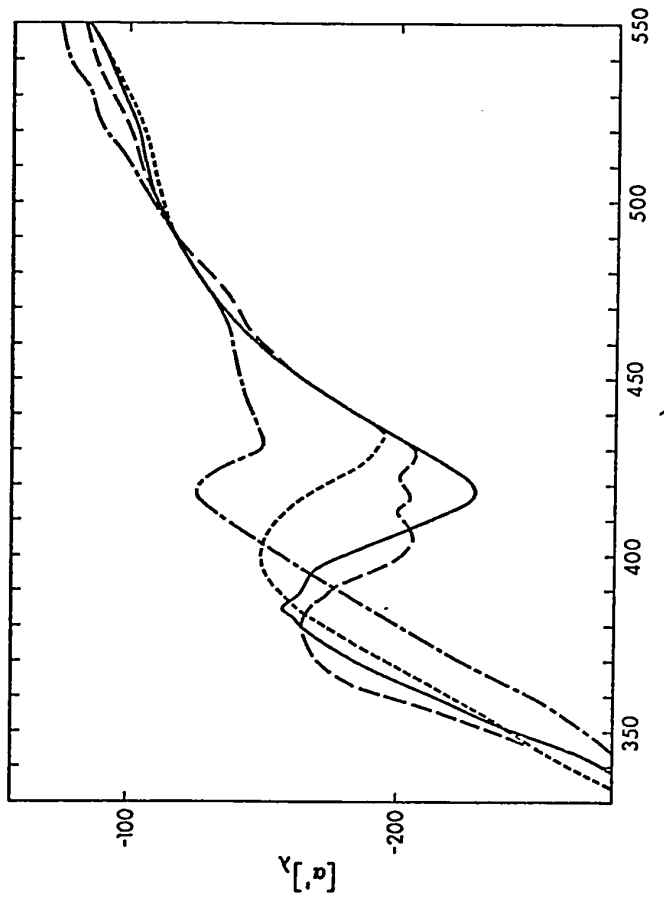

Fig. 2-2. Optical rotatory dispersion in the Soret region of lactoperoxidase (—), and its fluoride (– – –), cyanide (— - —), and azide (----) complexes.

difference being within experimental error. It is concluded that the binding of these ligands to lactoperoxidase has no significant effect on the amount of α-helical conformation of the enzyme. Lactoperoxidase is calculated to contain 17% α-helix at pH 7.

The data in Fig. 2-1 indicate the presence of Cotton effects due to side-chain chromophores in the 260-290 nm region. They were studied in some detail but were difficult to resolve. However, it is possible that they could interfere with the measurement of peptide optical activity.

The numerical values of the α-helical content of lactoperoxidase and its derivatives should be viewed with reservation. The trough method is attractive in that it provides a direct detection of the helical and random coiled conformations, but it neglects the complications due to the possible existence of β-forms of poly-peptides with different rotatory characteristics (Iizuka and Yang, 1966; Sarkar and Doty, 1966; Davidson et al., 1966) and also to the Cotton effects of side-chain chromophores on the enzyme itself (Vournakis et al., 1968). In addition, there may be forms of helices other than the α-helix which show different rotatory characteristics. The rotatory strength of the 3_{10} helix has been shown to differ from that of the α-helix and to display a chain-length dependence (Woody and Tinoco, 1967). Urry (1967) has shown that the heme chromophore may be another possible

source of optical activity in the peptide region. Finally, the difference in reference scales for 100% helical PGA should be recalled.

The O.R.D. curves of lactoperoxidase and its derivatives in the Soret region are interesting in that negative Cotton effects are displayed. It has been reported that cytochrome c peroxidase also shows a negative Cotton effect in the Soret region (Willick et al., 1969). The Soret O.R.D. curves of LP at pH 3.9 and pH 4.5 are identical to that at pH 7.0. The amplitudes of the Soret Cotton effects are as follows: LP, 71°; LP-F, 40°; LP-N_3, 56°; and LP-CN, 26°; the enzyme concentration was 4.96×10^{-5} M in all solutions. The reduction of the amplitude of the Soret Cotton effect for the complexes of lactoperoxidase may indicate some conformational change in the immediate environment of the heme group induced by the bound ligand.

While the absorption spectrum of catalase is similar to that of horseradish peroxidase and to spectra of ferrihemoglobin and ferrimyoglobin, the O.R.D. spectrum of catalase is remarkably different. Horseradish peroxidase, ferrihemoglobin, and ferrimyoglobin each exhibit a positive Cotton effect in the Soret region while in catalase the effect is negative. Ullmer and Vallee (1963) have correlated this observation with an orientation of the heme group in catalase different from the other heme

proteins mentioned. Marked differences in the accessibility of the heme group in catalase and horseradish peroxidase are suggested by the fact that the rates of catalysis by catalase decrease sharply as the size of the substrate increases, a phenomenon not observed with horseradish peroxidase. The O.R.D. data for catalase and horseradish peroxidase seem pertinent to these observations, since they demonstrate that catalase and horseradish peroxidase provide different asymmetric environments for their respective heme moieties. In this regard, it is indicated that lactoperoxidase also has a heme group with a different accessibility to substrate than the heme group of horseradish peroxidase, because of its negative Cotton effect. A steric effect is also exhibited by lactoperoxidase similar to catalase. The rate of reaction decreases with increasing substrate size in the reaction between lactoperoxidase and hydrogen peroxide, methyl hydrogen peroxide, and ethyl hydrogen peroxide (Chance, 1949).

Chapter 3

The Kinetics of the Formation of the Primary Lactoperoxidase-Hydrogen Peroxide Compound

Introduction

Lactoperoxidase (LP) is known to combine with hydrogen peroxide to form certain compounds capable of oxidizing various inorganic and organic reducing agents. George (1953) and Yamazaki and Souzu (1960) have suggested that free radicals should be formed from substrates as intermediates in peroxidatic oxidation. The proposed mechanism as applied to LP is

$$LP + H_2O_2 \xrightarrow{k_1} LP\text{-}I \tag{1}$$

$$LP\text{-}I + AH_2 \xrightarrow{k_2} LP\text{-}II + AH\cdot \tag{2}$$

$$LP\text{-}II + AH_2 \xrightarrow{k_3} LP + AH\cdot \tag{3}$$

$$2AH\cdot \xrightarrow{k_4} A + AH_2 \text{ (or AH-AH)} \tag{4}$$

where LP-I and LP-II are the oxidized forms of the enzyme referred to as compounds I and II respectively, AH_2 is the substrate reducing agent, AH· the free radical intermediate (such as semiquinone in the oxidation of hydroquinone), and A is the oxidized product. Chance (1949) determined the value of k_1 at pH 7.0 to be 2×10^7 $M^{-1}sec^{-1}$. Our interest lay in determining the pH dependence of k_1. This chapter describes in greater detail the work already published (Maguire et al., 1971).

Application of the steady-state approximation to the reaction scheme leads to the following relations:

$$\frac{d[LP]}{dt} = -k_1[LP][H_2O_2] + k_3[LP\text{-}II][AH_2] = 0 \quad (5)$$

$$\frac{d[LP\text{-}I]}{dt} = k_1[LP][H_2O_2] - k_2[LP\text{-}I][AH_2] = 0 \quad (6)$$

$$\frac{d[LP\text{-}II]}{dt} = k_2[LP\text{-}I][AH_2] - k_3[LP\text{-}II][AH_2] = 0 \quad (7)$$

$$\frac{d[AH\cdot]}{dt} = k_2[LP\text{-}I][AH_2] + k_3[LP\text{-}II][AH_2] - 2k_4[AH\cdot]^2 = 0 \quad (8)$$

The enzyme conservation relation is

$$[LP] + [LP\text{-}I] + [LP\text{-}II] = [LP]_0 \quad (9)$$

From Equations 7 and 8,

$$[AH\cdot]^2 = \frac{k_3}{k_4}[LP\text{-}II][AH_2] \quad (10)$$

Also

$$\frac{d[Products]}{dt} = k_4[AH\cdot]^2 \quad (11)$$

Therefore

$$\frac{d[Products]}{dt} = k_3[LP\text{-}II][AH_2] \quad (12)$$

An expression for [LP-II] can be derived in terms of $[LP]_0$ by using the enzyme conservation relation Eq. 9 and the steady-state Eqs. 5-7:

$$[LP\text{-}II] = \frac{k_1k_2[LP]_0[H_2O_2]}{(k_1k_2 + k_1k_3)[H_2O_2] + k_2k_3[AH_2]} \quad (13)$$

Therefore

$$\frac{-d[H_2O_2]}{dt} = \frac{d[\text{Products}]}{dt} = \frac{k_1k_2k_3[LP]_0[H_2O_2][AH_2]}{(k_1k_2 + k_1k_3)[H_2O_2] + k_2k_3[AH_2]} \quad (14)$$

The term $[LP]_0$ refers to the total concentration of LP in solution. If the concentration of the hydrogen donor, AH_2, is sufficiently large that the last term in the denominator of Eq. 14 is dominant, then

$$\frac{-d[H_2O_2]}{dt} = k_1[LP]_0[H_2O_2]$$

$$= k_{1obs}[H_2O_2] \quad (15)$$

If the initial concentration of lactoperoxidase ($[LP]_0$) is known, k_1 may be obtained from the relation

$$k_1 = \frac{k_{1obs}}{[LP]_0} \quad (16)$$

It should be noted that Eqs. 15 and 16 are independent of any radical recombination steps. For example, if reaction step (4) were

$$AH\cdot + AH_2 \longrightarrow \text{Products} \quad (17)$$

the expression for $-d[H_2O_2]/dt$ would be the same. There are three necessary and sufficient conditions that allow the derivation of our kinetic results. The first condition is that there should be one obligatory step in the reaction scheme as in step (1). The second condition is that there should be no other step in which LP or H_2O_2 appear as

reactants, and the third condition is that the substrate concentration should be in excess of the concentration of hydrogen peroxide, and sufficient to make step (1) rate-determining. The finding that the rate is first order in $[LP]_0$ and $[H_2O_2]$, and independent of $[AH_2]$ or the nature of AH_2 constitutes a check that the above conditions exist in our experiments.

Experimental Section

Lactoperoxidase with a P.N. of 0.65 - 0.90 was isolated from cow's milk by the procedure of Morrison and Hultquist (1963) and lyophilized. Stock solutions of lactoperoxidase for the experiments were prepared by dialyzing the lyophilized material against a pH 7.0 phosphate buffer of ionic strength 0.05. The stock solutions were stored at 5° and were used within three days. Lactoperoxidase concentrations were determined from absorbance measurements at 412 nm, using a molar absorptivity of 1.14×10^5 $M^{-1}cm^{-1}$ (Morrison et al., 1957). All kinetic measurements were performed at 25° using a Cary model 14 spectrophotometer. Slidewires for the absorbance ranges 0-0.2 and 0-2 were used. Doubly distilled water was used to prepare all solutions. Inorganic chemicals were of reagent grade and were used without further purification. Hydroquinone was obtained from the Baker and Adamson Chemical Company, guaiacol from the Sigma Chemical Company. Hydrogen peroxide concentration determinations were performed according to the method of Ovenston

and Rees (1950). An Orion model 801 digital pH meter in conjunction with a Fisher combination electrode was used for all pH measurements.

Steady state methods were employed in the determination of k_1. A very large excess of reducing agent was present in solution, sufficient to make reactions (2) and (3) much faster than reaction (1). Reaction (4) is also presumed much faster than (1). Therefore reaction (1) is rate-limiting in the oxidation-reduction cycle.

The rate of reaction (1) was followed spectrophotometrically by observation of the rate of oxidation of an appropriate hydrogen donor. The scheme depicted in Eq. (1) to (4) may be simplified, but since the rate-limiting step is reaction (1), neglect of other free radical intermediates in the oxidation of AH_2 to A does not complicate the predicted, and observed, first-order kinetics for reaction (1). It should be noted that if the concentration of hydrogen donor is not sufficiently large, the rate of appearance of product will be governed by rather complicated kinetics.

Hydroquinone was used as the hydrogen donor in the pH region 3.0 to 7.0; guaiacol from pH 7.0 to 10.8. Guaiacol was used at high pH because hydroquinone apparently undergoes a rapid rate of oxidation in basic solution to benzoquinone and polymerization products.

When hydroquinone was used as the hydrogen donor, the

usual concentrations were: [LP], 2×10^{-9} M; [H_2O_2], 2×10^{-5} M; and [hydroquinone], 1×10^{-3} M. The reaction was followed spectrophotometrically at 290 nm. Experimentally determined molar absorptivities at 290 nm are, 3.16×10^{3} $M^{-1}cm^{-1}$ for hydroquinone and 3×10^{2} $M^{-1}cm^{-1}$ for benzoquinone. Within experimental error the observed absorbance decreases were the same as those calculated from the molar absorptivities and the concentration of hydrogen peroxide.

When guaiacol was used, the usual concentrations were: [LP], 2×10^{-9} M; [H_2O_2], 2×10^{-5} M; and [guaiacol], 10^{-1} M. The reaction was followed spectrophotometrically at 470 nm. The product of the oxidation of guaiacol is not known with certainty. There are absorption maxima at 470 and 420 nm which decay after some time, indicating further reaction. Booth and Saunders (1956) claim that the color production at 470 nm is due, at least in part, to the production of 3, 3'-dimethoxy-[bi-2,5-cyclohexadienyl-1-ylidene]-4,4'-dione. The molar absorptivity of the product of the oxidation is unknown; however, the absorbance changes during the course of the reaction were proportional to the concentrations of hydrogen peroxide used, in the range 5×10^{-6} M to 4×10^{-5} M.

The rate of autoxidation of hydroquinone or guaiacol, negligible in their respective pH regions, was compensated for by the presence of the same hydrogen donor in the reference and reaction cuvettes. The hydrogen donor

was added to the reaction cuvette first, then usually hydrogen peroxide, followed by lactoperoxidase. The order of addition of hydrogen peroxide and enzyme made no difference to the total absorbance change or rate constant observed.

The problem of the kinetic results being vulnerable to contributions in absorbance (either positive or negative) from the slower secondary reactions (for example, further reactions of benzoquinone, perhaps polymerization) was minimal on the time scale of the primary oxidation. For example, using the concentrations specified above, we did not notice any further reaction of benzoquinone for at least an hour, whereas the primary oxidation of hydroquinone to benzoquinone was over in less than half that time. In other words, a good "infinity" value of absorbance was obtained. These remarks also apply to the use of guaiacol as the reducing agent.

Buffers used in the order of increasing pH were: citric acid-sodium citrate, formic acid-sodium formate, acetic acid-sodium acetate, potassium dihydrogen phosphate-disodium hydrogen phosphate, tris(hydroxymethyl)aminomethane hydrochloride-tris(hydroxymethyl)aminomethane, glycine-sodium glycinate, and sodium bicarbonate-sodium carbonate. Overlapping buffer systems were used to check for possible buffer effects. All buffers were of an ionic strength of 0.05.

The values of k_{lobs} were obtained by analyzing all first order curves with a non-linear least squares program on an

IBM 360/67 computer. About fifty points were taken per curve over about six half-lives. Values of k_1 were then obtained using Equation (16). Order studies (plots of k_{1obs} *vs.* $[LP]_0$) were performed at pH's 3.01, 4.95, and 7.01 with hydroquinone as the hydrogen donor, and at pH's 7.00 and 9.07 with guaiacol as the hydrogen donor.

Results

Figure 3-1 shows a typical experimental first order curve for the oxidation of hydroquinone. The oxidation of guaiacol also followed first order kinetics. Values of k_1 were found to be independent of the P.N. of the lactoperoxidase. Table 3-1 contains values of k_1 (with standard deviations) as a function of pH with hydroquinone as the reducing agent; Table 3-2 contains values of k_1 (with standard deviations) as a function of pH with guaiacol as the reducing agent; and Table 3-3 contains values of k_1 (with standard deviations) as a function of the pH of formate buffer, with hydroquinone as the reducing agent. It is seen that guaiacol gives the same results as hydroquinone for k_1, within experimental error, in the pH region at which kinetic experiments overlapped, e.g., at pH 5.0 and 7.0. A concentration effect of guaiacol on the observed rate constant was found in the 10^{-2} M region as illustrated in Fig. 3-2. To facilitate discussion, k_1' will be defined as that second order rate constant which is obtained by dividing k_{1obs} by $[LP]_0$. Only in the limit

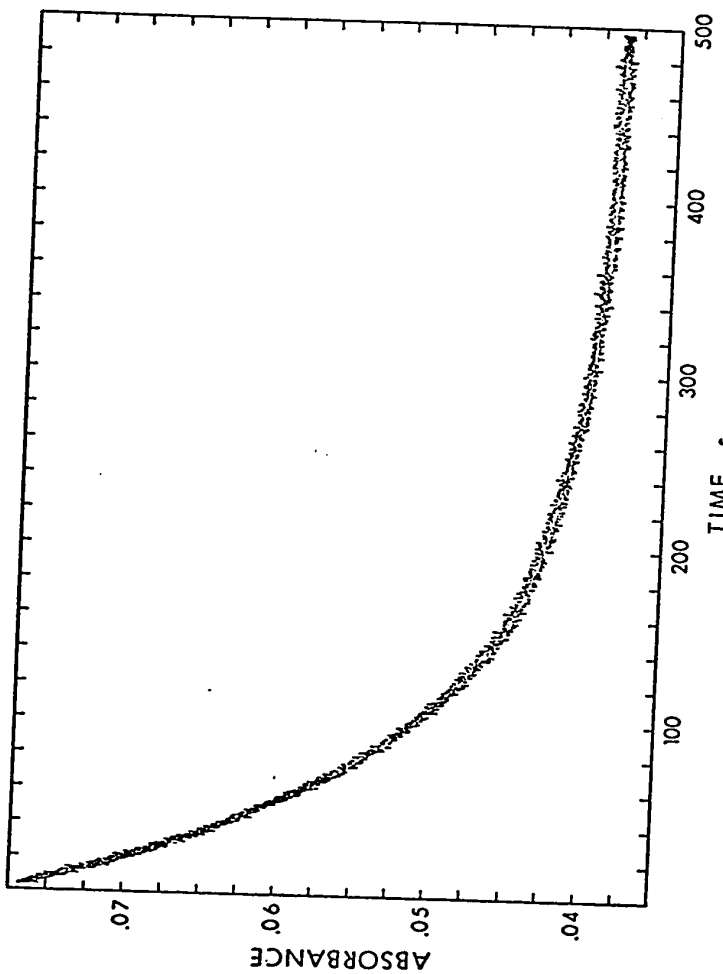

Fig. 3-1. A typical experimental first-order (exponential) curve for the oxidation of hydroquinone in pH 4.4 acetate buffer, reproduced from the original recording spectrophotometer trace. The experimental conditions are: $[LP]_0 = 1.0 \times 10^{-9}$ M, $[H_2O_2]_0 = 1.86 \times 10^{-5}$ M, and $[HQ]_0 = 9.59 \times 10^{-4}$ M. The rate constant obtained from this curve has the value $k_{1obs} = (1.18 \pm 0.01) \times 10^{-2}\ s^{-1}$. The time course for guaiacol oxidation is similar in that it is also represented by a first-order (exponential) decay.

Table 3-1

k_1 as a Function of pH with Hydroquinone as Reducing Agent. Reaction conditions are described in the Experimental section, and k_1 is calculated using Equation (16). Values of k_1 obtained at pH 4.95 and 7.01 are results of order plots (k_{1obs} vs. $[LP]_0$).

pH	k_1 ($M^{-1}sec^{-1}$)	Buffer*	Number of determinations
3.10	$(8.4 \pm 0.7) \times 10^6$	CIT	6
3.37	$(7.6 \pm 0.6) \times 10^6$	CIT	6
3.62	$(9.7 \pm 0.9) \times 10^6$	CIT	6
3.83	$(9.9 \pm 0.9) \times 10^6$	CIT	6
3.96	$(8.7 \pm 0.8) \times 10^6$	CIT	6
4.14	$(7.0 \pm 0.4) \times 10^6$	CIT	6
4.38	$(7.6 \pm 0.9) \times 10^6$	CIT	6
4.59	$(8.8 \pm 0.5) \times 10^6$	CIT	6
3.81	$(5.7 \pm 0.3) \times 10^6$	AC	6
3.94	$(9.6 \pm 0.5) \times 10^6$	AC	6
4.14	$(1.1 \pm 0.1) \times 10^7$	AC	6
4.37	$(1.2 \pm 0.1) \times 10^7$	AC	6
4.53	$(1.1 \pm 0.1) \times 10^7$	AC	6
4.77	$(9.9 \pm 1.3) \times 10^6$	AC	6
4.95	$(1.1 \pm 0.1) \times 10^7$	AC	6
5.16	$(1.4 \pm 0.1) \times 10^7$	AC	18
5.36	$(1.1 \pm 0.1) \times 10^7$	AC	6
5.59	$(1.5 \pm 0.1) \times 10^7$	AC	6
5.78	$(1.4 \pm 0.1) \times 10^7$	AC	6
5.96	$(1.1 \pm 0.2) \times 10^7$	AC	6
6.15	$(1.2 \pm 0.1) \times 10^7$	P	10
6.38	$(1.0 \pm 0.1) \times 10^7$	P	10
6.59	$(1.2 \pm 0.1) \times 10^7$	P	10
6.80	$(1.1 \pm 0.1) \times 10^7$	P	10

continued

Table 3-1 continued.

pH	k_1 ($M^{-1}sec^{-1}$)	Buffer*	Number of determinations
7.01	$(9.4 \pm 0.8) \times 10^6$	P	52
7.17	$(1.2 \pm 0.1) \times 10^7$	P	10
7.37	$(1.0 \pm 0.1) \times 10^7$	P	10

*Buffer key: CIT, citrate; AC, acetate; P, phosphate.

Table 3-2

k_1 as a Function of pH with Guaiacol as Reducing Agent.

Reaction conditions are described in the Experimental section, and k_1 is calculated using Equation (16). Values of k_1 obtained at pH 7.00 and 9.07 are results of order plots (k_{1obs} vs. $[LP]_0$).

pH	k_1 ($M^{-1}sec^{-1}$)	Buffer*	Number of determinations
4.97	$(1.0 \pm 0.1) \times 10^7$	AC	6
7.00	$(1.1 \pm 0.1) \times 10^7$	P	15
8.07	$(1.0 \pm 0.1) \times 10^7$	T	6
9.04	$(1.0 \pm 0.1) \times 10^7$	G	6
9.07	$(1.0 \pm 0.1) \times 10^7$	T	15
9.93	$(9.9 \pm 0.2) \times 10^6$	G	6
10.02	$(9.8 \pm 0.2) \times 10^6$	CAR	6
10.76	$(9.7 \pm 0.3) \times 10^6$	CAR	12

*Buffer key: AC, acetate; P, phosphate; T, tris; G, glycinate; CAR, carbonate.

Table 3-3

k_1 as a Function of pH of Formate Buffer with Hydroquinone as Reducing Agent.

Reaction conditions are described in the Experimental section, and k_1 is calculated using Equation (16). The value of k_1 at pH 3.01 was determined by an order plot (k_{1obs} *vs.* $[LP]_0$).

pH	k_1 ($M^{-1}sec^{-1}$)	Number of determinations
3.01	$(7.7 \pm 1.0) \times 10^4$	14
3.18	$(1.2 \pm 0.1) \times 10^5$	6
3.27	$(1.9 \pm 0.1) \times 10^5$	6
3.40	$(1.9 \pm 0.1) \times 10^5$	6
3.50	$(3.0 \pm 0.1) \times 10^5$	6
3.60	$(2.9 \pm 0.1) \times 10^5$	6
3.74	$(4.7 \pm 0.3) \times 10^5$	6
3.79	$(4.2 \pm 0.1) \times 10^5$	6
4.03	$(6.4 \pm 0.4) \times 10^5$	6
4.25	$(9.9 \pm 0.3) \times 10^5$	6
4.39	$(1.2 \pm 0.1) \times 10^6$	6
4.67	$(1.7 \pm 0.1) \times 10^6$	3
4.96	$(3.4 \pm 0.1) \times 10^6$	6

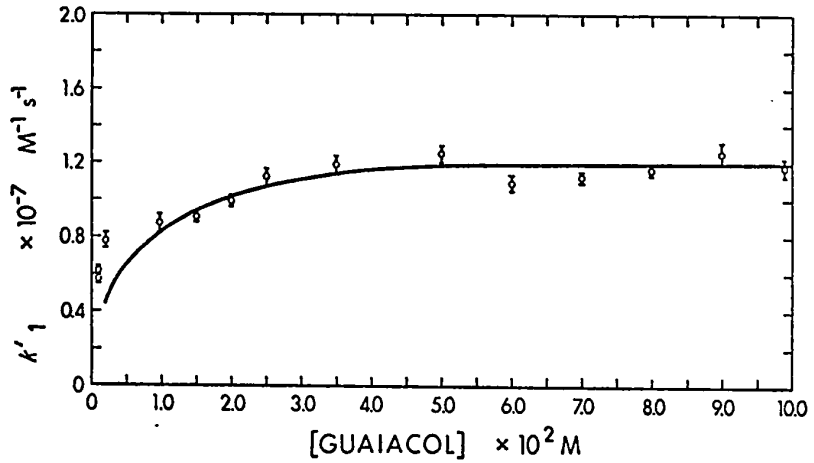

Fig. 3-2. A plot of k_1' as a function of guaiacol concentration at pH 7.00. Reaction conditions are described in the Experimental section, and values of k_1' are calculated as described in the Results section. Each point represents five experimental determinations. The error bars on the points represent standard deviations. The plateau begins at a concentration of guaiacol of about 5×10^{-2} M and extends through 10^{-1} M.

of sufficiently high concentration of reducing agent is k_{1obs} a true first order rate constant, and k_1' equal to k_1, the true second order rate constant for compound I formation (Eq. 16). Figure 3-2 shows that at pH 7.00, the value of k_1' increases with increasing guaiacol concentration until the latter reaches about 5×10^{-2} M; the value of k_1' then becomes independent of guaiacol concentration to 10^{-1} M and hence equal to k_1. At pH 10.02, k_1' becomes independent of guaiacol concentration (hence equal to k_1) when the latter reaches about 7×10^{-2} M as shown in Fig. 3-3. At pH 10.76, where guaiacol is ionized, a number of experiments were performed in which the ionic strength was varied from 0.1 to 0.3 and guaiacol concentrations were about 0.1 M. No ionic strength effect on the rate constant k_1 was observed.

Figure 3-4 is a plot of k_{1obs} *vs.* $[LP]_0$ at pH 7.01. From the slope, k_1 is calculated by least squares analysis to be $(9.4 \pm 0.8) \times 10^6$ $M^{-1}sec^{-1}$. The intercept is zero within experimental error, showing first order behaviour; this was the case for all such plots of k_{1obs} *vs.* $[LP]_0$ at other pH values.

A plot of k_1 *vs.* pH over the pH range 3.0 to 10.8 is shown in Fig. 3-5. The filled-in circles represent values of k_1 obtained when experiments were performed in formate buffer. The line through the rest of the points was obtained by least-squares analysis. The slope of the line

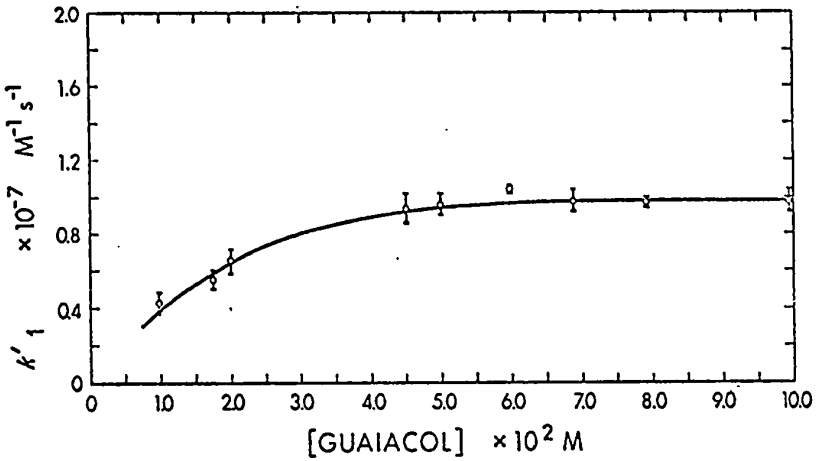

Fig. 3.3. A plot of k_1' as a function of guaiacol concentration at pH 10.00. Reaction conditions are described in the Experimental section, and values of k_1' are calculated as described in the Results section. Each point represents five experimental determinations. The error bars on the points represent standard deviations. At this pH, the plateau begins at a concentration of guaiacol of about 7×10^{-2} M and extends through 10^{-1} M.

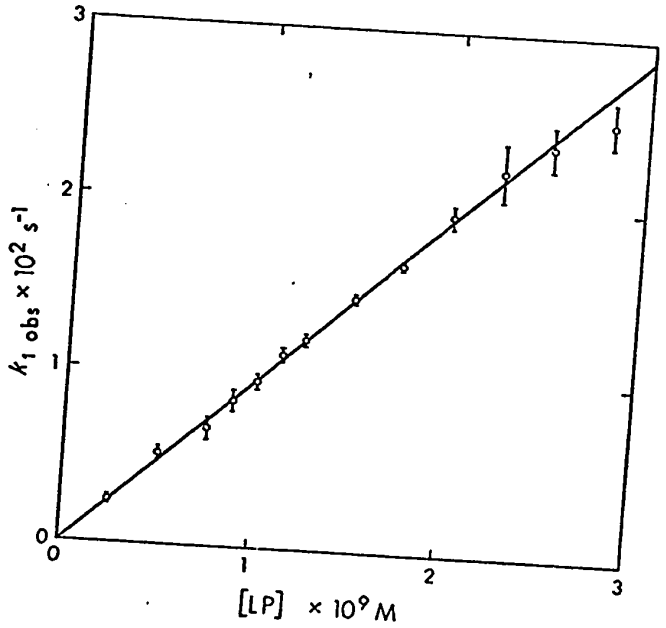

Fig. 3-4. A plot of k_{lobs} vs. [LP] at pH 7.01 using hydroquinone as reducing agent. This plot is typical of the five order plots performed at the pH's described in the Experimental section. The slope, equal to k_1, has a value of $(9.4 \pm 0.8) \times 10^6\ M^{-1}s^{-1}$, calculated by least-squares analysis.

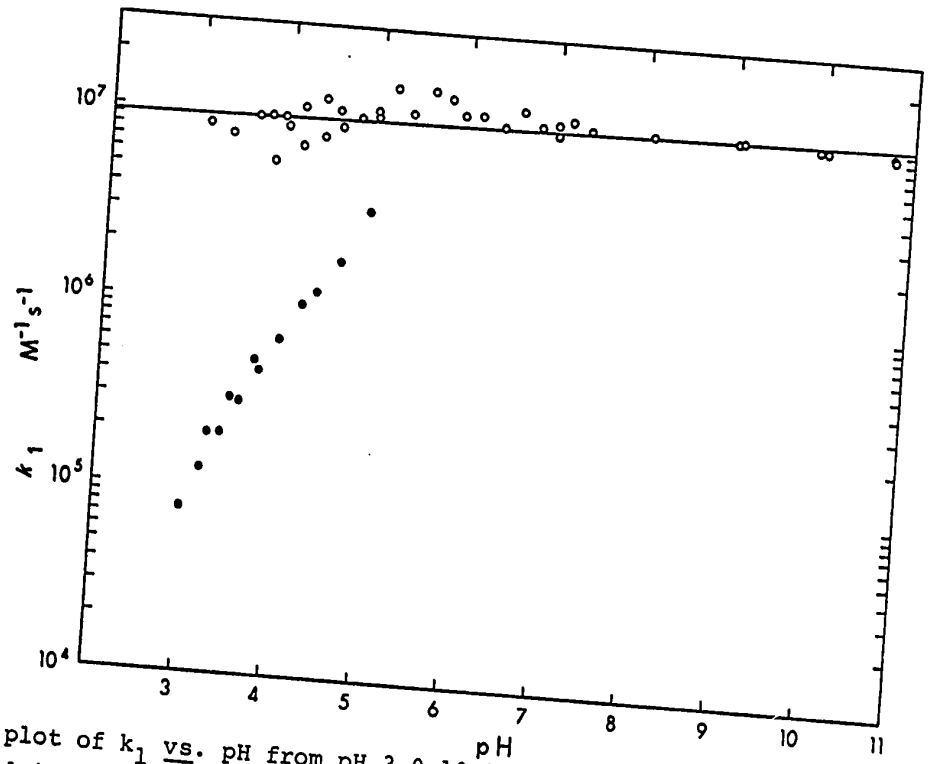

Fig. 3.5. A plot of k_1 vs. pH from pH 3.0-10.8. The filled-in circles represent values of k_1 obtained in formate buffer. The values of k_1 are those in Tables 3-1,3-2 and 3-3. The slope of the line through the open circles is zero within experimental error, $(1.1 \pm 1.2) \times 10^5$.

is zero within experimental error, indicating a constant value of k_1 of $(9.2 \pm 0.9) \times 10^6$ $M^{-1}sec^{-1}$. In formate buffer however, the value of k_1 decreases from 3.4×10^6 $M^{-1}sec^{-1}$ at pH 4.96 to 7.7×10^4 $M^{-1}sec^{-1}$ at pH 3.01.

It was necessary to store the lactoperoxidase in stock solutions at pH 7.0. It was noticed that if the LP were stored at pH 3.0, for example, the values of k_1 progressively decreased with time, indicating deactivation of the lactoperoxidase at this extreme of pH.

Discussion

It is seen from Fig. 3-5 that the rate constant for the formation of lactoperoxidase compound I from lactoperoxidase and hydrogen peroxide is essentially pH-independent over the pH region investigated. The value of $(9.2 \pm 0.9) \times 10^6$ $M^{-1}sec^{-1}$ is to be compared with Chance's (1949, 1950) value for k_1 of 2×10^7 $M^{-1}sec^{-1}$, slightly more than twice the value reported here. One possible reason for the discrepancy may be that the value for the extinction coefficient at 412 nm is incorrect by a factor of two. This would cause the concentrations of LP to be incorrect by a factor of two, giving rise to a rate constant k_1 that is different by a factor of two. However, although the extinction coefficient may be in error, it would appear unlikely that it would be in error by this amount. Another possibility, that the enzyme cycle could be affected by the attack of a free radical on, for example, LP, thereby reducing the amount of LP in the cycle, could be discounted on the grounds that first order kinetics

are observed throughout the course of a particular reaction. There could be some unforeseen source of error in our results, but to the best of our knowledge, the results yield accurate values of k_1. On the other hand, Chance (1949, 1950) gives little experimental detail concerning the conditions under which his value of 2×10^7 $M^{-1}sec^{-1}$ was obtained. No mention is made of the number of determinations or the reproducibility of the value of k_1 obtained; Chance (1950) does mention obtaining values of k_1 as low as 9×10^6 $M^{-1}sec^{-1}$ and as high as 2.7×10^7 $M^{-1}sec^{-1}$. There is also no mention made of ionic strength or temperature, although Morell (1954) claims that Chance's experiments were performed at temperatures varying from 25° to 30°. For these reasons, it is concluded that the value of k_1 reported here is more reliable.

There is some evidence that k_2 and/or k_3 of the reaction scheme Eqs. 1-4 are not pH-independent, as is k_1. This is implied by the k_1' vs. [guaiacol] plots at pH 7.00 (Fig. 3-2) and pH 10.02 (Fig. 3-3). The concentration region in which k_1' becomes independent of guaiacol concentration (hence equal to k_1) occurs at higher guaiacol concentration at pH 10.02 than at pH 7.00, as shown in the Results section. This indicates that it is more difficult to attain the conditions under which reaction (1) is rate-limiting at this higher pH, i.e., the concentration of guaiacol must be increased in order to maintain pseudo-first order conditions. This could be explained by a decrease in some other rate constant(s) in the cycle with

increasing pH. In fact, it will be shown in the next two chapters that k_3 does decrease with increasing pH for the reactions between LP-II and either iodide ion or p-cresol.

Figure 3-5 also shows the effect of formate buffer on the kinetics of the formation of compound I. It was determined that in pH 3.60 formate solution there is no shift in the lactoperoxidase Soret band maximum, but there is a 7% decrease in the molar absorptivity compared to standards either in pure water or pH 7.0 phosphate solution. Fig. 3-6 and 3-7 are plots of k_1' vs. [hydroquinone] obtained from experiments performed in pH 3.6 acetate and pH 3.6 formate solutions. In pH 3.6 acetate buffer, k_1' becomes independent of hydroquinone concentration (hence equal to k_1) at about 4×10^{-4} M, while in pH 3.6 formate buffer this occurs at a higher hydroquinone concentration of about 5×10^{-4} M.

More striking is the vertical difference between the two plateaus of Fig. 3-6 and Fig. 3-7. The value of k_1 (i.e., the limiting value of k_1') in pH 3.6 formate buffer is 3.5×10^5 $M^{-1}sec^{-1}$ while the value of k_1 in pH 3.6 acetate buffer is 7.5×10^6 $M^{-1}sec^{-1}$, a factor of 20 higher. This difference can be thought of as a measure of the extent of binding of formate ion to lactoperoxidase, and can be used to calculate a dissociation constant for the lactoperoxidase-formate ion complex. In the equation $K = [LP][HCO_2^-]/[LP\text{-}HCO_2^-]$, $[HCO_2^-] = 0.05$ (the ionic strength

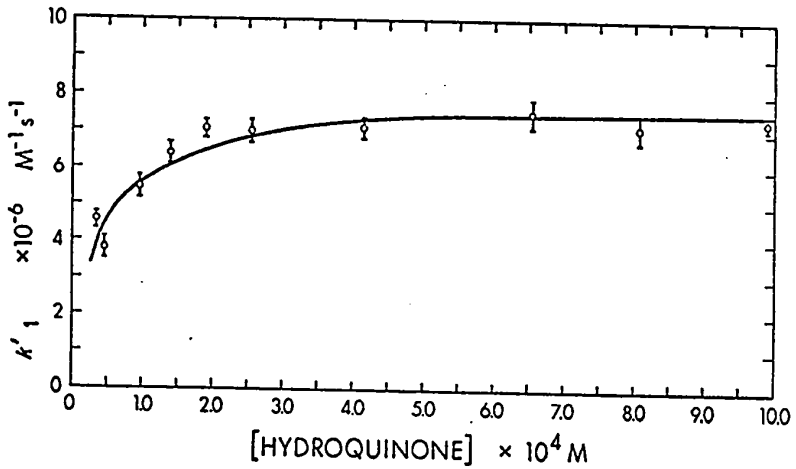

Fig. 3.6. A plot of k_1' as a function of hydroquinone concentration in pH 3.59 acetate buffer. Reaction conditions are described in the Experimental section, and values of k_1' are calculated as described in the Results section. Each point represents five experimental determinations. The error bars on the points represent standard deviations. The plateau begins at a concentration of hydroquinone of about 4×10^{-4} M.

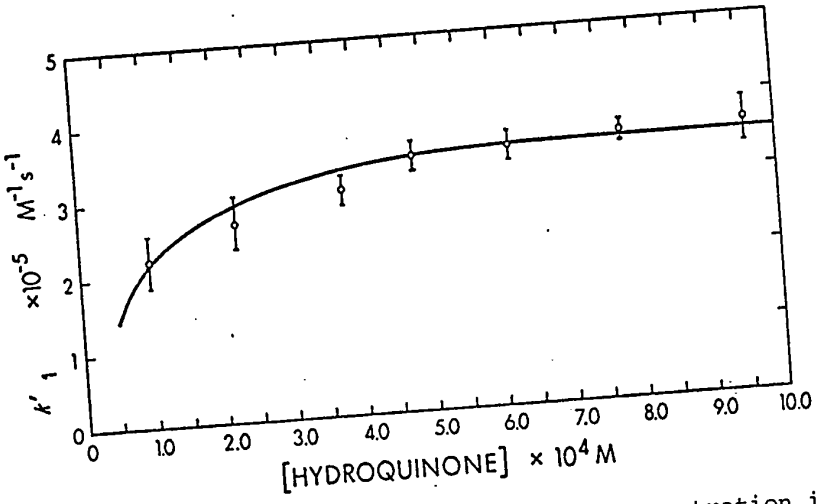

Fig. 3-7. A plot of k'_1 as a function of hydroquinone concentration in pH 3.62 formate buffer. Reaction conditions are described in the Experimental section, and values of k'_1 are calculated as described in the Results section. Each point represents five experimental determinations. The error bars on the points represent standard deviations. The plateau begins at a concentration of hydroquinone of about 5×10^{-4} M.

of all buffers in this study), and the ratio $[LP]/[LP\text{-}HCO_2^-]$ was calculated from the ratio of rate constants k_1 obtained in formate buffer and another buffer at the same pH (such as acetate or citrate, again at the same ionic strength of 0.05). The dissociation constant thus calculated varies from 4.2×10^{-4} M at pH 3.01 tc 1.85×10^{-2} M at pH 4.96. Fig. 3-8 is a plot of the negative logarithm of the dissociation constant (pK) vs. pH. The slope, obtained by least squares analysis, is $-(0.77 \pm 0.03)$. A value of less than unity for the slope may indicate that there is a pK of the free enzyme in this region. Chance (1952a) has demonstrated that the binding of formate buffer to catalase involves only a small perturbation of the Soret spectrum, and also that the dissociation constant of the catalase-formate ion complex varies from 5×10^{-4} M at pH 5.6 to 1.6×10^{-5} M at pH 4.1. Chance (1952a) further demonstrates that a plot of the negative logarithm of the dissociation constant (for the catalase formate-ion complex) vs. pH is linear with a slope of -1, indicating that for catalase there is no ionizing group on the enzyme which affects the binding reaction.

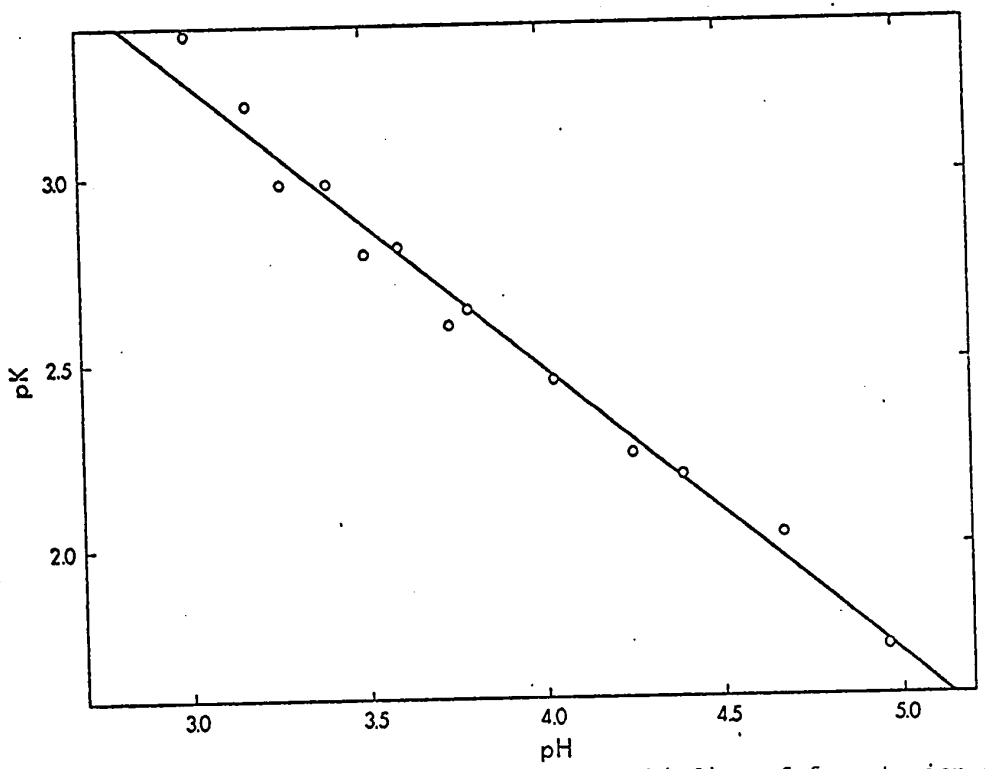

Fig. 3-8. A plot of pK as a function of pH for the binding of formate ion to lactoperoxidase. The equilibrium constant was calculated from the relation $K = [LP][HCO_2^-]/[LP\text{-}HCO_2^-]$, where $[HCO_2^-] = 0.05$ M over the pH range 3.01-4.96, and the quantity $[LP]/[LP\text{-}HCO_2^-]$ is the ratio of rate constants k_1 obtained in formate buffer and another buffer, at the same pH (such as acetate or citrate buffer at the same ionic strength). The slope of the line is $-(0.77 \pm 0.03)$.

Chapter 4

The Kinetics of the Oxidation of Iodide Ion By Lactoperoxidase Compound II

Introduction

The horseradish peroxidase-catalyzed oxidation of iodide ion was first observed by Bach (1904, 1907) at the turn of the century. In the thyroid gland a peroxidase with physical properties similar to those of the horseradish enzyme oxidizes iodide ion as a step in thyroid hormone biosynthesis (Hosoya and Morrison, 1967a). Hosoya and Morrison (1967b) have demonstrated that lactoperoxidase is more active in catalyzing the oxidation of iodide ion than is thyroid peroxidase, myeloperoxidase, or horseradish peroxidase. The chloroperoxidase of Caldariomyces fumago plays a similar role in the synthesis of caldariomycin, which contains chlorine (Hager et al., 1966). Hence the peroxidase-catalyzed oxidation of halide ions is of particular interest. The present study was undertaken for two reasons. One reason was that a study of this sort would be preliminary to a study of a system in which the kinetics of the iodination of tyrosine would be studied, thereby providing a close analogy to the action of thyroid peroxidase in the thyroid gland. Another reason this study was undertaken was to probe the active site of compound II of lactoperoxidase in the hope that the log k-pH profile might provide clues as to the identity of

amino acid groups on the enzyme which participate in the reaction. This chapter describes in greater detail the work in press (Maguire and Dunford, 1972).

Experimental Section

Lactoperoxidase was obtained as a lyophilized powder from Calbiochem. In this form, the samples exhibited a P.N.[1] of about 0.6. The enzyme was purified by gel filtration on Sephadex G-200 Superfine at 4° using a phosphate buffer of pH 7 and ionic strength 0.05 as eluant. Enzyme fractions obtained in this way which exhibited P.N.'s greater than 0.8 were used in this study. The concentration of lactoperoxidase was determined spectrophotometrically at 412 nm using a molar absorptivity of 1.14×10^5 $M^{-1}cm^{-1}$ (Morrison et al., 1957).

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[1]Abbreviations used are: LP, lactoperoxidase; LP-I, the primary lactoperoxidase-hydrogen peroxide compound; LP-II, the secondary lactoperoxidase-hydrogen peroxide compound; HRP-II, the secondary horseradish peroxidase-hydrogen peroxide compound; P.N., purity number, the ratio of the absorbance of a solution of lactoperoxidase at 412 nm to that at 280 nm; μ, ionic strength; k_{obs}, the pseudo-first order rate constant for the reaction of LP-II with iodide ion; k_1, k_2: second and third order rate constants, respectively, for the reaction of LP-II with iodide ion; [ ], molar concentration; ΔV, ΔA: changes in voltage and absorbance, respectively.

The buffers used in this study, prepared from reagent grade chemicals and of an ionic strength of 0.05, are listed in Table 4-1. An Orion model 801 digital pH meter in conjunction with a Fisher combination electrode was used for all pH measurements.

Potassium iodide was obtained from Alfa Inorganics and the McArthur Chemical Company, and sodium iodide from Orion Research, Inc. The three iodide samples were found to exhibit identical chemical behavior within experimental error.

Solutions of hydrogen peroxide were prepared by diluting a 30% solution of hydrogen peroxide obtained from the Fisher Scientific Company. Concentrations of hydrogen peroxide were determined according to the method of Ovenston and Rees (1950). The water used in all solutions was distilled once from alkaline permanganate and once from glass.

The rate of reaction of LP-II with iodide ion was followed spectrophotometrically at 412 nm, the wave-length of maximum absorbance by native lactoperoxidase. The concentration of iodide ion was at least ten times that of LP, maintaining pseudo-first order conditions. LP-II was prepared in most cases by the addition of one equivalent of hydrogen peroxide to a LP solution, and had a half-life of over 20 minutes at most pH's. In tris-HCl buffer, about 1.2 to 1.5 equivalents of hydrogen peroxide were required, which may be due to the presence of some reducing

agent in this buffer. LP and LP-II were found to exhibit an isosbestic point at 422 nm, and LP-II an absorption maximum at 432 nm, in agreement with the spectra obtained by Chance (1950).

The reactions between LP-II and iodide ion at 25° were studied using a Cary 14 recording spectrophotometer (equipped with 0-0.2 and 0-2 absorbance slidewires), and a stopped-flow apparatus constructed in this laboratory which has a dead-time of 6 milliseconds. This stopped-flow apparatus is nearly identical to that described by Hasinoff (1970) except that the cell compartment is constructed of polypropylene instead of plexiglass. The stopped-flow data were obtained as an amplified photomultiplier voltage print-out digitalized at 30 equally spaced intervals of time. The optical detection system has been described elsewhere (Ellis and Dunford, 1968).

In stopped-flow experiments, one driving syringe contained the LP-II in unbuffered aqueous solution, and the other syringe contained iodide ion in the appropriate buffer. This procedure minimized the possibility of enzyme denaturation at the low pH's at which some experiments were performed. In addition the total absorbance change was kept small ($\Delta A < 0.04$) so that the relative voltage changes observed, ΔV, were proportional to ΔA.

The kinetic data were analyzed as described by Roman et al. (1971). At each pH and for a given

concentration of iodide ion, 3-5 experiments were performed on the Cary spectrophotometer, or about 10 experiments were performed if the stopped-flow apparatus was used. When it was possible, concentrations of iodide ion were used which would yield reaction rates which overlapped the time scales pertaining to the Cary spectrophotometer and the stopped-flow apparatus; using both instruments, experiments at the same concentration of iodide ion (and the same pH) yielded values for a pseudo-first order rate constant, k_{obs}, which were equal within experimental error.

Blank reaction rate experiments were conducted at various pH's in the absence of LP, and in no case was an appreciable rate measured (by observing absorbance changes at 353 nm, an absorbance maximum of tri-iodide ion) for the uncatalyzed reaction between iodide ion and hydrogen peroxide.

The products produced in the reaction between LP-II and iodide ion (iodine atoms or molecular iodine) may react further with lactoperoxidase, perhaps with aromatic amino acid residues. It was noticed in experiments performed using the stopped-flow apparatus that there exists a second, much slower, reaction. At pH 5, this reaction is 50 times slower than the reaction between LP-II and iodide ion, and so caused no detectable interference in measurements of the rate of the reaction of LP-II with iodide. Calculation of rate constants using the initial

time course of the LP-II—iodide reaction led to values which were slightly higher (ca. 3%) than those rate constants obtained by computer analysis of the whole first order curve. Because this value is within experimental error, it was not necessary to apply the correction, and it was concluded that this second, unidentified, reaction did not interfere with observation of the reaction between LP-II and iodide ion.

Results

The kinetics of the reaction between LP-II and iodide under conditions of high iodide ion concentration in relation to the concentration of LP-II are consistent with the differential rate expression

$$-\frac{d[\text{LP-II}]}{dt} = k_{obs}[\text{LP-II}] \qquad (1)$$

Linear semilogarithmic plots (over four or more half-lives) of ΔV (or ΔA) vs. time proved the validity of Equation 1. An example of an experimental curve is shown in Fig. 4-1; Fig. 4-2 shows a semilogarithmic plot of the curve in Fig. 4-1, and also plots of curves obtained at two other pH values.

Fig. 4-3 is a plot of k_{obs} vs. the concentration of iodide ion, using data obtained at pH 6.95. The non-linearity is evidence that a higher order in concentration of iodide ion is involved. Values of k_{obs} as a function of the concentration of iodide ion were found to fit the relation

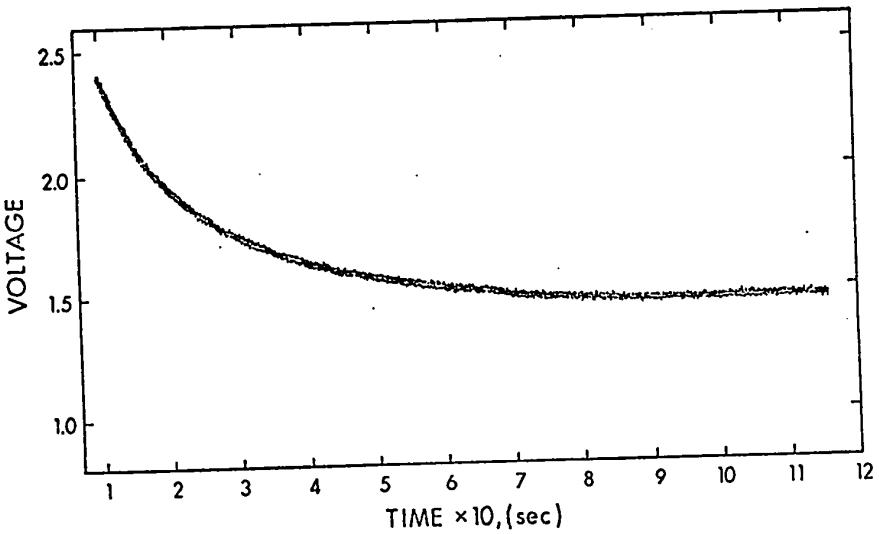


Fig. 4-1. An oscilloscope trace of voltage vs. time for the reaction of LP-II with iodide ion at pH 4.17 citrate, μ = 0.05. The reaction was observed at a monochromator setting of 412 nm. The initial concentrations of iodide ion and LP-II were 1.0×10^{-5} M and 9.1×10^{-7} M, respectively. Data points from this trace are plotted in Fig. 4-2.

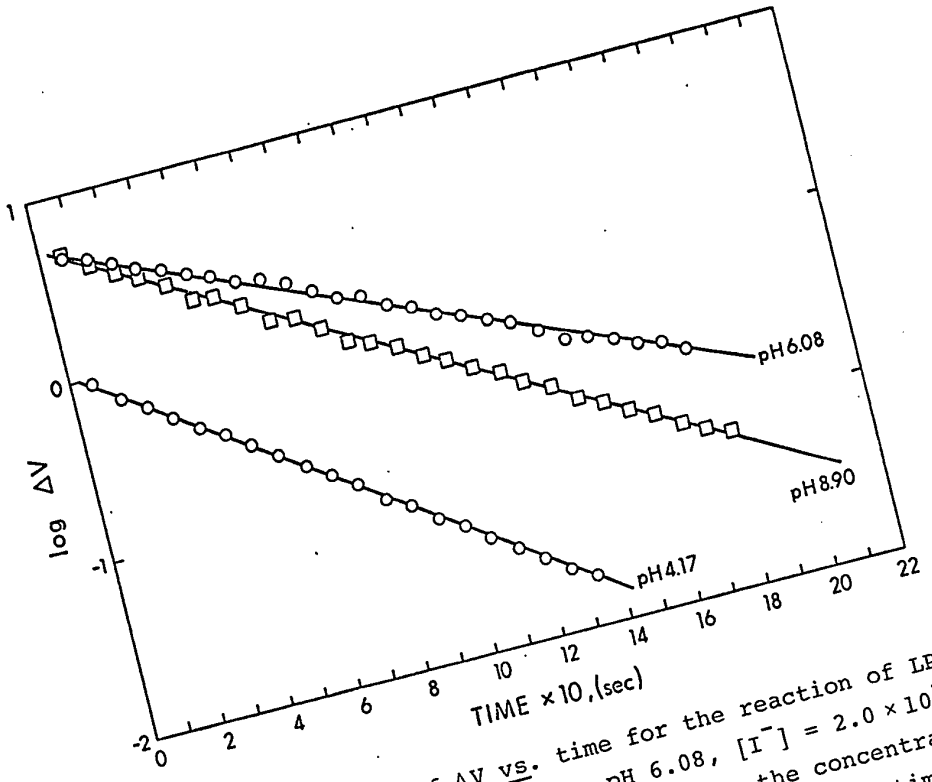


Fig. 4-2. Semilogarithmic plots of ΔV vs. time for the reaction of LP-II with iodide ion at three pH values. Experimental conditions: pH 6.08, $[I^-] = 2.0 \times 10^{-4}$ M; pH 8.90, $[I^-] = 4.5 \times 10^{-2}$ M; pH 4.17, $[I^-] = 1.0 \times 10^{-5}$ M. In all cases the concentration of LP-II was about 9×10^{-7} M. Linear semilogarithmic plots of ΔV (or ΔA) vs. time (over more than four half-lives) over the pH range 2.8-10.1 proved the validity of Eq. 1.

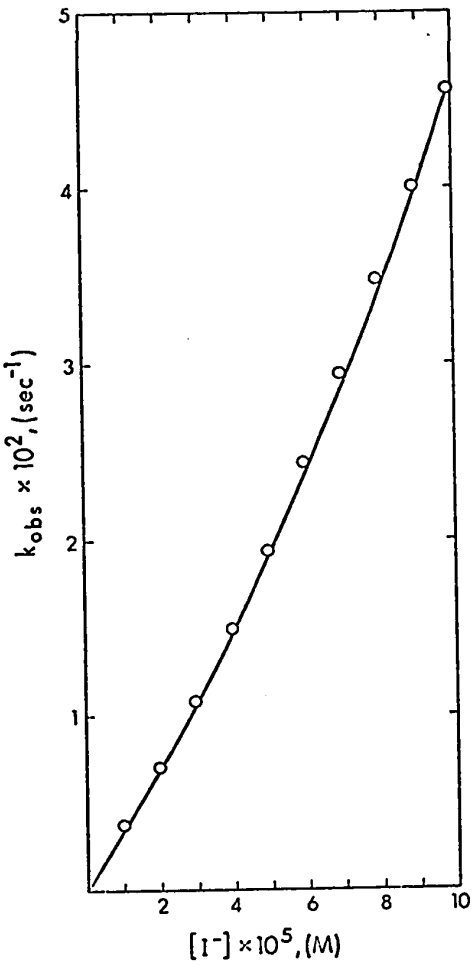


Fig. 4-3. A plot of k_{obs} vs. $[I^-]$ at pH 6.95 phosphate, $\mu = 0.05$. The curvature shows that the reaction is not simply first order in the concentration of iodide ion.

$$k_{obs} = k_1[I^-] + k_2[I^-]^2 \qquad (2)$$

where k_1 and k_2 are second and third order rate constants respectively. A plot of $k_{obs}/[I^-]$ *vs.* $[I^-]$ yields k_2 from the slope and k_1 from the intercept. A plot of $k_{obs}/[I^-]$ *vs.* $[I^-]$ using data obtained at pH 6.95 is shown in Fig. 4-4. The plot curves up at low concentrations of iodide ion because of the spontaneous decay of LP-II. The values of the rate constants k_1 and k_2 were determined by least squares analysis. The rate constant k_1 varies from $(2.6 \pm 0.4) \times 10^{-1}$ $M^{-1}sec^{-1}$ to $(4.20 \pm 0.44) \times 10^{6}$ $M^{-1}sec^{-1}$ over the pH range 10.07 - 2.86, while the rate constant k_2 varies from $(1.41 \pm 0.17) \times 10^{3}$ $M^{-2}sec^{-1}$ to $(2.72 \pm 0.24) \times 10^{10}$ $M^{-2}sec^{-1}$ over the same pH interval.

The rate constants k_1 and k_2 are plotted logarithmically *vs.* pH in Fig. 4-5 and 4-6, and listed in Table 4-1. The slope (obtained by least squares analysis) of the log k_2 *vs.* pH plot is -1.03 ± 0.10; the slope of the log k_1 *vs.* pH plot is -0.98 ± 0.04. The error in k_1 is greater than the error in k_2 because of the interference of the spontaneous rate of decay of LP-II at the low concentrations of iodide ion in plots such as Fig. 4-4.

A slight perturbation of the Soret spectrum of lactoperoxidase was observed when the concentration of iodide ion was in very large excess over that of lactoperoxidase. Fig. 4-7 shows a difference spectrum at pH 7 of LP + I^- *vs.* LP. The same difference spectrum is obtained at pH 4.6.

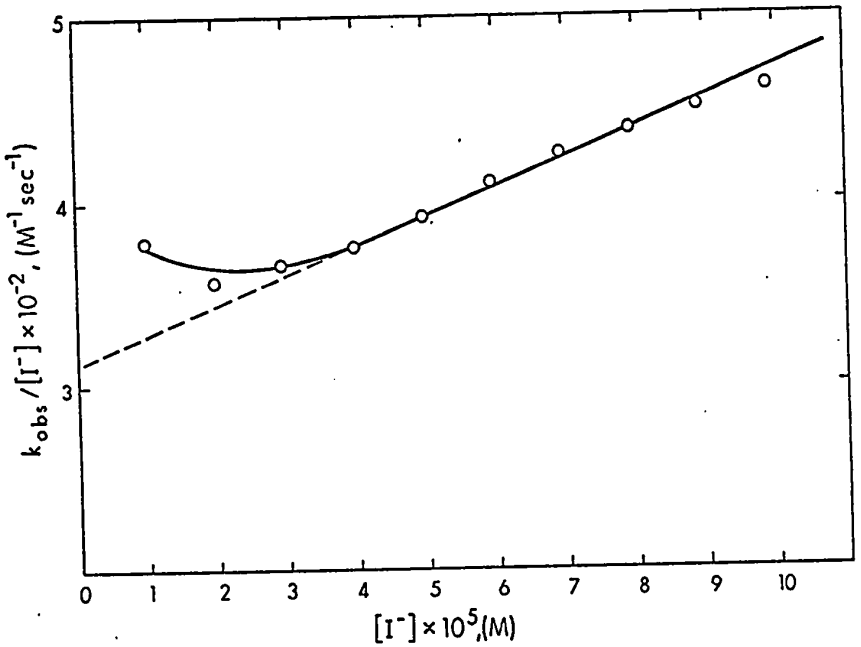


Fig. 4-4. A plot of $k_{obs}/[I^-]$ vs. $[I^-]$ at pH 6.95 for the LP-II—iodide reaction. The straight line was obtained by least squares analysis. The slope, k_2, with its standard deviation is $(1.48 \pm 0.18) \times 10^6$ $M^{-2}sec^{-1}$. The intercept, k_1, is $(3.19 \pm 0.29) \times 10^2$ $M^{-1}sec^{-1}$.

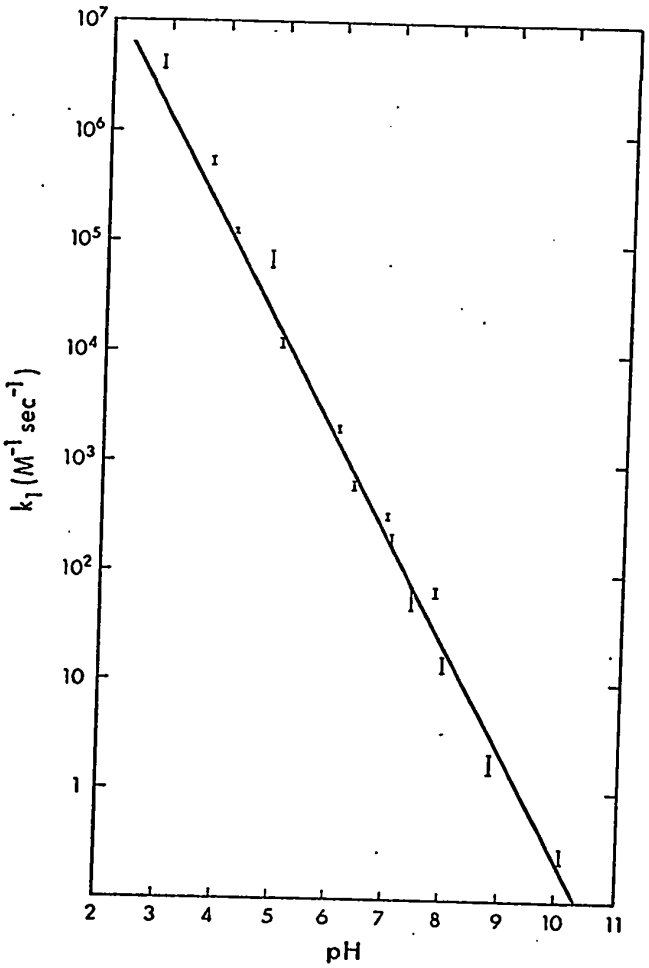


Fig. 4-5. A semilogarithmic plot of k_1 vs. pH for the reaction of LP-II with iodide ion. The error bars on the points are standard deviations obtained by analysis of plots such as Fig. 4-4 at each pH. The solid line is a least squares fit, with a slope of $-(0.98 \pm 0.04)$.

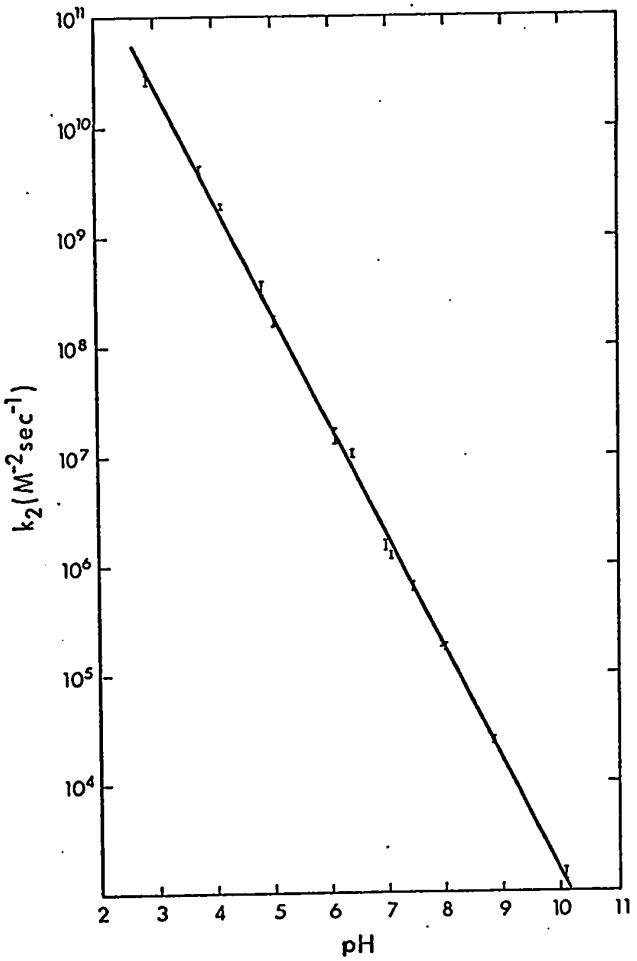


Fig. 4-6. A semilogarithmic plot of k_2 vs. pH for the reaction of LP-II with iodide ion. The error bars on the points are standard deviations obtained by analysis of plots such as Fig. 4-4 at each pH. The solid line is a least squares fit, with a slope of $-(1.03 \pm 0.10)$.

Table 4-1. Rate Constants with Standard Deviations for the LP-II-Iodide Reaction at 25.0° and Ionic Strength 0.05.

pH	$[I^-]$ (M)	k_1 ($M^{-1}sec^{-1}$)	k_2 ($M^{-2}sec^{-1}$)	Buffer[a]
2.86	$7 \times 10^{-6} - 2 \times 10^{-5}$	$(4.20 \pm 0.55) \times 10^6$	$(2.72 \pm 0.24) \times 10^{10}$	C
3.76	$1 \times 10^{-5} - 5 \times 10^{-5}$	$(5.36 \pm 0.48) \times 10^5$	$(4.15 \pm 0.37) \times 10^9$	C
4.17	$1 \times 10^{-5} - 5 \times 10^{-5}$	$(1.24 \pm 0.07) \times 10^5$	$(1.91 \pm 0.09) \times 10^9$	C
4.81	$2 \times 10^{-5} - 2 \times 10^{-4}$	$(7.02 \pm 1.19) \times 10^4$	$(3.42 \pm 0.51) \times 10^8$	C
5.01	$1 \times 10^{-5} - 8 \times 10^{-5}$	$(1.19 \pm 0.12) \times 10^4$	$(1.70 \pm 0.17) \times 10^8$	A
6.08	$7 \times 10^{-5} - 8 \times 10^{-4}$	$(2.03 \pm 0.14) \times 10^3$	$(1.50 \pm 0.24) \times 10^7$	P
6.35	$5 \times 10^{-5} - 5 \times 10^{-4}$	$(6.20 \pm 0.56) \times 10^2$	$(1.03 \pm 0.13) \times 10^7$	P
6.95	$4 \times 10^{-5} - 1 \times 10^{-4}$	$(3.19 \pm 0.29) \times 10^2$	$(1.48 \pm 0.18) \times 10^6$	P
7.04	$2 \times 10^{-4} - 5 \times 10^{-3}$	$(2.01 \pm 0.24) \times 10^2$	$(1.19 \pm 0.07) \times 10^6$	P
7.41	$5 \times 10^{-5} - 2 \times 10^{-4}$	$(5.99 \pm 1.44) \times 10^1$	$(6.21 \pm 0.62) \times 10^5$	T
7.94	$3 \times 10^{-4} - 7 \times 10^{-3}$	$(6.62 \pm 0.73) \times 10^1$	$(1.81 \pm 0.05) \times 10^5$	T
7.98	$3 \times 10^{-4} - 1 \times 10^{-3}$	$(1.42 \pm 0.21) \times 10^1$	$(1.69 \pm 0.13) \times 10^5$	T
8.81	$3 \times 10^{-4} - 8 \times 10^{-4}$	(1.82 ± 0.38)	$(2.39 \pm 0.05) \times 10^4$	T
10.07	$4 \times 10^{-4} - 4 \times 10^{-3}$	(0.26 ± 0.04)	$(1.41 \pm 0.17) \times 10^3$	Car

[a]Buffer key: C, citric acid-sodium citrate; A, acetic acid-sodium acetate; P, potassium dihydrogen phosphate-disodium hydrogen phosphate; T, tris(hydroxymethyl) aminomethane hydrochloride-tris(hydroxymethyl)aminomethane; Car, sodium bicarbonate-sodium carbonate.

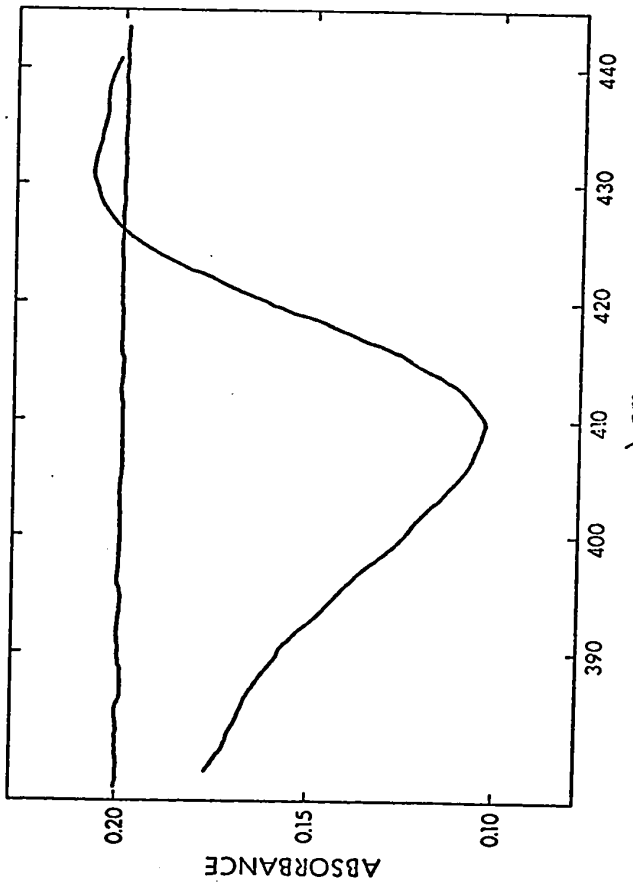


Fig. 4-7. A difference spectrum of LP plus iodide ion vs. LP at pH 7. There is a maximum at 431 nm and a minimum at 410 nm. The baseline is LP vs. LP balanced to 0.2 absorbance units. The concentrations of LP and iodide ion are 1.02×10^{-5} M and 1.06 M, respectively.

There is a maximum in the difference spectrum at 431 nm, and a minimum at 410 nm, an observation made previously by Morrison et al. (1970). It is not clear if there is binding of iodide ion to the iron of lactoperoxidase. Perhaps the perturbation of the Soret spectrum is due to a structural alteration induced by the high concentration of salt in the enzyme solution; we observed that nitrate ion at the same concentration produced the same sort of difference spectrum. In addition, we were unable to remove cyanide ion bound to LP (by observing a shift of the Soret maximum of LP-CN at 432 nm back to 412-413 nm) by adding iodide ion up to very high concentrations (ca. 0.25 M). In general hemoprotein halide complexes form more readily at low pH; however, our spectral results give no indication of such a trend for iodide and indeed provide little evidence for any binding of iodide. It can at least be said that if iodide ion binds to the iron of lactoperoxidase, the extent of binding is much less than that of fluoride (Segal et al., 1968) and cyanide (Dolman et al., 1968).

Discussion

The plots of log k_1 vs. pH and log k_2 vs. pH are linear with a slope of -1 within experimental error over the pH range of the study. This behavior can be explained by a kinetically important ionization on either the enzyme or substrate (Dixon and Webb, 1964) which is outside the pH range of this study to low pH. The pK_a of hydriodic acid

has been estimated to be about -9 (Bell, 1959). Using this value, it can be shown that the rate constant k_1 for the reaction of LP-II with HI would exceed the maximum value of the diffusion-controlled limit (10^{10} $M^{-1}sec^{-1}$) by about eight orders of magnitude. For example, if the system were

$$\begin{array}{ccccc} \text{LP-II} & + & \text{HI} & \xrightarrow{k_a} & \\ & & K_L \uparrow\downarrow & & \text{Products} \\ \text{LP-II} & + & I^- & \xrightarrow{k_b} & \end{array}$$

Then $$\frac{d[\text{Products}]}{dt} = k_a[\text{LP-II}][\text{HI}] + k_b[\text{LP-II}][I^-]$$

$$= \frac{k_a[\text{LP-II}][I^-]\text{total}}{1 + \frac{K_L}{[H^+]}} + \frac{k_b[\text{LP-II}][I^-]\text{total}}{1 + \frac{[H^+]}{K_L}} \quad \ldots(3)$$

which is to be compared with the experimentally determined equation

$$\frac{d[\text{Products}]}{dt} = k_1[\text{LP-II}][I^-]\text{total} \quad (4)$$

Therefore $$k_1 = \frac{k_a}{1 + \frac{K_L}{[H^+]}} + \frac{k_b}{1 + \frac{[H^+]}{K_L}} \quad (5)$$

If we suppose that HI is the reactive species, then $k_b = 0$, and

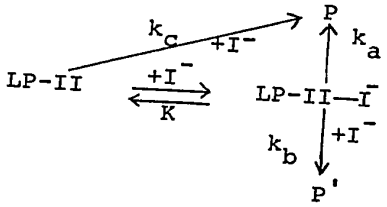
$$k_1 = \frac{k_a}{1 + \frac{K_L}{[H^+]}} \quad (6)$$

At pH 3, k_1 is about 10^6 $M^{-1}sec^{-1}$ and therefore

$$k_a = 10^6\left(1 + \frac{10^9}{10^{-3}}\right) = 10^{18} \quad (7)$$

which clearly exceeds the diffusion-controlled limit. The same is true at any pH value. Thus the kinetic results can be explained if LP-II exists in two forms with the protonated form reacting much more rapidly than the unprotonated form with iodide ion.

A simple mechanism which incorporates the effects of first and second order concentrations of iodide ion (but not the ionization in the enzyme) is:



where P and P' are the products.

$LP\text{-}II\text{-}I^-$ is a complex formed between LP-II and iodide ion, and K is its dissociation constant. One may be able to make some choice as to which steps are more important in the mechanism. From the work of Roman et al., (1971) it is known that HRP-II does not form a long-lived complex with iodide ion. However, the second order rate constant k_1 for the reaction of LP-II with iodide is larger by a factor of 12 over the comparable constant for HRP-II. This indicates greater efficiency in the LP-II oxidation and therefore favors the formation of the LP-II-iodide complex. The direct reaction, with rate constant k_c, cannot be excluded on the basis of a purely kinetic argument however. The reaction which is second order in iodide points clearly to the importance of a complex between LP-II and iodide ion.

The probability of termolecular collisions would appear too small to account for a significant proportion of the total rate; this possibility has not been shown in the mechanism. The alternative involves the reaction between iodide ion and the LP-II-iodide complex, an example of substrate activation.

It is possible to place limits on the value of the dissociation constant, K_a, of the kinetically important ionizing group on the enzyme. From the lack of curvature at low pH of the log k_1 *vs.* pH plot, an upper limit can be assigned of $pK_a < 2.8$. A lower limit of pK_a is obtained by extrapolation of the log k_1 *vs.* pH plot to the diffusion-controlled limit. Depending upon whether one uses 10^8 or 10^{10} $M^{-1}sec^{-1}$ for this diffusion-controlled rate (Alberty and Hammes, 1958) one obtains $pK_a > 1.2$ or $pK_a \geq -0.8$. This pK_a value lies outside the range of ionization constants for the common amino acids found in proteins, and may be due to the influence of the heme group on the ionization of an acid, or the ionizing group may be a component of the heme group. An acid group bound in the fifth or sixth coordination position of the heme iron appears an attractive possibility, but an acid group obtained from protonation of the porphyrin ring cannot be excluded.

The reaction of LP-II with iodide is similar to the HRP-II-iodide reaction in that the catalytic importance of a single acid group on the enzyme is clearly demonstrated in both reactions (Roman *et al.*, 1971). However, the

faster rate and the importance of a term second-order in iodide in the LP-II reaction, point clearly to the pitfalls if one attempts to extrapolate results from one enzymatic reaction to the analogous reaction for a related enzyme.

Chapter 5

The Kinetics of the Oxidation of p-Cresol by Lactoperoxidase Compound II

Introduction

As stated in previous chapters, lactoperoxidase catalyzes the oxidation of a wide variety of compounds by hydrogen peroxide according to the following generally agreed upon reaction scheme:

$$LP + H_2O_2 \longrightarrow LP\text{-}I$$

$$LP\text{-}I + AH_2 \longrightarrow LP\text{-}II + AH\cdot$$

$$LP\text{-}II + AH_2 \longrightarrow LP + AH\cdot$$

$$2AH\cdot \longrightarrow \text{Products}$$

where LP represents the native enzyme, LP-I and LP-II are the oxidized forms of the enzyme referred to as compounds I and II, respectively, and AH_2 is the oxidizable substrate.

Previous work on the kinetics of reactions of compound II has involved the oxidation of iodide ion (Maguire and Dunford, 1972), described in Chapter 4. The present study was undertaken with a view to determining the effect of a neutral molecule on the reduction of compound II. p-Cresol was chosen because of the remarkable efficiency of oxidation of phenols by peroxidase systems (Saunders et al., 1964), and because of its resemblance to the amino acid tyrosine, while having fewer acid-base groups. A peroxidase system has been suggested to be active in the thyroid gland

(Hosoya and Morrison, 1967a), which produces the hormone thyroxine by coupling iodinated tyrosine residues. This chapter describes in greater detail material submitted for publication.

Experimental

Lactoperoxidase (LP) was obtained from Calbiochem as a lyophilized powder, and purified by gel filtration at 4° on a Sephadex G-200 Superfine column with a pH 7.0 phosphate buffer as eluant. The ratio of the absorbances at 412 nm and 280 nm (P.N.) was never less than 0.8 for any enzyme sample used in the kinetic experiments. Solutions of the enzyme were stored in the cold at concentrations of about 10^{-4} M, and were diluted immediately before use. Enzyme concentrations were determined spectrophotometically at 412 nm, using a molar absorptivity of 1.14×10^5 $M^{-1}cm^{-1}$ (Morrison et al., 1957).

Practical grade p-cresol was obtained from Eastman Organic Chemicals, and was purified by sublimation.[1] Solutions of p-cresol were prepared by weighing and the concentrations checked spectrophotometrically at 277 nm. All other substances were of reagent grade and were used without further purification. Hydrogen peroxide, 30% by weight obtained from the Fisher Scientific Co., was stored

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[1]We are grateful to Dr. M. K. Evett for the purification of the p-cresol.

as a 5×10^{-2} M solution, and diluted before use; its concentration was checked periodically by the method of Ovenston and Rees (1950). Water was distilled from alkaline potassium permanganate and then redistilled.

Kinetic investigations were carried out at 25° using a stopped-flow apparatus essentially the same as that described previously (Hasinoff, 1970) except that a polypropylene cell was used instead of one constructed of plexiglass. In a typical experiment, a 2×10^{-6} M solution of LP in pure water was converted to compound II (LP-II) immediately before use by the addition of one equivalent of hydrogen peroxide. The compound I (LP-I) initially formed was converted by reducing impurities in the enzyme solution to LP-II almost immediately. This solution of LP-II was allowed to react in the stopped-flow apparatus with a solution of p-cresol in the appropriate buffer of ionic strength 0.1. The final reaction mixture had an ionic strength of 0.05. At high pH, the p-cresol made a contribution towards both the ionic strength and the buffering capacity, and the former was allowed for by suitable adjustment of the ionic strength of the buffer.

The reduction of LP-II to LP was monitored at 412 nm (the Soret band maximum of native lactoperoxidase) by following the amplified photomultiplier voltage *vs.* time trace on a 564B Tektronix storage oscilloscope. The data were recorded in the form of a four figure digital print-out at 30 equally spaced intervals of time, by using an

analog-to-digital converter. Pseudo first order conditions were achieved by keeping the concentration of p-cresol in at least a ten-fold excess over that of LP-II, which was below 10^{-6} M. The resulting absorbance changes were less than 0.04, and could hence be regarded as proportional to the observed voltage changes. An average rate constant with standard deviation was obtained from 5-10 traces for each set of conditions. After reaction the solutions were collected for pH measurement, which was carried out with an Orion digital pH meter in conjunction with a Fisher combination electrode.

The degree of dissociation of p-cresol at the same salt concentrations as were used in the kinetic experiments was measured spectrophotometrically, using a Cary 14 spectrophotometer with the cell compartments maintained at 25°. The pH was maintained with carbonate-bicarbonate and glycinate buffers, which were also added to the reference solution. Absorbance measurements were made at 295 nm, close to the maximum in the difference spectrum between the acidic and basic forms of p-cresol. Fifteen solutions were studied over the pH range 9.39-11.32, and absorbance measurements were also made for solutions 0.01 M in perchloric acid and sodium hydroxide.

Results

The aqueous solutions of p-cresol exhibited maximum molar absorptivities of 1.70×10^3 $M^{-1}cm^{-1}$ at 277 nm in

0.01 M perchloric acid and 2.54×10^3 $M^{-1}cm^{-1}$ at 295 nm in 0.01 M sodium hydroxide. These values compare favourably with the maximum values of 1.71×10^3 $M^{-1}cm^{-1}$ at 277 nm and 2.55×10^3 $M^{-1}cm^{-1}$ at 295 nm, respectively, found for a sample of 99.96% purity (Herington and Kynaston, 1957).

The effective pK_a of p-cresol under the conditions of the present work may be defined in terms of concentrations and the operational pH scale by the relation

$$pK_a = \log\frac{[AROH]}{[ARO^-]} + pH \qquad (1)$$

where AROH and ARO^- represent the protonated and unprotonated forms of p-cresol, respectively. Non-linear least squares analysis of the spectrophotometric data, using the measured value of 80 $M^{-1}cm^{-1}$ for the molar absorptivity of p-cresol at 295 nm in acid solution, yielded a pK_a value of 10.11 ± 0.01. The largest deviation from the best fit curve was of 4% absorbance, and the predicted molar absorptivity of the anion was 2.71×10^3 $M^{-1}cm^{-1}$ compared with 2.54×10^3 $M^{-1}cm^{-1}$ obtained by direct experimental measurement. This value of the pK_a is in agreement with the value 10.10 obtained by the use of Davies' equation (1938) together with the value at zero ionic strength found by Chen and Laidler (1962).

All reaction traces were simple exponential curves, showing the reaction to be kinetically first order in concentration of LP-II. The results were analyzed by a

non-linear least squares method on a computer with equal weighting of all voltage readings.

Over the entire pH region of this study, the observed first order rate constant depended on the concentration of p-cresol in a simple linear fashion:

$$k_{obs} = k[PC] \qquad (2)$$

where k_{obs} is the experimental first order rate constant, k is a second order rate constant, and [PC] is the total concentration of p-cresol. This is shown in Fig. 5-1, a plot to check the order of the reaction with respect to p-cresol at pH 7.87, from which k is $(1.17 \pm 0.04) \times 10^6$ $M^{-1}sec^{-1}$ and the intercept is 0.74 ± 1.13 sec^{-1}. The linearity of the plot confirms that the reaction is first order with respect to the concentration of p-cresol. The rate of spontaneous decay of LP-II was negligible compared to the fast rates observed in the presence of p-cresol. At pH values below 10, the range of concentration of p-cresol that could be employed was restricted to one order of magnitude; the lower limit was 10^{-5} M due to the restriction of observing pseudo first order conditions, and the upper limit was 10^{-4} M because the rates of reaction became too fast to study using the stopped-flow apparatus. Table 5-1 contains values of k (with standard deviation) as a function of pH over the entire pH range.

The plot of log k vs. pH in Fig. 5-2 is well defined, and can be seen with the aid of Dixon's rules (Dixon and

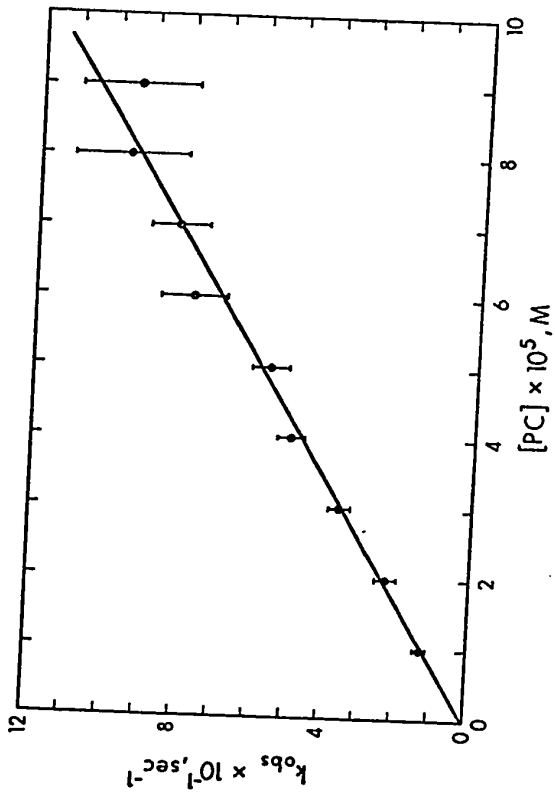


Fig. 5-1. A plot of k_{obs} vs. [PC] at pH 7.87 Tris. The data were treated by linear least squares analysis, which yielded the slope, k, equal to $(1.17 \pm 0.04) \times 10^6$ $M^{-1}sec^{-1}$, and the intercept equal to (0.74 ± 1.13) sec^{-1}, zero within experimental error.

Table 5-1. Values of k (with standard deviation) as a function of pH.

$k, M^{-1}sec^{-1}$	pH	Buffer[1]
$(9.48 \pm 0.78) \times 10^5$	2.08*	GH
$(1.67 \pm 0.22) \times 10^6$	2.53	GH
$(1.63 \pm 0.21) \times 10^6$	2.71	GH
$(1.94 \pm 0.25) \times 10^6$	2.93	F
$(2.32 \pm 0.64) \times 10^6$	2.96*	F
$(1.83 \pm 0.28) \times 10^6$	3.15	F
$(1.85 \pm 0.32) \times 10^6$	3.35	F
$(2.30 \pm 0.28) \times 10^6$	3.78	F
$(2.17 \pm 0.22) \times 10^6$	3.99	F
$(2.66 \pm 0.14) \times 10^6$	4.00*	F
$(2.27 \pm 0.14) \times 10^6$	4.22	F
$(2.40 \pm 0.23) \times 10^6$	4.52	F
$(2.13 \pm 0.40) \times 10^6$	4.75	F
$(2.09 \pm 0.13) \times 10^6$	4.96*	A
$(2.23 \pm 0.24) \times 10^6$	4.96	A
$(2.01 \pm 0.15) \times 10^6$	5.19	A
$(2.05 \pm 0.36) \times 10^6$	5.20	F
$(2.06 \pm 0.09) \times 10^6$	5.37	A
$(1.93 \pm 0.13) \times 10^6$	5.53	A
$(1.66 \pm 0.10) \times 10^6$	5.83	P
$(1.52 \pm 0.19) \times 10^6$	6.00	P
$(1.61 \pm 0.03) \times 10^6$	6.01*	P
$(1.56 \pm 0.10) \times 10^6$	6.21	P
$(1.31 \pm 0.28) \times 10^6$	6.38	P
$(1.47 \pm 0.16) \times 10^6$	6.62	P
$(1.38 \pm 0.11) \times 10^6$	6.79	P
$(1.28 \pm 0.03) \times 10^6$	6.97*	P
$(1.30 \pm 0.08) \times 10^6$	6.99	P
$(1.27 \pm 0.09) \times 10^6$	7.20	P
$(1.09 \pm 0.17) \times 10^6$	7.34	T

continued

Table 5-1 continued

$(1.09 \pm 0.15) \times 10^6$	7.61	T
$(9.00 \pm 1.89) \times 10^5$	7.81	T
$(1.17 \pm 0.04) \times 10^6$	7.87*	T
$(1.02 \pm 0.06) \times 10^6$	8.03	T
$(1.12 \pm 0.08) \times 10^6$	8.16	P
$(1.10 \pm 0.12) \times 10^6$	8.27	T
$(8.30 \pm 1.57) \times 10^5$	8.48	T
$(9.50 \pm 0.89) \times 10^5$	8.68	T
$(8.81 \pm 0.79) \times 10^5$	9.06	C
$(7.82 \pm 0.40) \times 10^5$	9.24	C
$(6.57 \pm 1.31) \times 10^5$	9.43	C
$(6.35 \pm 0.68) \times 10^5$	9.46*	C
$(5.40 \pm 0.25) \times 10^5$	9.59	C
$(4.65 \pm 0.25) \times 10^5$	9.69	G
$(3.37 \pm 0.06) \times 10^5$	9.87	G
$(3.17 \pm 0.16) \times 10^5$	9.90*	G
$(2.26 \pm 0.19) \times 10^5$	10.05	G
$(1.71 \pm 0.17) \times 10^5$	10.16	G
$(1.53 \pm 0.23) \times 10^5$	10.20	G
$(1.05 \pm 0.05) \times 10^5$	10.33	G
$(5.28 \pm 0.46) \times 10^4$	10.54	C
$(5.09 \pm 0.53) \times 10^4$	10.54	G
$(5.10 \pm 0.37) \times 10^4$	10.55	G
$(1.60 \pm 0.15) \times 10^4$	10.86	G
$(4.85 \pm 0.42) \times 10^3$	11.15*	C
$(4.26 \pm 0.58) \times 10^3$	11.18	G

[1]Buffer key: GH, glycine hydrochloride-glycine; F, formic acid-sodium formate; A, acetic acid-sodium acetate; P, potassium dihydrogen phosphate-disodium hydrogen phosphate; T, tris(hydroxymethyl)aminomethane hydrochloride-tris(hydroxymethyl)aminomethane; C, sodium bicarbonate-sodium carbonate; G, glycine-sodium glycinate.

*pH values marked by an asterisk are those at which order studies in [PC] were performed. All other rate constants are results of determinations at a single concentration of p-cresol.

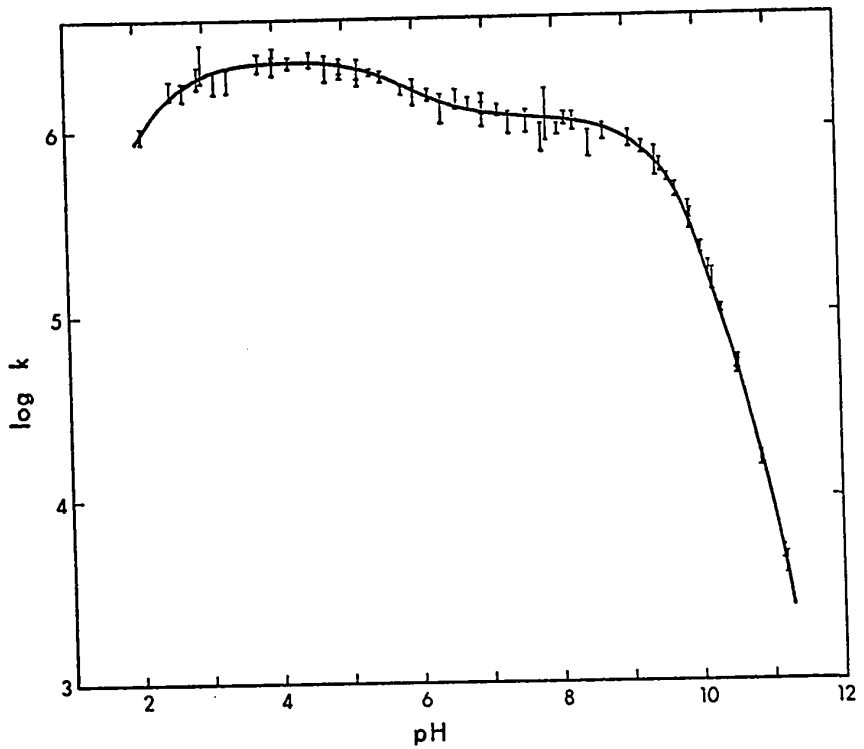
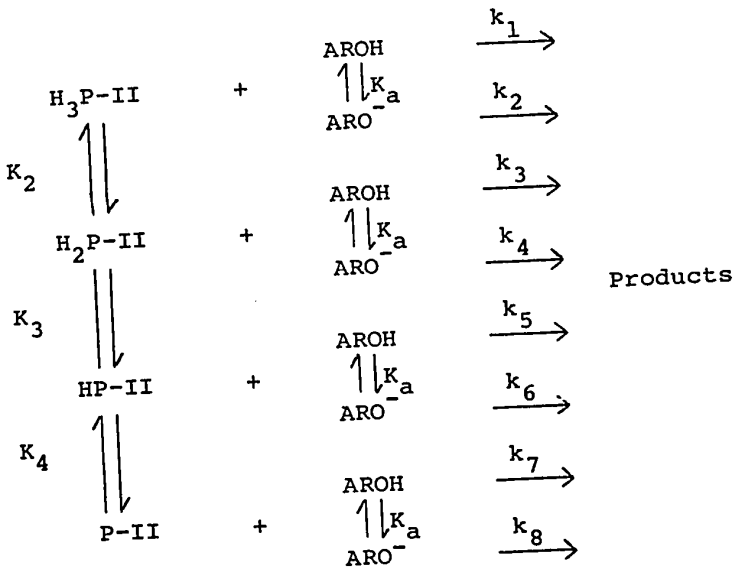


Fig. 5-2. A plot of log k vs. pH for the reaction between LP-II and p-cresol. The solid line is a curve calculated by computer for the fitting of the data to Eq. 3. The error bars represent standard deviations in the rate constant, k.

Webb, 1964) to depend on the acid dissociation of the substrate at pH 10 and the ionization of three kinetically important enzyme groups having pK_a values of around 2, 6, and 9.5. The minimum reaction scheme consistent with the data is given by Scheme I, in which P-II, HP-II, H_2P-II, and H_3P-II are the four kinetically distinct states of protonation of compound II, the K's are acid dissociation constants, and the k's are bimolecular rate constants:

Scheme I

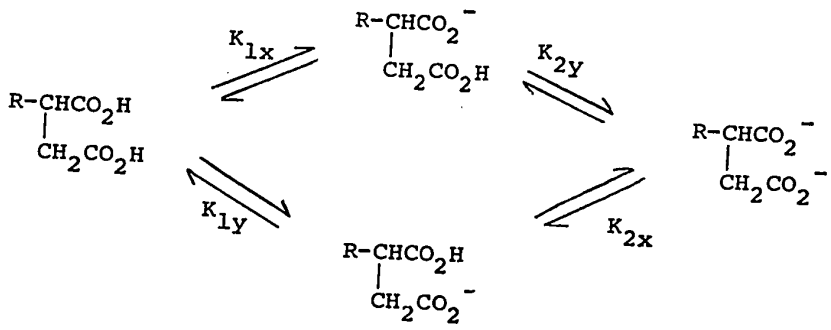


The vertical arrows denote fast non-rate determining proton transfers. The corresponding kinetic expression is

$$k = \left[\frac{k_1[H^+]^4}{K_aK_2K_3K_4} + \left(k_3 + \frac{k_2K_a}{K_2}\right)\frac{[H^+]^3}{K_aK_3K_4} + \left(k_5 + \frac{k_4K_a}{K_3}\right)\frac{[H^+]^2}{K_aK_4} + \left(k_7 + \frac{k_6K_a}{K_4}\right)\frac{[H^+]}{K_a} + k_8\right] \Bigg/ \left(1 + \frac{[H^+]}{K_4} + \frac{[H^+]^2}{K_3K_4} + \frac{[H^+]^3}{K_2K_3K_4}\right)\left(1 + \frac{[H+]}{K_a}\right)$$

.....(3)

In a reaction involving two or more ionizations such as that depicted by Scheme I, molecular rather than group ionizations are necessarily implied (Dixon and Webb, 1964). Group ionization constants, of which the molecular ionization constants consist, cannot be determined from the analysis of rate data alone. If the pK values of the group ionization constants are widely separated, the ionization constants measured indicate the true value of the group ionization constants. The example of the unsymmetrical dibasic acid given by Dixon and Webb (1964) illustrates clearly the distinction between group and molecular ionization constants:



Each group may ionize either from the undissociated molecule or from the anion, in other words in the first or second stage; we therefore need four ionization constants to represent the ionization of an unsymmetrical dibasic acid completely. The relationship between the molecular ionization constants K_1 and K_2, and the group ionization constants K_{1x}, K_{2x}, K_{1y}, K_{2y} is given by the following expressions:

$$K_1 = K_{1x} + K_{1y} \tag{4}$$

$$K_2 = \frac{K_{2x}K_{2y}}{K_{2x} + K_{2y}} \tag{5}$$

Two pathways of Scheme I such as

$$H_3P\text{-II} + ARO^- \xrightarrow{k_2} \tag{6}$$

and

$$H_2P\text{-II} + AROH \xrightarrow{k_3} \tag{7}$$

are kinetically indistinguishable and are combined together in one term of the expression relating k to the specific rate constants, ionization constants, and hydrogen ion concentrations. The only unambiguous rate constants obtainable from Eq. 3 are k_1 and k_8. The differential rate law for the reaction represented by k_2 is

$$-\frac{d[H_3P\text{-II}]}{dt} = k_2[H_3P\text{-II}][ARO^-] \tag{8}$$

and for the reaction represented by k_3 is

$$-\frac{d[H_2P\text{-}II]}{dt} = k_3[H_2P\text{-}II][AROH] \quad (9)$$

$$\text{but } [H_2P\text{-}II] = K_2\frac{[H_3P\text{-}II]}{[H^+]} \quad (10)$$

$$\text{and } [AROH] = \frac{[H^+][ARO^-]}{K_a} \quad (11)$$

Equations (10) and (11) substituted in equation (9) give

$$-\frac{d[H_2P\text{-}II]}{dt} = \frac{k_3K_2}{K_a}[H_3P\text{-}II][ARO^-] \quad (12)$$

which when compared with Eq. (8) is seen to be of identical form. Hence the two reactions (6) and (7) are kinetically indistinguishable.

The k vs. pH data were analyzed using a non-linear least squares program (described in Appendix 2). The smooth curve in Fig. 5-2 shows the best fit to Eq. 3, and is defined by the first column of parameters in Table 5-2.

An alternative and algebraically more simple method of analyzing the log k vs. pH plot which avoids the need to consider kinetically indistinguishable or insignificant rate processes is provided by the use of transition state acid dissociation constants. As is shown elsewhere (Critchlow and Dunford, submitted for publication), an acid dissociation in the transition state gives rise to an increase in slope in the plot of log k vs. pH, whereas an acid dissociation in the ground state causes a decrease in slope. The number and approximate pK_a values of such ionizations may therefore

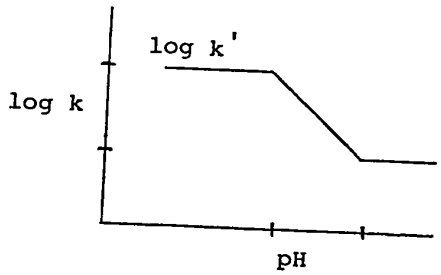
Table 5-2. Parameter values[a] and standard deviations for the variation of k with pH, obtained by non-linear least squares fit of data to Eqs. (3) and (15).

	Equation 3 pK_a variable	Equation 15 pK_a variable	Equation 15 pK_a invariable
k_1	0[b]		
$(k_3 + \frac{k_2K_a}{K_2})$	$(2.30\pm0.05)\times10^6$		
$(k_5 + \frac{k_4K_a}{K_3})$	$(1.09\pm0.03)\times10^6$		
$(k_7 + \frac{k_6K_a}{K_4})$	0[b]		
k_8	0[b]		
pK_2	2.3±0.1	2.3±0.1	2.2±0.1
pK_3	5.8±0.1	5.8±0.1	5.9±0.1
pK_4	9.7±0.1	9.7±0.1	9.8±0.1
pK_a	10.3±0.1	10.3±0.1	10.11
$pK_1^{\pm}$		6.1±0.1	6.2±0.1
k'		$(2.39\pm0.05)\times10^6$	$(2.37\pm0.05)\times10^6$

[a]Rate constants in units of $M^{-1}sec^{-1}$.

[b]Zero within experimental error.

be read off the plot by an extension of Dixon's rules (Dixon and Webb, 1964) in the same way as the pK values of the enzyme or enzyme-substrate complex. The essential significance of this treatment may be arrived at more simply by considering the experimental log k—pH profile in a less complicated plot, as in



The change of slope on passing from the horizontal portion of the plot at high pH to the portion of unit negative slope must be caused by a change in the reaction route from one involving a given set of reactants to one involving a different set containing a total of one more proton. The transition state at medium (and low) pH therefore contains one more proton than that for reaction at high pH, and the point at which the curve begins to rise in going to lower pH may be associated formally with a transition state acid dissociation. The subsequent return to a horizontal relationship at lower pH is of course due to the protonation of the ground state and is covered by Dixon's rules (Dixon and Webb, 1964).

The use of this treatment in a description of the mechanism of a reaction requires that each transition-state acid dissociation constant ($pK_a^{\ddagger}$) be paired with that for the ground-state dissociation (pK_a) considered to involve the same ionizing group, although both members of each pair may not be found in the pH range of the study. The pK difference between the ground- and transition-states, which may be regarded as expressing the sensitivity of the rate to the ionization in question, provides a qualitative measure of the change in environment accompanying the activation process. For example, if protonation of a particular protein residue facilitates a reaction, then $pK_a^{\ddagger} > pK_a$, as is the case with the simple reaction considered above. Conversely, if $pK_a^{\ddagger} < pK_a$, then protonation of the residue will retard the rate of the reaction.

In the actual derivation of an equation describing the observed rate constant as a function of pH, use is made of dimensionless Michaelis functions (Dixon and Webb, 1964), and the final result is (Critchlow and Dunford, submitted for publication):

$$k_{obs} = \frac{k' f_n^{\ddagger}}{f_i^E f_j^A f_k^B \ldots f_z^Z} \qquad (13)$$

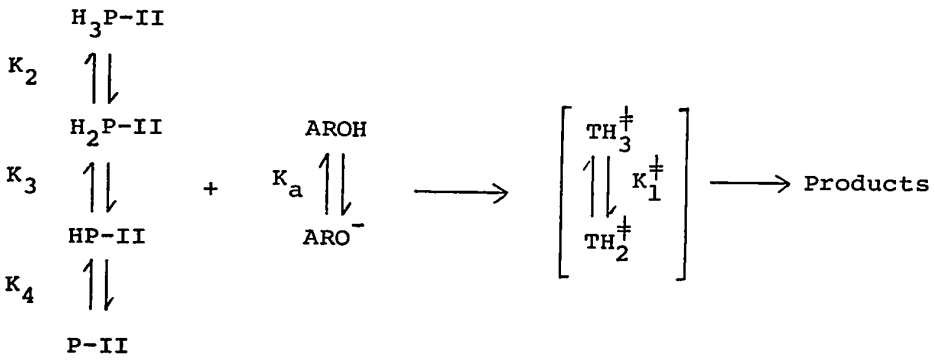
where k' is a pH-independent rate constant, the f's are Michaelis functions, E is the enzyme, A, B, ... Z are reactants, and the subscripts refer to particular states

of ionization of the enzyme, substrates, and transition state. For example, the corresponding equation for the simple case shown above is

$$k_{obs} = k' \frac{(1 + K^{\ddagger}/[H^+])}{(1 + K_a/[H^+])} \qquad (14)$$

For the oxidation of p-cresol by LP-II, consideration of Fig. 5-2 shows a single kinetically important transition state ionization of $pK_a^{\ddagger}$ value about 6, so that the reaction may be represented by Scheme II,

Scheme II



where the species in square brackets represent transition state complexes in different states of protonation, $K_1^{\ddagger}$ is the associated transition state acid dissociation constant, and the other symbols have been defined for Scheme I. The resulting kinetic expression is

$$k = k' \frac{1 + K_1^{\ddagger} / [H^+]}{\left(\frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]} + \frac{K_3 K_4}{[H^+]^2}\right)\left(1 + \frac{K_a}{[H^+]}\right)} \qquad (15)$$

where k' is the pH-independent rate constant associated with the pH-independent part of the curve between pH 3 and pH 5. The significance and advantages of this style of treatment of pH-rate effects in terms of transition state acid dissociation constants are discussed in more detail elsewhere (Critchlow and Dunford, submitted for publication), but the readily apparent advantage of simplicity can be seen by comparing Eqs. 3 and 15.

Non-linear least squares treatment of the data using Eq. 15 resulted in the same smooth curve in Fig. 5-2. The best fit values of the parameters, with pK values identical to those obtained by the former treatment, are given by the second column of parameter values in Table 5-2. The value of pK_a for p-cresol obtained kinetically is different from the spectrophotometric result mentioned previously; however, a second analysis with a fixed value of $pK_a = 10.11$ produced only small changes in the other parameters, as shown in the third column of Table 5-2. A good fit to the experimental data was still obtained, so that the difference in pK_a values may not be significant.

Discussion

A number of conclusions may be drawn from the nature

of the pH-rate profile for the oxidation of p-cresol by compound II of lactoperoxidase. Critchlow and Dunford (submitted for publication) have performed an analogous study of the oxidation of p-cresol by compound II of horseradish peroxidase (HRP-II), and they obtained a pH-rate profile very similar to that obtained in this study. They also obtained evidence for an acid group on the enzyme with a pK_a value of 2.3. As they have suggested, the decrease in rate associated with this pK_a may arise from an initial step in the acid catalyzed splitting of the porphyrin ring from the apoprotein, perhaps by protonation of the acceptor atom of a hydrogen bond involved in the heme-protein stabilizing linkage. The pK_a of LP-II at pH 5.8 is notable for its closeness to the transition state $pK_1^{\ddagger}$ at pH 6.1, which may mean protonation of the same group; if such is the case, protonation of this group would then exert only a slight accelerating influence on the rate of reaction, indicating that the group is positioned close to the active center of reaction, but plays little part in the catalytic process.

It is also possible to draw some conclusions about the nature of the reactive form of p-cresol. Inspection of the pH-rate profile shows that if p-cresol were reacting in the anionic form, the second order rate constant would exceed the diffusion controlled limit (10^{10} $M^{-1}sec^{-1}$ or less for an enzyme-substrate system in which, also, the direction of approach of the substrate molecule is probably important) below pH 6.5. Thus the $pK_1^{\ddagger}$ at pH 6.1 cannot be paired to

the pK_a of p-cresol at pH 10.3 and therefore the transition state $pK^{\ddagger}$ pertaining to the protonation of p-cresol must occur outside the experimentally covered pH range to high pH. This indicates that the acid form of the substrate is the reactive species over the entire pH range covered. The same conclusion is reached when Scheme I is used. In order to decide which of the kinetically equivalent pathways included in each of the two non-zero rate terms of Eq. 3 in Table 5-2 is responsible for the reaction it is necessary to make use of the maximum value which can be placed on the rate of a diffusion controlled process. Since $K_a/K_2 = 10^{-8}$ and $K_a/K_3 = 3 \times 10^{-5}$, it follows that even for the maximum diffusion controlled limit (10^{10} $M^{-1}sec^{-1}$) the predominant pathways are those described by k_3 and k_5. Thus the protonated forms of compound II would appear to react with the neutral form of the substrate.

It is possible that the transition state $pK_1^{\ddagger}$ at pH 6.1 could be paired to any of the three enzyme ground state pK's; however, if the 5.8 - 6.1 pairing scheme is accepted, the pK at 9.7 can only be paired (again by using the concept of a second order rate constant exceeding the diffusion controlled limit) with a transition state $pK^{\ddagger}$ lying outside the observed pH range at high pH. This is equivalent to saying that the group of pK 9.7 must be present in its acid form for reaction to occur. The value of $pK_4 = 9.7$ is notable in that it is 1 pH unit higher than the value of 8.6 obtained from the studies of the reaction of HRP-II with

ferrocyanide (Hasinoff and Dunford, 1970) and p-cresol (Critchlow and Dunford, submitted for publication), which may indicate that the identity of the ionizing group is different, or that its ionization constant is shifted through some sort of interaction such as hydrogen bonding in the acid form. In spite of this, the similarity of the pH-rate profiles for the oxidation of p-cresol by LP-II and HRP-II gives some indication that the mechanisms of reaction in both cases are the same, except for the unproductive binding observed in the latter case. This work can also be compared to the reaction between LP-II and iodide ion, in which the second order rate constant varies with unit negative slope over the experimental pH range, from 4.2×10^{6} $M^{-1}sec^{-1}$ at pH 2.9 to 2.6×10^{-1} $M^{-1}sec^{-1}$ at pH 10.1 (Maguire and Dunford, 1972). Thus the second order rate constant for p-cresol oxidation by LP-II is appreciably greater than the rate constant for iodide oxidation over most of the pH range, except for the low pH region. The radical difference in the pH-rate profiles for these two substrates (three enzyme pK_a's observed in the LP-II—p-cresol reaction, none in the pH range 2.9 - 10.1 observed for the LP-II—iodide reaction) may be a result of the lack of steric interactions between iodide ion and LP-II, or it may be the result of the operation of a different mechanism. Since p-cresol reacts in its unionized form, electrostatic interactions cannot be a factor in its reaction.

Chapter 6

The Rate of Exchange of Water with Iron of Horseradish Peroxidase Detected by O^{17} Nuclear Magnetic Resonance

Introduction

Horseradish peroxidase is an enzyme which catalyzes oxidations in the presence of hydrogen peroxide and has been given the following designation by the Enzyme Commission of the International Union of Biochemistry (1961): 1.11.1.7 Donor: H_2O_2 oxidoreductase. The first isolation of horseradish peroxidase (HRP), in partially purified form, was carried out by Bach and Chodat (1903). It was crystallized by Theorell (1942) using electrophoretic techniques. HRP is now available commercially in various degrees of purity from a number of firms. HRP has a molecular weight of 40,200 (Maehly, 1955), and it has been found that about 18% by weight of HRP is carbohydrate material (Shannon et al., 1966; Theorell and Akeson, 1942), although the nature of the linkage to the protein is not known. HRP, like hemoglobin myoglobin, and catalase, contains as a prosthetic group protoporphyrin IX, shown in Figure 1-1a of Chapter 1. The hematin nature of HRP was established by Keilin and Mann (1937), and the importance of the heme group was demonstrated by Theorell (1940) who found that neither the protein nor the prosthetic group independently has any appreciable peroxidatic activity. In its natural form, the iron of the heme group of

HRP is in the ferric state. To date there has been no X-ray crystallographic work reported for HRP, so evidence regarding the nature of the heme-protein forces is mostly based on a comparison of the properties of HRP with those of hemoglobin and myoglobin. There has been a considerable amount of speculation in regard to the identity of the ligands occupying the fifth and sixth coordination positions on the heme iron of HRP. It is generally agreed that a water molecule occupies the sixth coordination position (Brill and Williams, 1961; Brill, 1966; Nicholls, 1962).

Nuclear magnetic resonance (NMR) methods have been extensively applied to the investigation of biological systems (Kowalsky and Cohn, 1964). High frequency NMR (Kowalsky, 1962), relaxation time measurements (Jardetsky, 1964), and halide ion probe techniques (Stengle and Baldeschweiler, 1966; Marshall, 1968) have been used to determine the structure, conformation, and motion of enzymes and proteins in solution. While interpretation of the NMR spectrum of a macromolecule is limited by its inherent complexity, much information can be obtained from the NMR spectrum of a small molecule that is able to probe the environment of the macromolecule by rapid exchange between free solution and attachment to the macromolecule. This study was undertaken in an attempt to determine the exchange rate and activation parameters involved in the interaction of the ferric iron of the heme group of HRP with water.

Let us consider the Bloch equations in the rotating frame of coordinates (Pople et al., 1959):

$$\frac{du}{dt} + \frac{u}{T_2} + (\omega_0-\omega)v = 0 \qquad (1)$$

$$\frac{dv}{dt} + \frac{v}{T_2} - (\omega_0-\omega)u = -\gamma H_1 M_0 \qquad (2)$$

If a complex moment of magnetization, G, is defined as

$$G = u + iv \qquad (3)$$

then

$$\frac{dG}{dt} = \frac{du}{dt} + i\frac{dv}{dt} \qquad (4)$$

or

$$\frac{dG}{dt} + \left[\frac{1}{T_2} - i(\omega_0-\omega)\right]G = -i\gamma H_1 M_0 \qquad (5)$$

If exchange of a nucleus between two sites A and B is allowed for, then

$$\frac{dG_A}{dt} + \left[\frac{1}{T_{2A}} - i(\omega_{0A}-\omega)\right]G_A = -\frac{G_A}{\tau_A} + \frac{G_B}{\tau_B} - i\gamma H_1 M_{0A}$$

$$\ldots(6)$$

$$\frac{dG_B}{dt} + \left[\frac{1}{T_{2B}} - i(\omega_{0B}-\omega)\right]G_B = -\frac{G_B}{\tau_B} + \frac{G_A}{\tau_A} - i\gamma H_1 M_{0B}$$

$$\ldots(7)$$

where τ_A and τ_B are the lifetimes of a nucleus in each environment.

If a steady state is assumed in which $\frac{dG_A}{dt} = \frac{dG_B}{dt} = 0$, and if it is assumed that A is the dominant site and that the contribution M_{0B} is negligible (this could correspond to water exchange between bulk solvent (site A) and a paramagnetic ion (site B)), then

$$G = \frac{i\gamma H_1 M_{0A}}{\lambda_A - \frac{\lambda_B}{\tau_A\tau_B(\lambda_B^2 + \Delta\omega_B^2)} - i\left(\Delta\omega_A + \frac{\Delta\omega_B}{\tau_A\tau_B(\lambda_B^2 + \Delta\omega_B^2)}\right)} \tag{8}$$

where

$$\lambda_A = \frac{1}{T_{2A}} + \frac{1}{\tau_B} \qquad \lambda_B = \frac{1}{T_{2B}} + \frac{1}{\tau_B}$$

$$\Delta\omega_A = \omega_{0A} - \omega \qquad \Delta\omega_B = \omega_{0B} - \omega$$

This derivation follows that of Swift and Connick (1962) for two sites.

When this equation is compared to that representing no exchange, and when respective real and imaginary parts are equated, the following relations result:

$$\frac{1}{T_{2OBS}} = \lambda_A - \frac{\lambda_B}{\tau_A\tau_B(\lambda_B^2 + \Delta\omega_B^2)} \tag{9}$$

$$\Delta\omega_{OBS} = \Delta\omega_A + \frac{\Delta\omega_B}{\tau_A\tau_B(\lambda_B^2 + \Delta\omega_B^2)} \tag{10}$$

An experimental situation is depicted in Figure 6-1 in which, say, the addition of a paramagnetic ion to water results in a frequency shift of the solvent peak. The half-width at half-height of the broadened peak is equal to $1/T_2$, and $1/T_{2A}$ is equal to the half-width at half-height of the original peak.

If we let

$$\frac{1}{T_{2p}} = \frac{1}{T_{2OBS}} - \frac{1}{T_{2A}} \tag{11}$$

then

$$\frac{1}{T_{2p}} = \frac{1}{\tau_A}\left[\frac{(T_{2B})^{-2} + (T_{2B}\tau_B)^{-1} + \Delta\omega_B{}^2}{\left((T_{2B})^{-1} + (\tau_B)^{-1}\right)^2 + \Delta\omega_B{}^2}\right] \tag{12}$$

The temperature effects predicted by equation (12) were investigated by Swift and Connick (1962), who considered the following limiting cases:

Case A

$$\Delta\omega_B{}^2 >> \frac{1}{T_{2B}{}^2}, \frac{1}{\tau_B{}^2} \tag{13}$$

then

$$\frac{1}{T_{2p}} = \frac{P_M}{\tau_B} \tag{14}$$

Relaxation occurs through a change in the precessional frequency and is rapid; $\frac{1}{T_{2p}}$ is controlled by the rate of chemical exchange. P_M is given by $\frac{n[M]}{55.5}$, where n is the

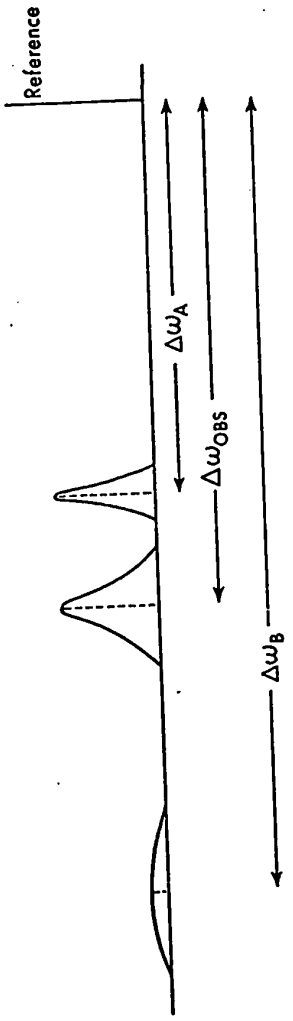


Fig. 6-1. A schematic diagram of the effect of a paramagnetic ion on H_2O^{17} resonance.

number of coordinated water molecules per ion and [M] is the molar concentration of the paramagnetic ion.

Case B

$$\frac{1}{\tau_B^2} >> \Delta\omega_B^2 >> \frac{1}{T_{2B}\tau_B} \tag{15}$$

then

$$\frac{1}{T_{2p}} = P_M \tau_B \Delta\omega_B^2 \tag{16}$$

Chemical exchange is rapid; $\frac{1}{T_{2p}}$ is controlled by the rate of relaxation through the precessional frequency.

Case C

$$\frac{1}{T_{2B}^2} >> \Delta\omega_B^2, \frac{1}{\tau_B^2} \tag{17}$$

then

$$\frac{1}{T_{2p}} = \frac{P_M}{\tau_B} \tag{18}$$

Relaxation by T_{2B} is fast; $\frac{1}{T_{2p}}$ is controlled by the rate of chemical exchange.

Case D

$$\frac{1}{T_{2B}\tau_B} >> \frac{1}{T_{2B}^2}, \Delta\omega_B^2 \tag{19}$$

then

$$\frac{1}{T_{2p}} = \frac{P_M}{T_{2B}} \tag{20}$$

Chemical exchange is rapid; $\frac{1}{T_{2p}}$ is controlled by the T_{2B} relaxation process.

The temperature dependence of τ_B is given by

$$\tau_B = \left(\frac{kT}{h}\right)^{-1} \exp\left(\frac{\Delta H^{\ddagger}}{RT} - \frac{\Delta S^{\ddagger}}{R}\right) \qquad (21)$$

$$\tau_B = \frac{1}{k_1} \qquad (22)$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation for the first order reaction of exchange of water from a cation, and k_1 is the rate constant associated with such a process.

The temperature effects predicted by the preceding four cases are illustrated schematically in Figure 6-2 as log $\left(\frac{1}{T_{2p}}\right)$ as a function of $\frac{1}{T°K}$. From consideration of experimental data plotted in this way, one may be able to determine which particular "kinetic region" describes the experimental system, and thus it may be possible to calculate τ_B, hence the rate constant for exchange of water molecules with the ferric iron of horseradish peroxidase.

Experimental Methods

H_2O^{17} containing 4.56 atom % O^{17} was obtained from Miles Laboratories. HRP was obtained from the Boehringer-Mannheim Corporation with a P.N. of 0.6 (for HRP, P.N. is the ratio of the absorbance at 403 nm to that at 280 nm). The enzyme was purified to a P.N. of 2.8 by gel filtration (on Sephadex G-200 Superfine, using pH 7.0 phosphate buffer of ionic strength 0.05 at 4°), and then dialysis against doubly distilled water, followed by lyophilization. For HRP, samples exhibiting P.N. of 3.0 are commonly considered pure.

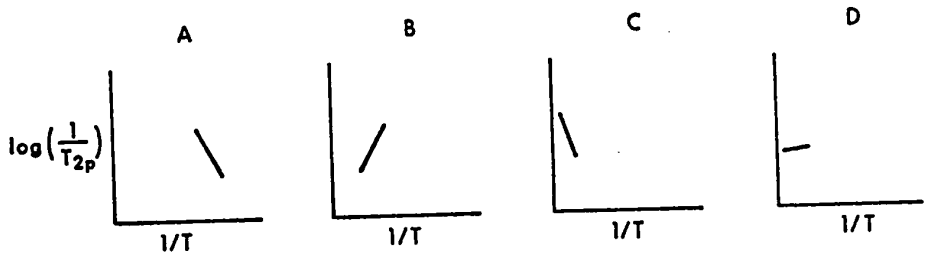


Fig. 6-2. Schematic plots of log $(1/T_{2p})$ vs. 1/T°K for the four "kinetic cases" considered by Swift and Connick (1962).

Solutions for NMR analysis were prepared by dissolving the lyophilized material in the H_2O^{17}.

HRP solutions (with an external reference of H_2O^{17}) at six pH values from 5.6 to 10.0 were examined by NMR at temperatures from 4° to 55°. The sample volume was about 2.5 ml. A typical HRP concentration was 2.20×10^{-4} M, as determined spectrophotometrically (Cary 14) with an extinction coefficient of 9.1×10^4 $M^{-1}cm^{-1}$ at 403 nm (Keilin and Hartree, 1951).

The NMR spectra were recorded on a Varian model HA-60I spectrometer equipped with a Hewlett-Packard 5100 B Frequency Synthesizer and a standard variable temperature probe and temperature controller. The temperature of the sample was controlled by a stream of nitrogen, previously heated or cooled, and calibrated with a thermocouple using ice-water as a reference. Operating conditions were 14,000 gauss and 8.116 MHz. Resonance was recorded as the derivative of the absorption mode (the separation of the two peaks of the derivative curve is $2/\sqrt{3}T_2$), and linewidth calibrations were made with the frequency synthesizer.

An H_2O^{17} reference solution was used before and after each sample measurement at each temperature. There was no detectable shift in the resonance line due to the paramagnetic ion introduced. Table 6-1 contains values of $1/T_{2p}'$ ($1/T_{2p}' = 1/T_{2p} \times 1/[\text{metal ion}]$ at six different values of pH and eight to ten temperatures. Figures 6-3 to 6-8 are linear least squares plots of log ($1/T_{2p}'$) vs. 1/T°K for each pH value. The estimated error limit per point is ±10%.

Table 6-1

$(1/T_{2p}')$ vs. T°C

pH 10.15		pH 8.87		pH 7.92	
T°C	$(1/T_{2p}')\times10^{-5}$	T°C	$(1/T_{2p}')\times10^{-5}$	T°C	$(1/T_{2p}')\times10^{-5}$
4	2.23	4	1.71	4	2.05
15	0.80	15	1.35	15	1.98
20	0.54	20	0.95	20	1.82
25	0.74	25	1.05	25	2.04
30	0.81	30	1.05	30	2.02
32	0.85	32	0.98	32	1.95
34.5	0.83	34.5	1.23	34.5	1.97
39	0.66	39	0.85	39	1.71
44.5	0.59	44.5	0.74	44.5	1.79
50	0.64	50	0.76	50	1.53

pH 6.85		pH 5.94		pH 5.61	
T°C	$(1/T_{2p}')\times10^{-6}$	T°C	$(1/T_{2p}')\times10^{-6}$	T°C	$(1/T_{2p}')\times10^{-6}$
4	0.88	4	0.41	4	0.79
15	0.50	15	0.26	15	0.54
20	0.42	20	0.17	20	0.45
25	0.33	25	0.15	25	0.48
30	0.32	30	0.15	32	0.32
32	0.29	32	0.15	39	0.23
34.5	0.29	34.5	0.16	44.5	0.15
39	0.28	39	0.14	50	0.11
44.5	0.27	44.5	0.14		
50	0.22	50	0.15		

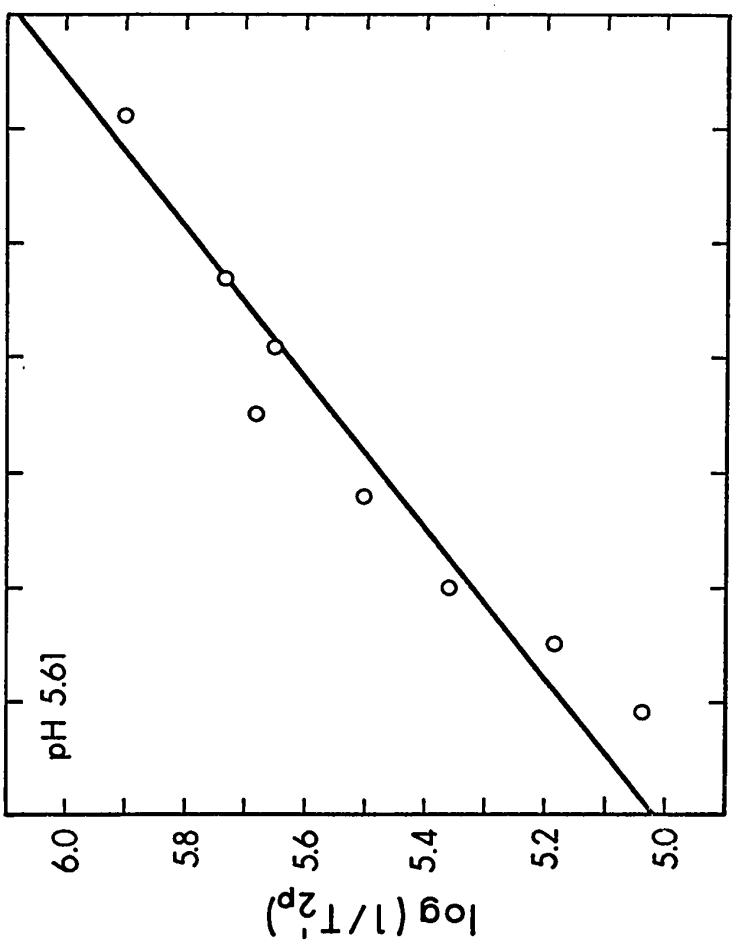


Fig. 6-3: log $(1/T_{2p}')$ vs. $1/T°K$ at pH 5.61.

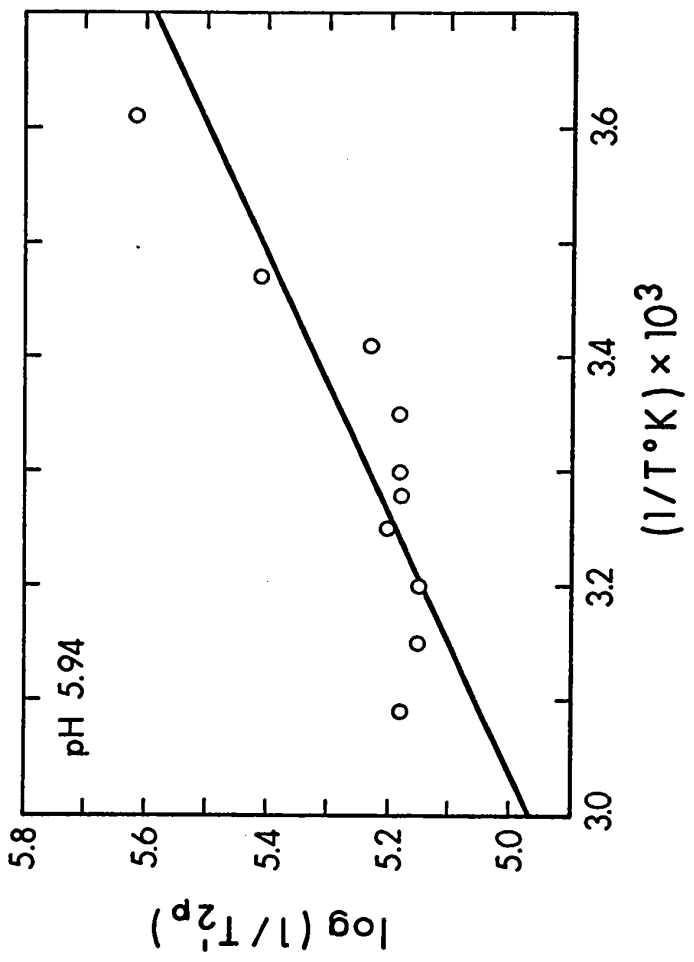


Fig. 6-4: log $(1/T_{2p}')$ vs. 1/T°K at pH 5.94.

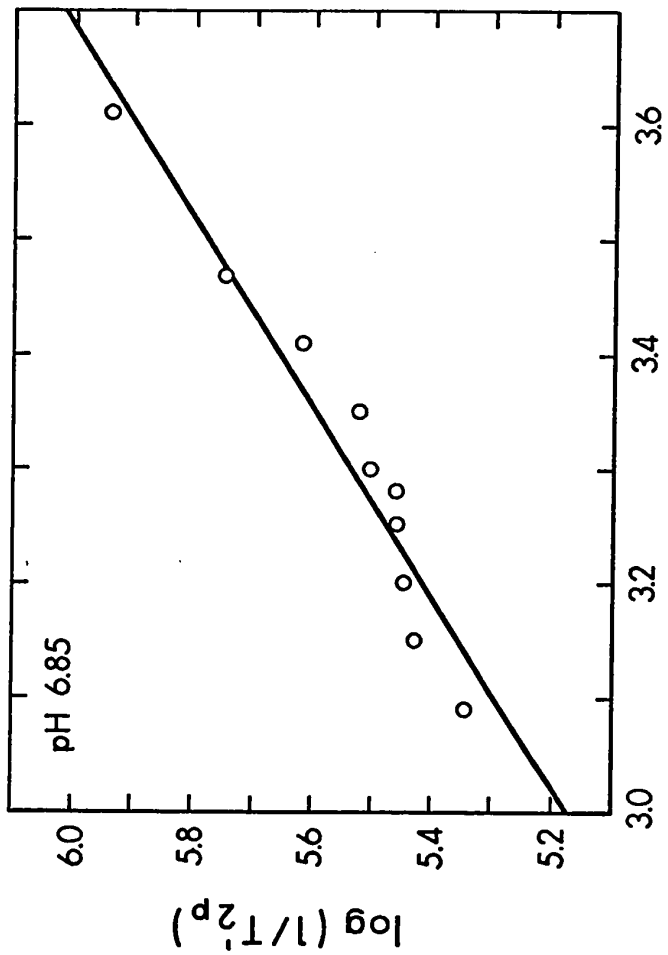


Fig. 6-5: log $(1/T_{2p}')$ vs. 1/T°K at pH 6.85.

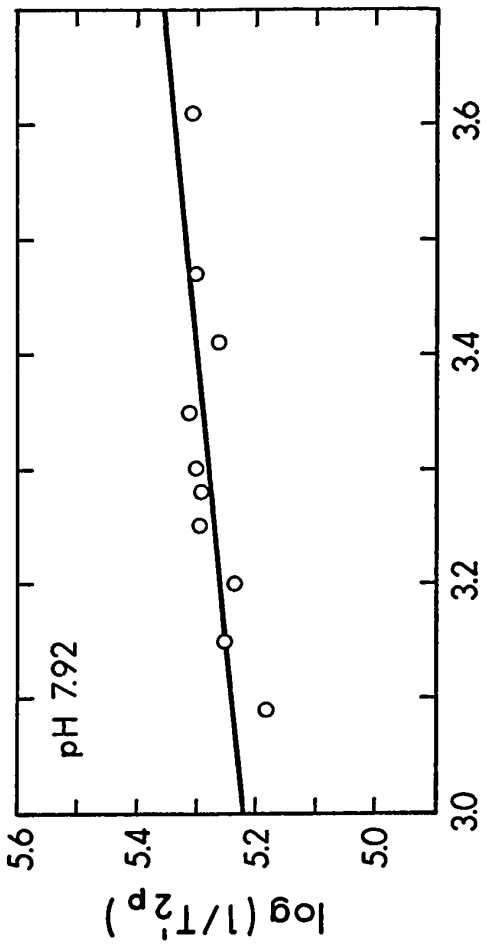


Fig. 6-6: log $(1/T_{2p}')$ vs. 1/T°K at pH 7.92.

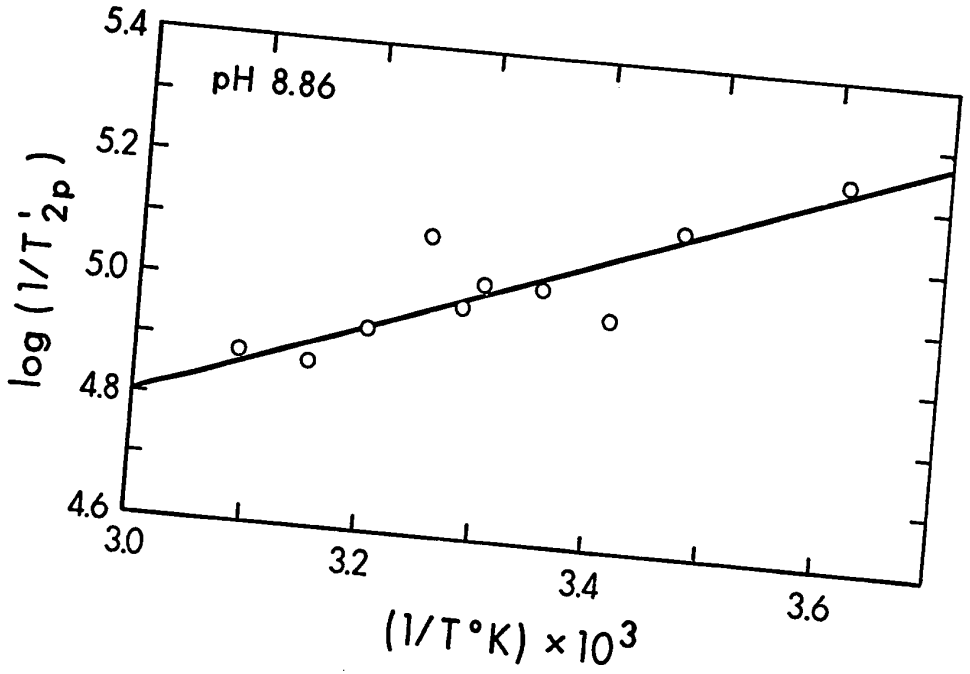


Fig. 6-7: log ($1/T_{2p}'$) vs. 1/T°K at pH 8.86.

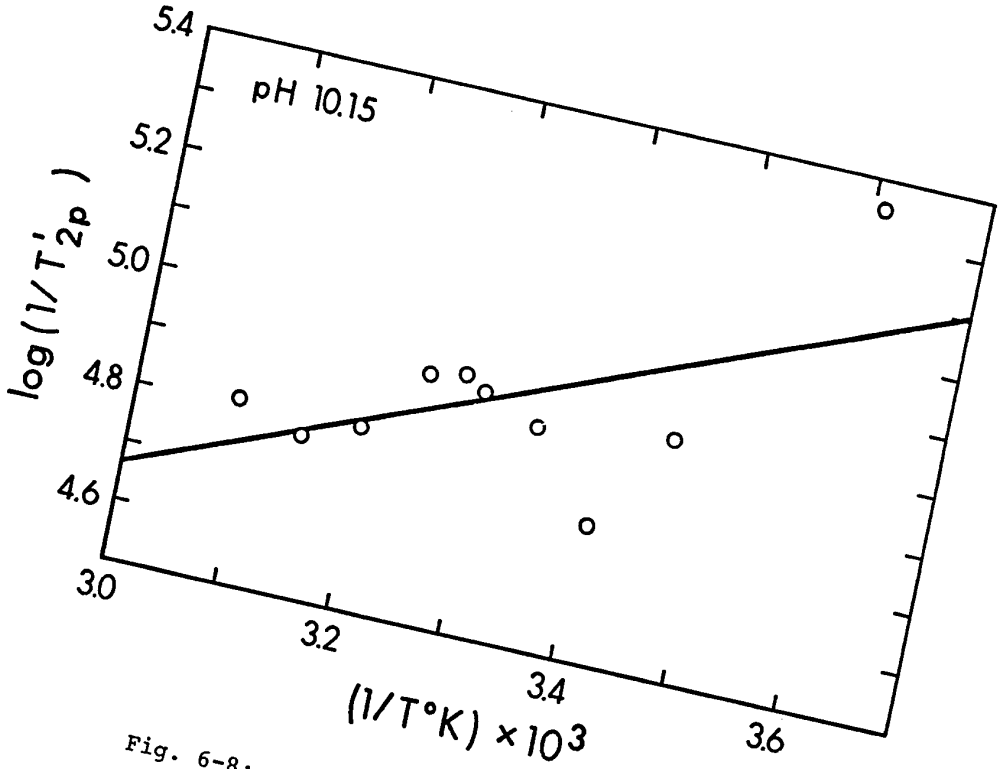


Fig. 6-8: log ($1/T_{2p}'$) vs. 1/T°K at pH 10.15.

Results and Discussion

A schematic derivative-of-absorption curve is shown in Fig. 6-9. As mentioned above, only one resonance line was seen; there was no observed shift in the line due to the paramagnetic ion.

The peak-to-peak separation of the experimental curves decreased, and the peaks became sharper, in every case, as the temperature of the samples was increased. The experimentally determined value of $1/T_2$ for the H_2O^{17} reference solution was 291 sec^{-1} at 25° and 276 sec^{-1} at 30°. Glasel (1966) determined a value of 195 sec^{-1} at 25°, while Garrett et al. (1967) determined a value of 143 sec^{-1} at 29°. However, Hindman et al. (1970) have performed T_1 measurements which indicate the presence of paramagnetic impurites in the solutions of Glasel et al. and Garrett. A correction to their data would decrease their values of $1/T_2$. In view of this finding, the results of this work would seem to indicate the presence of paramagnetic impurities or instrumental broadening. These errors, if systematic, should then cancel in the treatment of data because $1/T_2$ for the blank solution is subtracted from $1/T_2$ for the various sample solutions.

There is a fair degree of scatter in the points of the plots of log ($1/T_{2p}'$) vs. 1/T°K; however, the points define lines of positive slope. This would indicate that Swift and Connick's (1962) "Case B" or "Case D" (in the case of the pH 7.92 solution) is being observed. For Case B,

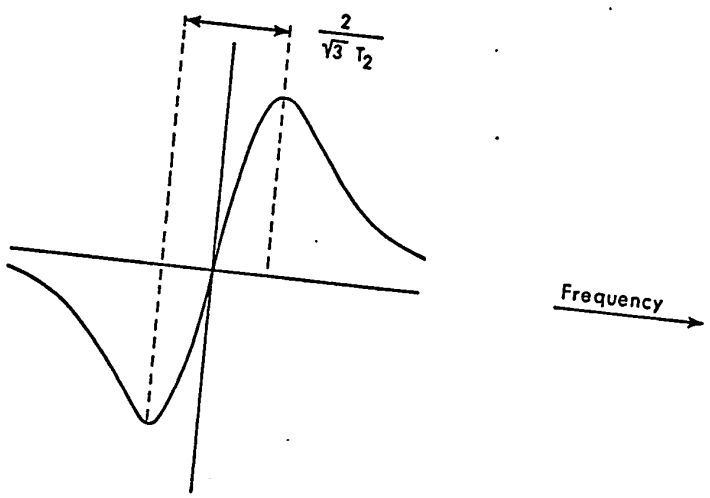


Fig. 6-9. A schematic derivative-of-absorption curve.

$$\frac{1}{T_{2p}} = P_M \tau_B \Delta\omega_B^2 \qquad (16)$$

If Case B were the situation, this could be shown by a study at another frequency. Since $\Delta\omega_B$ varies with the frequency, a doubling of the frequency would produce an increase in the value of $1/T_{2p}$ by a factor of four. Since the frequency was not varied in this study, there is no kinetic information available on this point because there is no observable shift caused by H_2O^{17}, which would allow a calculation of $\Delta\omega_B^2$, hence τ_B. In addition, P_M would have to be known, which involves a knowledge of the coordination number of the iron atom in HRP for water. This is presumed to be one, possibly two, the other four positions being occupied by the nitrogen atoms of the porphyrin ring.

If Case D were the situation, then no kinetic information concerning τ_B could be derived from the associated equation

$$\frac{1}{T_{2p}} = \frac{P_M}{T_{2B}} \qquad (20)$$

A calculation could be made of $1/T_2$ in this case, but τ_C, the correlation time, is not known for HRP, nor is the Fe-O distance.

Table 6-2 contains values for the activation energy for the exchange reaction, determined from the slopes of the six plots. There is no trend apparent; the average of all these values is 4.0 kcal $mole^{-1}$. This fairly small value (compared to values for ferric and other ions found by Swift and Connick (1962) and Luz and Shulman (1965) of 8-12 kcal $mole^{-1}$) may be due to outer sphere exchange of water.

Table 6-2

Activation Energies as a Function of pH of HRP - H_2O^{17} Solutions

pH	Activation Energy (kcal $mole^{-1}$)
5.61	6.8
5.94	4.1
6.85	5.2
7.92	0.8
8.87	3.0
10.15	4.3

Table 6-3 contains values for the lower limit of the rate constant for the exchange process, calculated according to the method of Connick and Poulson (1959). Again, there is no trend with pH, and an average value for the lower limit of k is about 3×10^6 sec^{-1} at 25°. Connick (1965) has reported a rate constant for exchange of water molecules from the first coordination sphere of ferric ion at 25° to be 3×10^3 sec^{-1}. The difference in values may be due to an exchange of water molecules between bulk water and an outer coordination sphere of the ferric iron of HRP, an easier process. The problem remains that of the large experimental broadening of resonance curves reported in this work which is incompatible with previous observations (Glasel, 1966; Garrett et al., 1967), and which may be reflected in serious errors in values of activation energies and lower limits for rate constants for the exchange process, but which does not affect the conclusion that the true values for the rate of exchange are not obtainable in this temperature region by the NMR line broadening technique.

Table 6-3

Lower Limits of the Rate Constant for Exchange as a Function of pH

pH	k_{lower} (sec^{-1})
5.61	2.8×10^5
5.94	8.2×10^6
6.85	1.9×10^5
7.92	1.2×10^5
8.87	5.8×10^6
10.15	4.1×10^6

Summary

The work on lactoperoxidase reported in this thesis contains the first detailed studies on individual rate constants in the lactoperoxidase oxidation-reduction cycle.

A measure of the secondary structure of lactoperoxidase has been established; it was shown that lactoperoxidase contains approximately 17% α-helix at pH 7.0. In addition, it was shown that anions such as fluoride, cyanide, and azide affect the ORD spectrum of the native enzyme, indicating that these ions may cause an alteration of the geometry of the heme group with respect to the enzyme.

A detailed pH study of the kinetics of the formation of lactoperoxidase compound I over a wide pH range was made, and the pH-independent rate constant is $(9.2 \pm 0.9) \times 10^6$ $M^{-1}sec^{-1}$. In addition, it was demonstrated that formate buffer binds to lactoperoxidase at an ionic strength of 0.05.

The reaction of LP-II with two substrates, p-cresol and iodide ion, was studied over a wide pH range. It was shown in the case of iodide ion oxidation that a rate controlling ionization on the enzyme occurs outside the experimental pH range at low pH. In addition, a second order dependence on the concentration of iodide ion was noted, and a complex between LP-II and iodide ion, which reacts further with iodide ion, was proposed to account for this observation. In the case of the oxidation of p-cresol by LP-II, it was shown that the rate is dependent on three ionizations of

groups in the enzyme with pK_a values of 2.3, 5.8, and 9.7, and the ionization of the substrate. It was also demonstrated that the neutral form of p-cresol was the reactive species over the whole pH range.

An investigation of the rate of exchange of water with the iron atom of the heme group of horseradish peroxidase was attempted using H_2O^{17} NMR. Experimental difficulties precluded obtaining accurate values of line widths, but the conclusion was made that the true values for the rate of exchange could not be obtained in the experimental temperature range.

The work on lactoperoxidase reported in this thesis provides the basis for further studies. For example, the kinetics of the transition from LP-I to LP-II using various reducing agents has yet to be studied. Preliminary experiments have shown several experimental difficulties. The spontaneous rate of decomposition of LP-I at pH 7 is associated with a rate constant of 4 sec^{-1} which apparently increases as the pH is either raised or lowered. The spontaneous rate also varies considerably with the state of purity of the enzyme. A study of the oxidation of iodide ion by LP-I would be important in view of the results of Björksten (1970) and Roman and Dunford (submitted for publication), which show that in the oxidation of iodide ion, HRP-I goes directly to HRP in a two-electron transfer, without the appearance of HRP-II in the reaction cycle.

Another problem of interest would be the simulation of the action of thyroid peroxidase, i.e., an investigation of a system in which LP catalyzes the oxidation of iodide ion and the iodination of tyrosine. However, this would require studies of the elementary processes, that is, the oxidation of iodide ion and tyrosine by the individual compounds I and II before a steady state reaction is undertaken. Prior knowledge of all of the individual rate constants would simplify evaluation of the steady state results.

As far as the nature of LP-II is concerned, the difference in the log k-pH profiles for the oxidation of iodide ion and p-cresol has been shown in this thesis. This difference was suggested to be due to either the absence of steric interactions in the case of the oxidation of iodide ion, or to the operation of a different mechanism. Further work with other substrates could shed light on the structure-reactivity relationship of the active site of LP-II. For example, an homologous series of phenols could be used as substrates, with substituents on the benzene ring that vary in size and electronegativity. On the other hand, other inorganic ions could be used as substrates, such as ferrocyanide, nitrite, and sulfite ions. The role that lactoperoxidase plays <u>in vivo</u> is not known, so that even if the substrates used for lactoperoxidase-catalyzed oxidations are of little physiological importance, a detailed study of their reactions may provide a meaningful picture of the

mechanism of oxidation by lactoperoxidase, and to a certain extent by peroxidases in general.

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Appendix 1

Purification of Lactoperoxidase

Lactoperoxidase was supplied by Dr. Martin Morrison of St. Jude Children's Research Hospital, Memphis, Tennessee, and by Calbiochem, Los Angeles. The lactoperoxidase (LP) samples from both sources were obtained according to the method of Morrison and Hultquist (1963), described below.

All purification procedures were performed at 4°. Fifteen grams of the sodium form of carboxymethyl cellulose cation exchange resin (Bio-Rad) were added to each liter of raw skim milk, and the pH was adjusted to 7.0. The milk-resin suspension was stirred for 1 hour and then allowed to stand until the resin settled. The milk was then removed by decantation, and an additional 7.5 g of resin per liter was added. The pH was readjusted to 7.0 and the milk-resin suspension was stirred again for 1 hour and then allowed to settle. The combined resin was washed with distilled water in batches by alternately suspending the resin and allowing it to settle.

The resin was then washed into a chromatographic tube with distilled water. The LP was eluted with 0.5 M sodium acetate. The enzyme moved down the column as a dark green band, increasing in size as it descended. The green material which eluted was precipitated with ammonium sulfate and centrifuged, dissolved in a small volume of water, and dialyzed repeatedly against large volumes of distilled water.

The resulting solution was then applied to a column of Sephadex G-200 gel. The LP fractions collected exhibited P.N. > 0.8 (P.N. is the ratio of the absorbance of an LP solution at 412 nm to that at 280 nm). The use of P.N. was the criterion of purity of samples of lactoperoxidase used throughout the course of this research. A P.N. of 0.8 or greater signified that the sample was relatively pure. P.N. values exceeding 0.95 for LP are rare, and the suspicion arises that extensive chromatographic purification procedures cause the enzyme to lose activity. An attempt to isolate LP in this laboratory using the method outlined above yielded 63 mg LP (P.N. 0.7) from 5 gallons of milk.

One of the more common assay methods for the activity of lactoperoxidase samples involves the oxidation of guaiacol (Maehly and Chance, 1958). The major disadvantage of this procedure is that the color of the product fades rapidly, which may be responsible for the lack of reproducibility of this procedure. It appears that despite the proliferation of activity tests for peroxidases, the absorbance ratio P.N. is the most widely used criterion of purity.

Further purification of LP obtained from Dr. Morrison or Calbiochem was carried out, and the procedure will now be described. The LP from either source, which was obtained in a lyophilized (freeze-dried) form, possessed a P.N. of

about 0.6. It was purified to a P.N. of 0.8 or greater by Sephadex gel chromatography.

Sephadex is a modified dextran. The dextran macromolecules are cross-linked to give a three-dimensional network of polysaccharide chains. A gel filtration experiment can be described schematically in the following way. Molecules larger than the largest pores of the swollen Sephadex, i.e., above the exclusion limit, cannot penetrate the gel particles and therefore they pass through the bed in the liquid phase outside the Sephadex particles. They are thus eluted first. Smaller molecules, however, penetrate the gel particles to varying degrees depending on their size and shape. Molecules are therefore eluted from a Sephadex bed in the order of decreasing molecular size. As the molecules pass through the bed at different rates, they emerge at the outlet end of the column separated from each other. The zones eluted are somewhat wider than the applied sample, but by the use of suitable experimental conditions, this zone broadening can be limited. Sephadex gels and chromatographic columns are obtained from Pharmacia Fine Chemicals, Inc.

All procedures described were carried out in a cold room at 4°, unless otherwise specified. The gel, Sephadex G-200 Superfine, was swollen by suspension in pH 7.0 phosphate solution of ionic strength 0.05 for 3 days. A chromatographic column was prepared by pouring the gel into a glass column of dimensions 45 cm in length and 2.5 cm in diameter, equipped with a net at the bottom which allowed the passage

of water and enzyme, but not the gel particles. The quality of the gel packing was improved by the passage of 1 litre of pH 7.0 buffer through the column. This procedure took about 2 days, and the column was then ready for application of the enzyme. About 150 mg of lyophilized LP was dissolved in 2 ml of pH 7.0 phosphate buffer of ionic strength 0.05. The solution was clarified by passage through glass wool.

The enzyme solution was applied to the top of the Sephadex column with a disposable pipet. After the green band had descended under the top of the gel column, a buffer from a gravity bottle was allowed to drain into the column. The eluant was pH 7.0 phosphate buffer of ionic strength 0.05. The output of such a column was about 10 ml per hour, and fractions were collected every 10 min (about 1.5 ml per fraction) in test tubes with an LKB Ultrorac 7000 fraction collector. After the enzyme had eluted from the column, the test tubes containing the green enzyme (or brownish-green color at high LP concentrations, about 10^{-4}M) were collected, and their contents analyzed spectrophotometrically (using a Beckman DU at 25°) at 412 nm and 280 nm. The tubes were then stored in the cold prior to use. The results of one such purification are shown in Table A-1, where the absorbance values are of samples of 1 mm thickness, and the LP concentrations are obtained using the molar absorptivity $\varepsilon_{412} = 1.14 \times 10^5\ M^{-1}cm^{-1}$ (Morrison et al., 1957). Figure A-1 is a plot of P.N. (from Table A-1) vs. fraction number. Only those samples of LP of P.N. greater than 0.8 were used in kinetic experiments.

Table A-1: The results of a chromatographic purification of lactoperoxidase using Sephadex G-200 Superfine gel.

Fraction #	A_{412}[a]	A_{280}[a]	P.N.	[LP], M
73	0.06	0.10	0.65	5.70×10^{-6}
74	0.13	0.17	0.77	1.14×10^{-5}
75	0.24	0.31	0.80	2.10×10^{-5}
76	0.46	0.53	0.87	4.03×10^{-5}
77	0.74	0.84	0.88	6.49×10^{-5}
78	1.06	1.22	0.87	9.30×10^{-5}
79	1.31	1.50	0.87	1.15×10^{-4}
80	1.60	1.86	0.86	1.40×10^{-4}
81	1.90	2.00	0.95	1.67×10^{-4}
82	1.95	2.14	0.91	1.71×10^{-4}
83	1.54	1.68	0.92	1.35×10^{-4}
84	1.25	1.37	0.91	1.10×10^{-4}
85	0.85	0.93	0.91	7.46×10^{-5}
86	0.54	0.60	0.91	4.74×10^{-5}
87	0.34	0.38	0.89	2.98×10^{-5}
88	0.21	0.24	0.88	1.84×10^{-5}
89	0.13	0.16	0.86	1.14×10^{-5}
90	0.09	0.12	0.75	7.89×10^{-6}
91	0.06	0.08	0.73	5.26×10^{-6}
92	0.04	0.05	0.73	3.51×10^{-6}

[a]Absorbance values are obtained using 1 mm cells.

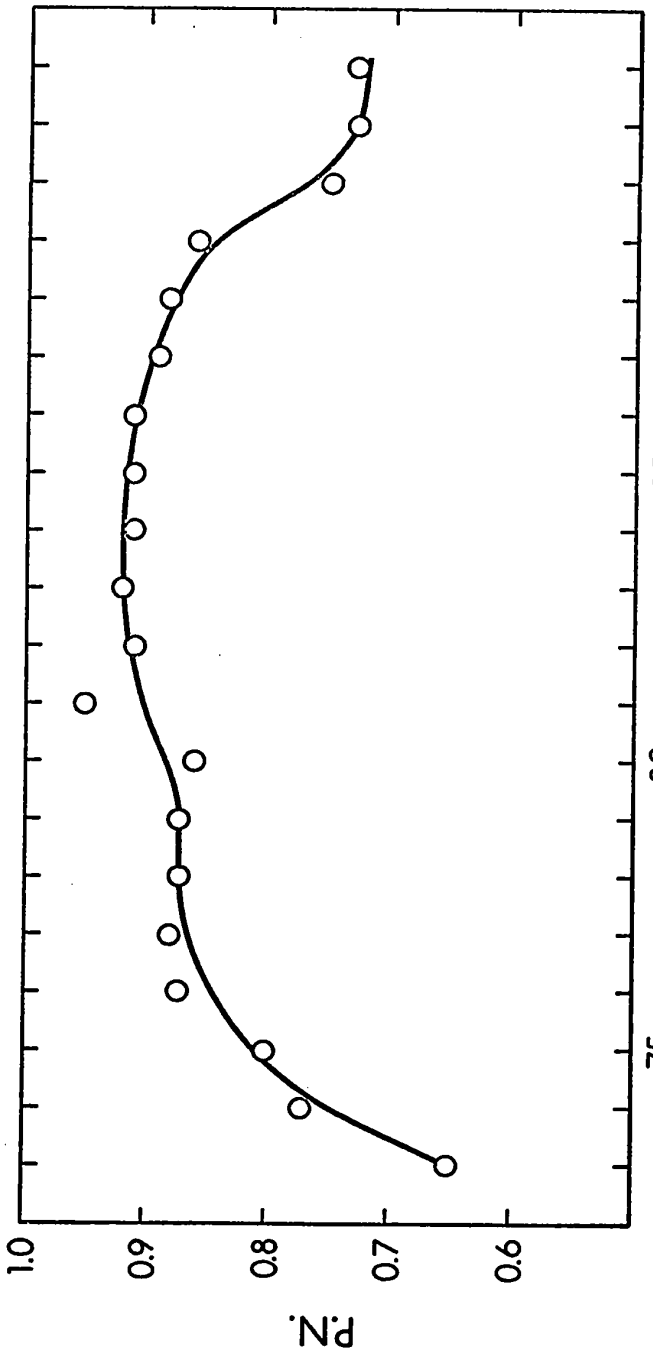

Fig. A-1. A plot of P.N. vs. fraction number for the results of Table A-1.

Appendix 2

Description of the Non-Linear Least Squares Program

This computer program, called "BRT2" was supplied by Dr. M. K. Evett (N.R.C.C. post-doctoral fellow at the University of Alberta, 1968-1970). The program uses Gauss's method to find the best least-squares values of parameters in non-linear functions of one dependent variable. A discussion of the minimization methods used is given by Wentworth (1965).

The program is listed at the end of this appendix. It contains some features that are not often used (e.g. CNTROl(11) and (12) discussed below). The subprograms OVERID, WRNGWY, and PCHANGE may be removed with their calls, or made dummies to reduce space requirements. In addition, the "restrained change mechanism" in subroutine GAUS can be removed.

There are three user-supplied subprograms for BRT2 that must be programmed for use on a particular equation that one may wish to solve. The first subprogram, called MANIP, allows one to modify the input variables. The second subprogram, YCOMP, calculates a value of the dependent variable as a function of the parameters and independent variables. The third subprogram, OUTDAT, is called, if needed, at the completion of the "fit" to allow computations with the parameters determined by YCOMP. These three subprograms are found at the end of the listing.

Examples of the use of these three subprograms will be

given:

MANIP – if the data were obtained as, for example, log k vs. pH, and the actual equation related log k with $[H^+]$, then MANIP could be used to convert the input variable pH to $[H^+]$ by the following cards

```
      DO 1  I=1,NPTS
1     Z(2,I)  =  EXP(-2.303*Z(2,I))
```

which is equivalent to saying that $[H^+] = e^{-2.303\ pH}$, and performing this operation for as many input points as is required.

YCOMP – if one wished to program a first-order kinetic expression involving absorbance, A, a rate constant, k, and time, t, which are related by

$$\frac{A - A_\infty}{A_0 - A_\infty} = e^{-kt}$$

then the expression is

```
YCOMP = (B(2)-B(3))*EXP(-B(1)*Z(2,N))+B(3)
```

where

YCOMP = A, the dependent variable

Z(2,N) = t, the independent variable

B(1) = k

B(2) = A_0

B(3) = A_∞

OUTDAT – if, for example, one obtained from YCOMP a value of k which was a pseudo first order rate constant, but wished to obtain the value of the true second order rate

constant by the relation

$$k' = k/[S]$$

where [S] is a concentration of some reactant, then the expression would merely be

$$K = B(1)/A$$

with an accompanying PRINT statement.

The program listing may be consulted for specification cards. The subprograms must be present even if only as dummies (with RETURN as the only executable statement).

The following is a list of the arrangement of data cards for the BRT2 program:

Card No.	Data	Format
1	CNTROL(I) I=3,19	I6,16I4
2	CNTROL(3), TITLE	I6,18A4,2A1
3	Number of parameters, NC	I5
	Number of parameters varied, NB	I5
	Parameter estimates, B(J) J=1,NC	7E10.4/(8E10.4)

The actual data cards follow card 3, and the data is right justified (E10.4) with the dependent variable, Z(1,N), in col. 1-10 and the independent variable, Z(2,N), in col. 11-20. At the end of all the data cards is a card with a zero in the sixth column. Note that the program will vary the first NB of the NC parameters.

The following is an explanation of the controls used by

the BRT2 program:

CNTROL(1) Number of parameters.

CNTROL(2) Number of parameters varied.

CNTROL(3) Number of data points. On CNTROL card make this number larger than the largest data set. Use the exact number on card 2; this number may be omitted from card 2 if each data set is terminated by a card with 999. in columns 7-10.

CNTROL(4) Number of parameters.

CNTROL(5) Total number of variables (independent +1).

CNTROL(6) Limit on the number of iterations.

CNTROL(7) =a+2b+4c+8d where a,b,c, and d are each 1 or 0 according to whether or not the corresponding printout is desired.

a. data
b. sum of squares and parameters for each iteration.
c. normal equation matrices.
d. restrained change mechanism.

CNTROL(8) Weighting factor. 1)1.0, 2)1/obs, 3)1/calc., 4)obs, 5)calc, 6)1/Z(M,N), 7)Z(M,N), where in the last two the weight is read in as the last independent variable in columns 21-30.

CNTROL(9) Formerly, OUTDAT was activated if the value of this control was non-zero. OUTDAT is now controlled through MANIP.

CNTROL(10) Corrections to parameters are scaled by 0.1*CNTROL(10). If CNTROL(10) is equal to zero, the scaling factor is 1.

CNTROL(11) Parameter change option. Reduces the number of parameters by one this many times when no reduction in the sum of squares is obtained.

CNTROL(12) If >0, upon termination - this many "over-rides" (i.e., the program ignores the fact that there is no reduction in the sum of squares). If <0, the program performs this many "wrong-ways" (i.e., the parameters are scaled and changed in the opposite direction).

CNTROL(16) Allows this many data sets to be run under one control card. May be -1 for automatic counting if sets are terminated by a card with STOP punched in columns 7-10.

CNTROL(17) This many sets of starting estimates are used.

Controls 13,14,15,18,19, and higher are internal program controls.

This program has been tested against a program used previously in this group (Ellis, 1968) on a number of problems, and has been found to give identical results; however, the BRT2 program has the advantages of several different forms of weighting of data points, as can be seen from "CNTROL(8)", and also the advantage that each data point can be individually weighted. The BRT2 program is listed on the following pages.

```
$SIGNON HAL  T=1M P=100 PRIO=H    'JIM MAGUIRE'
ON AT  15:13.37 ON 01-14-72  LAST ON AT  15:11.05 ON 01-09-72
$LIST *SOURCE*
     5      C      PROGRAM BRT2                                                                    BRT20000
     6             DIMENSION BTRIAL(20)                                                            BRT20010
     7             DIMENSION CORE(2000)                                                            BRT20020
     8             COMMON CNTROL                                                                   BRT20030
     9             DIMENSION NPRINT(4)                                                             BRT20040
    10             INTEGER IPRINT(2)/'NO','YES'/                                                   BRT20050
    11             INTEGER CNTROL(25),BDIM,TIT(20)                                                 BRT20060
    12             INTEGER ST/'STOP'/                                                              BRT20070
    13             INTEGER IFORM(3,10 )/' ','1.0',' ','1','/OBS',' ','1/','CALC',' ',BRT20080
    14            1' ','OBS',' ',' ','CALC',' ', '1/Z','(M+1',',N)','Z(','M+1',',N)',BRT20090
    15            19* '/                                                                           BRT20100
    16             BIN(K,J)=0.5-0.5*(-1.)**(K/2**(J-1))                                            BRT20110
    17     1       READ 12, (CNTROL(I),I=3,19)                                                     BRT20120
    18             IF (CNTROL(3).EQ.0) GO TO 11                                                    BRT20130
    19             KNTRL=CNTROL(10)                                                                BRT20140
    20             MDIM=CNTROL(5)                                                                  BRT20150
    21             NDIM=IABS(CNTROL(3))                                                            BRT20160
    22             IF (CNTROL(16).EQ.0) CNTROL(16)=1                                               BRT20170
    23             IF (CNTROL(17).EQ.0) CNTROL(17)=1                                               BRT20180
    24             III=CNTROL(16)                                                                  BRT20190
    25             NPRO=0                                                                          BRT20200
    26     2       NPRO=NPRO+1                                                                     BRT20210
    27             CNTROL(23)=NPRO                                                                 BRT20220
    28             READ 13, CNTROL(3),(TIT(I),I=1,20)                                              BRT20230
    29             IF (TIT(1).EQ.ST) GO TO 1                                                       BRT20240
    30             PRINT 14, TIT                                                                   BRT20250
    31             LAST=1                                                                          BRT20260
    32             NPROB=CNTROL(17)                                                                BRT20270
    33             PRINT 15                                                                        BRT20280
    34             DO 4 L=1,NPROB                                                                  BRT20290
    35             READ 16, IB,IC,(BTRIAL(I),I=1,IB)                                               BRT20300
    36             PRINT 17, L,IB,IC,(BTRIAL(I),I=1,IB)                                            BRT20310
    37      CALL TO MANIP ALLOWS PARAMETERS TO BE MANUPULATED.                                     BRT20320
    38             CALL MANIP (1,BTRIAL,CORE(LOCZ),MDIM,NDIM,BDIM)                                 BRT20330
    39             I=0                                                                             BRT20340
    40             LASTP2=LAST+2                                                                   BRT20350
    41             NEXT=LASTP2+IB-1                                                                BRT20360
    42             DO 3 K=LASTP2,NEXT                                                              BRT20370
    43             I=I+1                                                                           BRT20380
    44     3       CORE(K)=BTRIAL(I)                                                               BRT20390
    45             CORE(LAST)=IB                                                                   BRT20400
    46             CORE(LAST+1)=IC                                                                 BRT20410
    47     4       LAST=NEXT+1                                                                     BRT20420
    48             BDIM=CNTROL(4)                                                                  BRT20430
    49             LOCZ=4*BDIM+LAST                                                                BRT20440
    50             LOCA=LOCZ+MDIM*NDIM+1                                                           BRT20450
    51             NEXT=1                                                                          BRT20460
    52             DO 10 K=1,NPROB                                                                 BRT20470
    53             CNTROL(10)=KNTRL                                                                BRT20480
    54             CNTROL(24)=K                                                                    BRT20490
    55             CNTROL(2)=CORE(NEXT)                                                            BRT20500
    56             CNTROL(1)=CORE(NEXT+1)                                                          BRT20510
    57             M3=CNTROL(2)                                                                    BRT20520
    58             LOCB=LAST                                                                       BRT20530
    59             DO 5 I=1,M3                                                                     BRT20540
    60     5       CORE(LAST+I-1)=CORE(NEXT+1+I)                                                   BRT20550
    61             NEXT=NEXT+M3+2                                                                  BRT20560
```

```
      NB=CNTROL(1)                                                      BRT20570
      NPTS=IABS(CNTROL(3))                                              BRT20580
      NVARS=CNTROL(5)                                                   BRT20590
      LIMIT=CNTROL(6)                                                   BRT20600
      PRINT 19, (CNTROL(I),I=1,19),NB,NPTS,NVARS,LIMIT                  BRT20610
      IF (CNTROL(7).NE.0) GO TO 6                                       BRT20620
      PRINT 19                                                          BRT20630
      GO TO 8                                                           BRT20640
    6 PRINT 20                                                          BRT20650
      DO 7 J=1,4                                                        BRT20660
      L=1                                                               BRT20670
      IF (BIN(CNTROL(7),J).GT.0.) L=2                                   BRT20680
      NPRINT(J)=IPRINT(L)                                               BRT20690
    7 CONTINUE                                                          BRT20700
      PRINT 21, (NPRINT(J),J=1,4)                                       BRT20710
    8 LL=CNTROL(8)                                                      BRT20720
      PRINT 22, CNTROL(8),((IFORM(I,LL),I=1,3)                          BRT20730
      IF (CNTROL(10).EQ.0) PRINT 23                                     BRT20740
      ICHANG=10*CNTROL(10)                                              BRT20750
      IF (CNTROL(10).NE.0) PRINT 24, ICHANG                             BRT20760
      PRINT 25                                                          BRT20770
      LASPM3=LAST+M3-1                                                  BRT20780
      PRINT 26, (CORE(I),I=LAST,LASPM3)                                 BRT20790
      CNTROL(22)=1                                                      BRT20800
      PRINT 27, MDIM,NDIM,BDIM                                          BRT20810
CALL TO MANIP ALLOWS DATA TO BE READ IN AND MODIFIED IN THAT SUBROUTINE.BRT20820
      IF (CNTROL(3).LT.0.AND.K.EQ.1) CALL MANIP (2,CORE(LAST),CORE(LOCZ)BRT20830
     1,MDIM,NDIM,BDIM)                                                  BRT20840
      IF (NPTS.NE.IABS(CNTROL(3))) NPTS=IABS(CNTROL(3))                 BRT20850
      IF (CNTROL(3).GE.0.AND.K.EQ.1) CALL INDATA (CORE(LOCB),CORE(LOCZ),BRT20860
     1MDIM,NDIM,BDIM)                                                   BRT20870
      IF (BIN(CNTROL(7),2).EQ.0) GO TO 9                                BRT20880
      CALL PRINDA (CNTROL,CORE(LOCZ),MDIM,NDIM)                         BRT20890
    9 CALL GAUSS (CORE(LOCB),CORE(LOCZ),MDIM,NDIM,BDIM,CORE(LOCA))      BRT20900
      PRINT 14, TIT                                                     BRT20910
      CALL FINALE (CORE(LOCB),CORE(LOCZ),MDIM,NDIM,BDIM,CORE(LOCA))     BRT20920
      IF (CNTROL(21).EQ.0) PRINT 33                                     BRT20930
      IF (CNTROL(21).EQ.1) PRINT 28                                     BRT20940
      IF (CNTROL(21).EQ.2) PRINT 29                                     BRT20950
      IF (CNTROL(21).EQ.3) PRINT 30                                     BRT20960
      IF (CNTROL(21).EQ.4) PRINT 31                                     BRT20970
      IF (CNTROL(21).EQ.5) PRINT 32                                     BRT20980
                                                                        BRT20990
   10 CONTINUE                                                          BRT21000
      IF (NPRO.LT.IABS(III).OR.III.EQ.-1) GO TO 2                       BRT21010
      GO TO 1                                                           BRT21020
   11 PRINT 34                                                          BRT21030
      STOP                                                              BRT21040
C                                                                       BRT21050
   12 FORMAT (16,16I4)                                                  BRT21060
   13 FORMAT (16,18A4,2A1)                                              BRT21070
   14 FORMAT (1HI,20A4)                                                 BRT21080
   15 FORMAT(1H0,'0TRIAL READ-IN, ECHO CHECK FOLLOWS.'  )               BRT21090
   16 FORMAT (2I5,7E10.4/(8E10.4))                                      BRT21100
   17 FORMAT (3I5,7E15.5/(15X,7E15.5))                                  BRT21110
   18 FORMAT (32H0 NONLINEAR REGRESSION CONTROLS 1915//30H NUMBER OF PARBRT21120
     1AMETERS          112/30H NUMBER OF DATA POINTS          112/30H NUMBBRT21130
     2R OF VARIABLES          112/30H LIMIT ON NUMBER/ITERATIONS   112/)BRT21140
   19 FORMAT (' CNTROL(7) = 0. SO NO INTERMEDIATE OUTPUT ')             BRT21150
   20 FORMAT ('0 THE FOLLOWING INFORMATION IS TO BE PRINTED OUT'///' INPUBRT21160
     1T DATA   SUMSQ AND PARAMETER VALUES   NORMAL EQ MATRICES   RESTRAIBRT21170
```

```
      2NED CHANGE MECHANISM*)                                                  BRT21170
21     FORMAT (5X,A3,18X,A3,21X,A3,25X,A3)                                     BRT21180
22    FORMAT (' CNTROL(8) =',I3,' SO MINIMIZE LEAST SQUARES WEIGHTED BY        BRT21190
     1 (',3A4,' )')                                                           BRT21200
23    FORMAT (' CNTROL(10) = 0, SO UNCONSTRAINED PARAMETER CHANGES ')          BRT21210
24    FORMAT ( '  CNTROL(10) NONZERO, PARAMETERS CAN CHANGE BY AT MOST         BRT21220
     1',I4,' PERCENT')                                                        BRT21230
25     FORMAT (42H0 STARTING GUESSES FOR THE PARAMETERS B(J))                  BRT21240
26     FORMAT (1X,10E13.4)                                                     BRT21250
27     FORMAT (48H0 NONLINEAR REGRESSION ARRAY DECLARATIONS ARE   /4H  Z(BRT21260
     112,1H,I3,8H) AND B(12,1H)/1H0)                                          BRT21270
28     FORMAT ('0 NORMAL TERMINATION.  CONVERGENCE OF PARAMETERS ')            BRT21280
29     FORMAT ('0 TERMINATION DUE TO NEGLIGIBLE CHANGE IN SUM/SQUARES ')BRT21290
30     FORMAT ('0 TERMINATION DUE TO LIMIT ON NUMBER OF ITERATIONS ')          BRT21300
31     FORMAT ('0 TERMINATION DUE TO SINGULARITY OF NORMAL EQS ')              BRT21310
32    FORMAT ('0 NO SUM OF SQUARES REDUCTION IN LEAST SQUARES INTERVAL')BRT21320
33    FORMAT ('0 OPTION REDUNDANCY  - - -  EXPRESSION CHANGE INDICATED')BRT21330
34    FORMAT('1STANDARD JOB TERMINATION.')                                     BRT21340
      END                                                                      BRT21350
      SUBROUTINE INDATA (B,Z,MDIM,NDIM,BDIM)                                   INDA0000
      COMMON CNTROL(25)                                                        INDA0010
      DIMENSION B(1), Z(MDIM,NDIM)                                             INDA0020
      INTEGER CNTROL,BDIM                                                      INDA0030
      NVARS=CNTROL(5)                                                          INDA0040
      IBSET=CNTROL(24)                                                         INDA0050
      KTROL=CNTROL(14)+1                                                       INDA0060
      M=CNTROL(15)+1                                                           INDA0070
      IDSET=CNTROL(23)                                                         INDA0080
      NPTS=CNTROL(3)                                                           INDA0090
      IF (CNTROL(3).EQ.0) GO TO 8                                              INDA0100
      GO TO (1,3,7), KTROL                                                     INDA0110
C  NPTS IS THE NUMBER OF DATA POINTS                                           INDA0120
C  NVARS IS THE NUMBER OF VARIABLES TO BE READ IN, INCLUDING DEPENDENT         INDA0130
C  IDSET IS THE INDEX OF THE PARTICULAR DATA SET BEING CONSIDERED              INDA0140
C  IBSET IS THE INDEX OF THE FITTING FUNCTION LOOP OR SIMPLY THE B SET         INDA0150
C      INDEX                                                                   INDA0160
CNTROL(14)=BLANK, IF ALL DATA ARE IN STNADARD ARRANGEMENT (SEE WRITEUP)        INDA0170
C       1, IF ONE DESIRES TO READ INDEPENDENT VBLS ONLY ONCE FOR EACH          INDA0180
C       SET OF B PARAMETERS.                                                   INDA0190
C       2,IF THE INDEPENDENT VBLES ARE TO BE READ ONLY ONCE FOR EACH TIMEINDA0200
C  THE ENTIRE PROGRAM IS RUN.                                                  INDA0210
C  M ( CNTROL(15) ) IS BLANK IF ALL DATA ARE ON CARDS. OTHERWISE. (M=1)        INDA0220
C  DATA ARE EITHER-ALL ON TAPE, OR ONLY THE DEPENDENT DATA ON TAPE.            INDA0230
      IF (IABS(CNTROL(9)).EQ.1) PRINT 12, NPTS                                 INDA0240
      DO 2 I=1,NPTS                                                            INDA0250
      READ (5,13) (Z(J,I),J=1,NVARS)                                           INDA0260
      IF (IABS(CNTROL(9)).EQ.1) PRINT 14, I,(Z(J,I),J=1,NVARS)                 INDA0270
2     CONTINUE                                                                 INDA0280
      GO TO 11                                                                 INDA0290
3     CONTINUE                                                                 INDA0300
4     IF (IBSET.NE.1) GO TO 6                                                  INDA0310
      DO 5 I=1,NPTS                                                            INDA0320
      READ 13, (Z(J,I),J=2,NVARS)                                              INDA0330
5     READ (M,13) (Z(1,I),I=1,NPTS)                                            INDA0340
6     GO TO 11                                                                 INDA0350
7     CONTINUE                                                                 INDA0360
      IF (IDSET.NE.1) GO TO 6                                                  INDA0370
      GO TO 4                                                                  INDA0380
8     IF (IABS(CNTROL(9)).EQ.1) PRINT 12, NPTS                                 INDA0390
9     NPTS=NPTS+1                                                              INDA0400
```

```
182           READ (5,13) (Z(J,NPTS),J=1,NVARS)                                  INDA0410
183           IF (Z(1,NPTS).EQ.999.) GO TO 10                                    INDA0420
184           IF (IABS(CNTROL(9)).EQ.1) PRINT 14, NPTS,(Z(J,NPTS),J=1,NVARS)     INDA0430
185           GO TO 9                                                            INDA0440
186     10    NPTS=NPTS-1                                                        INDA0450
187           CNTROL(3)=NPTS                                                     INDA0460
188     CALL TO MANIP ALLOWS MODIFICATION OF INPUT DATA.                         INDA0470
189     11    CALL MANIP (3,B,Z,MDIM,NDIM,BDIM)                                  INDA0480
190           RETURN                                                             INDA0490
191     C                                                                        INDA0500
192        12 FORMAT (1H0,' DATA READ CHECK.  NPTS = ',I3/)                      INDA0510
193     13    FORMAT (8E10.4)                                                    INDA0520
194     14    FORMAT (1H ,I2,'.',8E12.4)                                         INDA0530
195           END                                                                INDA0540
196           SUBROUTINE SETUP (B,Z,A,SUMSQ,NDIM,NDIM,BDIM)                      SETU0000
197           COMMON CNTROL                                                      SETU0010
198           INTEGER CNTROL(25),BDIM                                            SETU0020
199           REAL A(BDIM,BDIM),B(BDIM,4),Z(MDIM,NDIM)                           SETU0030
200     CALCULATE THE TERMS IN THE MATRIX EQUATION     A * X = C                 SETU0040
201     COMPUTE ONLY THE SUM OF SQUARES IF CNTROL(10) NEGATIVE, SINCE THE GAUSS  SETU0050
202     CYCLE DID NOT REDUCE THE SUM OF SQUARES.....................             SETU0060
203           IF (CNTROL(10).LE.-1) GO TO 2                                      SETU0070
204           JJ=IABS(CNTROL(1))                                                 SETU0080
205           LL=CNTROL(8)                                                       SETU0090
206           DO 1 J=1,JJ                                                        SETU0100
207           B(J,3)=0.0                                                         SETU0110
208           DO 1 K=1,JJ                                                        SETU0120
209     1     A(J,K)=0.0                                                         SETU0130
210     2     NUMBER=IABS(CNTROL(3))                                             SETU0140
211           SUMSQ=0.0                                                          SETU0150
212           IHOLD=CNTROL(21)                                                   SETU0160
213           CNTROL(21)=68                                                      SETU0170
214     CNTROL(21) = 68 TELLS YCOMP THIS IS FIRST CALL OF AN ITERATION OR SUMSQ  SETU0180
215     CALCULATION                                                              SETU0190
216           ZERO=1./(NUMBER-JJ)                                                SETU0200
217           IF (CNTROL(10).EQ.-2) ZERO=1.                                      SETU0210
218           DO 19 N=1,NUMBER                                                   SETU0220
219           YC=YCOMP(N,B,Z,MDIM,NDIM,BDIM)                                     SETU0230
220           CNTROL(21)=IHOLD                                                   SETU0240
221           IF (NUMBER.NE.IABS(CNTROL(3))) GO TO 2                             SETU0250
222           ZN=Z(1,N)                                                          SETU0260
223           DELY=ZN-YC                                                         SETU0270
224           GO TO (9,3,4,5,6,7,8), LL                                          SETU0280
225     3     DELY=DELY/ZN                                                       SETU0290
226           GO TO 9                                                            SETU0300
227     4     DELY=DELY/YC                                                       SETU0310
228           GO TO 9                                                            SETU0320
229     5     DELY=DELY*ZN                                                       SETU0330
230           GO TO 9                                                            SETU0340
231     6     DELY=DELY*YC                                                       SETU0350
232           GO TO 9                                                            SETU0360
233     7     DELY=DELY/Z(MDIM,N)                                                SETU0370
234           GO TO 9                                                            SETU0380
235     8     DELY=DELY*Z(MDIM,N)                                                SETU0390
236     9     SUMSQ=SUMSQ+(DELY**2)/((NUMBER-JJ)*ZERO)                           SETU0400
237           IF (CNTROL(10).LE.-1) GO TO 19                                     SETU0410
238           DO 17 K=1,JJ                                                       SETU0420
239           B(K,4)=DERIV(K,N,B,Z,BDIM,MDIM,NDIM)                               SETU0430
240           GO TO (16,10,11,12,13,14,15), LL                                   SETU0440
241     10    B(K,4)=B(K,4)/ZN                                                   SETU0450
```

```
242          GO TO 16                                                                SETU0460
243       11 B(K,4)=B(K,4)/YC                                                        SETU0470
244          GO TO 16                                                                SETU0480
245       12 B(K,4)=B(K,4)*ZN                                                        SETU0490
246          GO TO 16                                                                SETU0500
247       13 B(K,4)=B(K,4)*YC                                                        SETU0510
248          GO TO 16                                                                SETU0520
249       14 B(K,4)=B(K,4)/Z(MDIM,N)                                                 SETU0530
250          GO TO 16                                                                SETU0540
251       15 B(K,4)=B(K,4)*Z(MDIM,N)                                                 SETU0550
252       16 B(K,3)=B(K,3)+B(K,4)*DELY                                               SETU0560
253       17 CONTINUE                                                               SETU0570
254          DO 18 J=1,JJ                                                           SETU0580
255          DO 18 K=J,JJ                                                           SETU0590
256          A(J,K)=A(J,K)+B(J,4)*B(K,4)                                            SETU0600
257       18 CONTINUE                                                               SETU0610
258       19 CONTINUE                                                               SETU0620
259          IF (CNTROL(10).LE.-1) RETURN                                           SETU0630
260          DO 20 K=2,JJ                                                           SETU0640
261          L=K-1                                                                  SETU0650
262          DO 20 J=1,L                                                            SETU0660
263       20 A(K,J)=A(J,K)                                                          SETU0670
264          RETURN                                                                 SETU0680
265          END                                                                    SETU0690
266          SUBROUTINE BTEST (CNTROL,B,BDIM)                                       BTES0000
267          INTEGER CNTROL(25),BDIM                                                BTES0010
268          REAL B(BDIM,4)                                                         BTES0020
269          JJ=IABS(CNTROL(1))                                                     BTES0030
270          XLIM=1.00E-05                                                          BTES0040
271          IF (CNTROL(9).EQ.3) XLIM=1.00E-02                                      BTES0050
272          DO 1 J=1,JJ                                                            BTES0060
273          DENOM=ABS(B(J,2))                                                      BTES0070
274          IF (DENOM.LT.1.0E-8) DENOM=1.0                                         BTES0080
275          TEST=ABS(B(J,3)/DENOM)                                                 BTES0090
276          IF (TEST.GT.XLIM) RETURN                                               BTES0100
277       1  CONTINUE                                                               BTES0110
278          IF (CNTROL(9).EQ.3) GO TO 2                                            BTES0120
279       2  CNTROL(21)=1                                                           BTES0130
280          RETURN                                                                 BTES0140
281          END                                                                    BTES0150
282          FUNCTION DERIV (K,N,B,Z,BDIM,MDIM,NDIM)                                DERI0000
283          INTEGER BDIM,CNTROL                                                    DERI0010
284          REAL B(BDIM,4),Z(MDIM,NDIM)                                            DERI0020
285          COMMON CNTROL(25)                                                      DERI0030
286       COMPUTE FINITE DIFFERENCE APPROXIMATIONS TO PARTIAL DERIVATIVES IN         DERI0040
287       CASE THIS ROUTINE IS NOT SUPPLIED BY USER.                                DERI0050
288          IHOLD=CNTROL(21)                                                       DERI0060
289          CNTROL(21)=67                                                          DERI0070
290       CNTROL(21) = 67 SUPPRESSES PRINTING OF CALCULATED QUANTITY YCOMP          DERI0080
291          J=K                                                                    DERI0090
292          H=0.001*ABS(B(J,1))                                                    DERI0100
293          REMARK=B(J,1)                                                          DERI0110
294          B(J,1)=REMARK+H                                                        DERI0120
295          Y2=YCOMP(N,B,Z,MDIM,NDIM,BDIM)                                         DERI0130
296          B(J,1)=REMARK-H                                                        DERI0140
297          Y1=YCOMP(N,B,Z,MDIM,NDIM,BDIM)                                         DERI0150
298          B(J,1)=REMARK                                                          DERI0160
299          DERIV=(Y2-Y1)/(2.0*H)                                                  DERI0170
300          CNTROL(21)=IHOLD                                                       DERI0180
301          RETURN                                                                 DERI0190
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302           END                                                              DER10200
303           SUBROUTINE DMPMAT (CNTROL,N,A,C)                                 DMPM0000
304           INTEGER CNTROL(25)                                               DMPM0010
305           REAL A(N,M),C(M)                                                 DMPM0020
306           JJ=IABS(CNTROL(1))                                               DMPM0030
307           IF (CNTROL(21).EQ.70) GO TO 1                                    DMPM0040
308           PRINT 4, CNTROL(20)                                              DMPM0050
309           GO TO 2                                                          DMPM0060
310     1     PRINT 5, CNTROL(20)                                              DMPM0070
311     2     DO 3 J=1,JJ                                                      DMPM0080
312     3     PRINT 6, J,C(J),(A(J,K),K=1,JJ)                                  DMPM0090
313           PRINT 7                                                          DMPM0100
314           RETURN                                                           DMPM0110
315     4     FORMAT (15H0 AT ITERATION I3,59H, IN THE MATRIX EQUATION  A X = C,DMPM0120
316          1 THE ROWS OF C AND A ARE /11H  J         C(J)                  **********DMPM0130
317          2**********  A(J,K)  ********************************************DMPM0140
318          3**************)                                                   DMPM0150
319          5 FORMAT ('H0 AFTER INVERSION AT ITERATION ',I4,'  IN THE MATRIX EQUDMPM0160
320          1ATION  AX = C.  THE ROWS OF C AND A ARE'/                       DMPM0170
321          311H  J         C(J)                    ********************  X(J,K)  *****DMPM0180
322          4**************************************************  )           DMPM0190
323     6     FORMAT (1H I2,E15.5,F20.5,4E15.5/(E38.5,4E15.5))                 DMPM0200
324     7     FORMAT (1X)                                                      DMPM0210
325           END                                                              DMPM0220
326           SUBROUTINE GAUSS (B,Z,MDIM,NDIM,BDIM,A)                          GAUS0000
327           COMMON CNTROL                                                    GAUS0010
328           INTEGER CNTROL(25),BDIM                                          GAUS0020
329           REAL A(BDIM,BDIM),B(BDIM,4),Z(MDIM,NDIM),S(24,6)                 GAUS0030
330           BIN(K,J)=0.5-0.5*(-1.)**(K/2**(J-1))                             GAUS0040
331           IF (CNTROL(22).EQ.0.) GO TO 1                                    GAUS0050
332           DETERM=1.                                                        GAUS0060
333           CNTROL(22)=0                                                     GAUS0070
334     1     NN=0                                                             GAUS0080
335           NOR=0                                                            GAUS0090
336           NWW=0                                                            GAUS0100
337           NWFLAG=0                                                         GAUS0110
338           NVSTOR=0                                                         GAUS0120
339           PCHGSS=0.                                                        GAUS0130
340           IF (CNTROL(10).LE.0) GO TO 2                                     GAUS0140
341           FAC=0.1*CNTROL(10)                                               GAUS0150
342           FACLIM=CNTROL(10)                                                GAUS0160
343           GO TO 3                                                          GAUS0170
344     2     FAC=1.0                                                          GAUS0180
345           FACLIM=FAC                                                       GAUS0190
346     3     OLFAC=0.                                                         GAUS0200
347           JJ=CNTROL(1)                                                     GAUS0210
348           LIMIT=CNTROL(6)                                                  GAUS0220
349           L=CNTROL(7)                                                      GAUS0230
350           CNTROL(20)=NN                                                    GAUS0240
351           CNTROL(21)=69                                                    GAUS0250
352           DO 4 J=1,JJ                                                      GAUS0260
353     4     B(J,2)=B(J,1)                                                    GAUS0270
354           SCALL=0.5                                                        GAUS0280
355           MARK10=CNTROL(10)                                                GAUS0290
356     5     CALL SETUP (B,Z,A,SUMSQ,MDIM,NDIM,BDIM)                          GAUS0300
357           IF (DETERM.NE.0.) GO TO 6                                        GAUS0310
358           PRINT 43                                                         GAUS0320
359           CALL DMPMAT (CNTROL,BDIM,A,B(1,3))                               GAUS0330
360           CNTROL(21)=4                                                     GAUS0340
361           GO TO 19                                                         GAUS0350
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      6     IF (CNTROL(10).LE.-1.AND.BIN(L,4).EQ.1.) GO TO 7                  GAUS0360
            IF (CNTROL(10).LE.-1) GO TO 26                                    GAUS0370
            IF (BIN(L,3).EQ.1.) CALL DMPMAT (CNTROL,BDIM,A,B(1,3))            GAUS0380
      7     IF (BIN(L,2).EQ.1.) PRINT 42                                      GAUS0390
            IF (NN.EQ.0) SOMIN=SUMSQ                                          GAUS0400
            IF (BIN(L,2).EQ.1.) PRINT 44, NN,SUMSQ,(B(J,1),J=1,JJ)            GAUS0410
            IF (CNTROL(10).LE.-1) GO TO 26                                    GAUS0420
            IF (CNTROL(20).LE.LIMIT) GO TO 8                                  GAUS0430
            CNTROL(21)=3                                                      GAUS0440
            GO TO 19                                                          GAUS0450
      CALCULATE SOLUTION FOR NORMAL EQUATIONS                                 GAUS0460
      8     IF (JJ.NE.1) GO TO 9                                              GAUS0470
            B(1,3)=B(1,3)/A(1,1)                                              GAUS0480
            A(1,1)=1./A(1,1)                                                  GAUS0490
            GO TO 10                                                          GAUS0500
      9     CALL MATINV (A,JJ,B(1,3),1,DETERM,BDIM)                           GAUS0510
      10    IF (BIN(L,3).EQ.0.) GO TO 11                                      GAUS0520
            IHOLD=CNTROL(21)                                                  GAUS0530
            CNTROL(21)=70                                                     GAUS0540
      CNTROL(21) = 70 HAS THE INVERSE MATRIX PRINTED OUT OF DMPMAT            GAUS0550
            CALL DMPMAT (CNTROL,BDIM,A,B(1,3))                                GAUS0560
            CNTROL(21)=IHOLD                                                  GAUS0570
      11    NN=NN+1                                                           GAUS0580
            CNTROL(20)=NN                                                     GAUS0590
            RESCAL=0.5                                                        GAUS0600
            IF (DETERM.EQ.0.) GO TO 5                                         GAUS0610
            DO 12 I=1,JJ                                                      GAUS0620
            S(I,4)=-1.                                                        GAUS0630
            IF (B(I,3).LT.0..AND.B(I,1).LT.0..OR.B(I,3).GT.0..AND.B(I,1).GT.0.GAUS0640
           1) S(I,4)=1.                                                       GAUS0650
      C..   S(I,2) CONTAINS THE PERMITTED CHANGE AND ITS REASSESSMENTS        GAUS0660
      C..   S(I,3) CONTAINS THE PREDICTED LSTSQ CORRECTION                    GAUS0670
      C..   S(I,4)=1. IF THE LSTSQ CORRECTION IS OF THE SAME SIGN AS S(J,1)   GAUS0680
      C..   AND =-1. OTHERWISE.                                              GAUS0690
            S(I,1)=B(I,1)                                                     GAUS0700
            S(I,3)=B(I,3)                                                     GAUS0710
            S(I,6)=S(I,4)                                                     GAUS0720
      12    S(I,2)=(FAC-OLFAC)*S(I,1)*S(I,4)                                  GAUS0730
            IF (BIN(L,3).EQ.0.) GO TO 13                                      GAUS0740
            PRINT 45, ((S(J,I),I=1,4),J=1,JJ)                                 GAUS0750
      CORRECT THE CURRENT VALUES OF THE PARAMETERS..                          GAUS0760
      13    CALL BTEST (CNTROL,B,BDIM)                                        GAUS0770
      C..IF CONVERGENCE DUE TO STEPPING DOWN B(J,3) AND NOT FITTING. OVERRIDE.GAUS0780
            IF (CNTROL(21).NE.1) GO TO 20                                     GAUS0790
      14    IF ((CNTROL(10).EQ.-1).AND.(FAC.NE.FACLIM)) GO TO 27              GAUS0800
            IF (CNTROL(10).GE.0.AND.NVSTOR.NE.0) GO TO 16                     GAUS0810
            IF (CNTROL(10).NE.-1) GO TO 19                                    GAUS0820
            CNTROL(21)=5                                                      GAUS0830
      C..OVERRIDE OPTION                                                      GAUS0840
            IF (CNTROL(12).LE.0) GO TO 15                                     GAUS0850
            CALL OVERID (L,NOP,SOMIN,JJ,A,S,BDIM)                             GAUS0860
            IF (NOP.GE.CNTROL(12)) GO TO 19                                   GAUS0870
            GO TO 39                                                          GAUS0880
      C..WRONGWAY OPTION                                                      GAUS0890
      15    IF (CNTROL(12).EQ.0) GO TO 16                                     GAUS0900
            CALL WRNGWY (L,NWW,SOMIN,JJ,A,S,B,NWFLAG,BDIM)                    GAUS0910
            IF (NWW.GT.IABS(CNTROL(12))) GO TO 16                             GAUS0920
            GO TO 5                                                           GAUS0930
      C..LABELS 940,960,1030 AND 1040 WERE COMMENTS                           GAUS0940
      C     ..  PARAMETER CHANGE OPTION                                       GAUS0950
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      16    IF (CNTROL(11).EQ.0.AND.CNTROL(12).EQ.0) GO TO 17                 GAUS0970
            IF (CNTROL(11).EQ.0) GO TO 19                                     GAUS0980
            CALL PCHANG (NVSTOR,SQMIN,PCHGSS,JJ,S,A,B,BDIM,NN,64,628)         GAUS0990
            DO 18 J=1,JJ                                                      GAUS1000
      17    B(J,1)=S(J,1)                                                     GAUS1010
      18    RETURN                                                            GAUS1020
      19    C..RESTRAINED CHANGE MECHANISM                                    GAUS1030
      20    IF ((MARK10.EQ.0.).OR.(CNTROL(10).EQ.-1)) GO TO 24                GAUS1040
            DO 22 J=1,JJ                                                      GAUS1050
            IF (ABS(B(J,3)).LE.AUS(FAC*S(J,1))) GO TO 21                      GAUS1060
            B(J,3)=S(J,4)*FAC*S(J,1)                                          GAUS1070
            IF (ABS(B(J,3)).EQ.AHS(S(J,1)).AND.S(J,4).EQ.-1.) B(J,3)=B(J,3)+SIGAUS1080
           1GN(1.0E-10,S(J,1))                                                GAUS1090
            GO TO 22                                                          GAUS1100
      21    S(J,2)=B(J,3)                                                     GAUS1110
            S(J,4)=0.                                                         GAUS1120
      22    CONTINUE                                                          GAUS1130
            DO 23 J=1,JJ                                                      GAUS1140
            IF (S(J,4).NE.0.) GO TO 24                                        GAUS1150
      23    CONTINUE                                                          GAUS1160
            FAC=FACLIM                                                        GAUS1170
      24    DO 25 J=1,JJ                                                      GAUS1180
      25    B(J,1)=(B(J,2)+B(J,3))                                            GAUS1190
            IF (CNTROL(10).NE.-1) CNTROL(10)=-3                               GAUS1200
            GO TO 5                                                           GAUS1210
      26    TEST=ABS((SUMSO-SQMIN)/SQMIN)                                     GAUS1220
            IF (TEST.GT.1.0E-10) GO TO 29                                     GAUS1230
            CNTROL(21)=2                                                      GAUS1240
     CNTROL(21) = 2  SIGNALS THAT SUM OF SQUARES CHANGES NEGLIGIBLY.          GAUS1250
            IF ((CNTROL(10).EQ.-1).AND.(FAC.NE.FACLIM)) GO TO 27              GAUS1260
            PRINT 46                                                          GAUS1270
            IF (NVSTOR.EQ.0) RETURN                                           GAUS1280
            GO TO 16                                                          GAUS1290
      27    RESCAL=0.5                                                        GAUS1300
            CNTROL(21)=69                                                     GAUS1310
      C..SET RESTRAINED CHANGE FACTOR                                         GAUS1320
            OLFAC=FAC                                                         GAUS1330
            FAC=2.*FAC                                                        GAUS1340
            IF (FAC.GT.FACLIM) FAC=FACLIM                                     GAUS1350
            CNTROL(10)=MARK10                                                 GAUS1360
            NN=NN+1                                                           GAUS1370
            DO 28 J=1,JJ                                                      GAUS1380
            B(J,3)=S(J,3)                                                     GAUS1390
      28    S(J,2)=(FAC-OLFAC)*S(J,1)*S(J,4)                                  GAUS1400
            GO TO 20                                                          GAUS1410
      C..RESTRAINED CHANGE PARTITION CONVERGENCE TEST                         GAUS1420
      29    IF (SUMSO.LT.SQMIN) GO TO 38                                      GAUS1430
            IF (NWFLAG.LE.0) GO TO 30                                         GAUS1440
            NWFLAG=0                                                          GAUS1450
            GO TO 39                                                          GAUS1460
      30    CNTROL(10)=-1                                                     GAUS1470
            DIFFAC=FAC-OLFAC                                                  GAUS1480
            SCAFAC=RESCAL*DIFFAC                                              GAUS1490
            RESCAL=SCALE*RESCAL                                               GAUS1500
            IF ((MARK10.NE.0).OR.BIN(L,4).NE.1) GO TO 33                      GAUS1510
            DO 31 J=1,JJ                                                      GAUS1520
      31    B(J,3)=SCALL*B(J,3)                                               GAUS1530
            CALL BTEST (CNTROL,B,BDIM)                                        GAUS1540
            IF (CNTROL(21).NE.1) GO TO 32                                     GAUS1550
            IF (BIN(L,4).EQ.1.) PRINT 47                                      GAUS1560
                                                                              GAUS1570
                                                                              GAUS1580
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482
483          GO TO 14
484       32 IF (BIN(L,4).EQ.1.) PRINT 48, SCAFAC,OLFAC                    GAUS1590
485          GO TO 24                                                      GAUS1600
486       33 DO 34 J=1,JJ                                                 GAUS1610
487          S(J,2)=SCALE*S(J,2)                                           GAUS1620
488          A(J,3)=S(J,2)                                                 GAUS1630
489       34 CONTINUE                                                      GAUS1640
490          INTER=CNTROL(9)                                               GAUS1650
491          IF (OLFAC.EQ.0) GO TO 35                                      GAUS1660
492          CNTROL(9)=3                                                   GAUS1670
493    CNTROL(9) = 3 INVOKES AN ALTERNATE CONVERGENCE LIMIT IN BTEST       GAUS1680
494       35 CALL BTEST (CNTROL,B,BDIM)                                    GAUS1690
495          CNTROL(9)=INTER                                               GAUS1700
496          DO 36 J=1,JJ                                                  GAUS1710
497          IF (ABS(S(J,3)).GT.FAC*S(J,1)) B(J,3)=S(J,2)+OLFAC*S(J,1)*S(J,4) GAUS1720
498       36 CONTINUE                                                      GAUS1730
499          IF (CNTROL(21).NE.1) GO TO 37                                 GAUS1740
500          IF (BIN(L,4).EQ.1.) PRINT 47                                  GAUS1750
501          GO TO 14                                                      GAUS1760
502       37 IF (BIN(L,4).EQ.1.) PRINT 49, SCAFAC,OLFAC                    GAUS1770
503          GO TO 13                                                      GAUS1780
504       38 SOMIN=SUMSQ                                                   GAUS1790
505          NOR=0                                                         GAUS1800
506          NWW=0                                                         GAUS1810
507          NWFLAG=0                                                      GAUS1820
508       39 IF (MARK10.GE.1) FAC=0.1*MARK10                               GAUS1830
509          NN=CNTROL(20)                                                 GAUS1840
510          OLFAC=0.                                                      GAUS1850
511          DO 40 J=1,JJ                                                  GAUS1860
512       40 B(J,2)=B(J,1)                                                 GAUS1870
513          IF (NVSTOR.NE.0.OR.CNTROL(11).EQ.1) GO TO 41                  GAUS1880
514          CNTROL(10)=MARK10                                             GAUS1890
515          GO TO 5                                                       GAUS1900
516       41 CNTROL(10)=0                                                  GAUS1910
517          GO TO 5                                                       GAUS1920
518    C                                                                   GAUS1930
519       42 FORMAT (114HOCYCLE    SUM OF SQUARES                         GAUS1940
520      1*********  B(J)  PARAMETERS **************************************GAUS1950
521       43 FORMAT ('0 THE FOLLOWING NORMAL EQUATIONS ARE SINGULAR ')      GAUS1960
522      44 FORMAT (1H012,2E21.7,4E15.7//(E45.7,4E15.7))                    GAUS1970
523       45 FORMAT ('0 S PARAMETERS '///(4E12.4))                          GAUS1980
524       46 FORMAT ('0 NEGLIGIBLE CHANGE IN SUM OF SQUARES')               GAUS1990
525       47 FORMAT ('0 PARTITION LESS THAN MESH LIMIT - - - RECYCLE RESTRAINT GAUS2000
526      1MECHANISM')                                                       GAUS2010
527       48 FORMAT ('0 NO REDUCTION IN SUM OF SQUARES. RESCALE PARAMETERS '///'GAUS2020
528        1 RESTRAINED CHANGE MECHANISM SET AT (',E11.4,'+',F7.4,') TIMES THEGAUS2030
529        2 PREDICTED LSTSQ CORRECTION.')                                  GAUS2040
530       49 FORMAT ('0 NO REDUCTION IN SUM OF SQUARES. RESCALE PARAMETERS '///'GAUS2050
531        1 RESTRAINED CHANGE MECHANISM SET AT (',E11.4,'+',F7.4,') TIMES THEGAUS2060
532        2 VALUE OF THE ORIGINAL PARAMETER')                              GAUS2070
533          END                                                           GAUS2080
534          SUBROUTINE OVERID (L,NOR,SOMIN,JJ,A,S,B,BDIM)                 GAUS2090
535          COMMON CNTROL                                                 OVER0000
536          INTEGER CNTROL(25),BDIM                                       OVER0010
537          DIMENSION A(BDIM,BDIM), S(BDIM,6), P(24), B(BDIM,4)           OVER0020
538          NOR=NOR+1                                                     OVER0030
539          IF (NOR.GT.CNTROL(12)) GO TO 2                                OVER0040
540          IF (BIN(L,2).EQ.1.) PRINT 4, NOR,SOMIN                        OVER0050
541          IF (NOR.NE.1) RETURN                                          OVER0060
             DO 1 J=1,JJ                                                   OVER0070
                                                                           OVER0080
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                                                                        OVER0090
                                                                        OVER0100
                      S(J,5)=A(J,J)                                     OVER0110
542     1             P(J)=S(J,1)                                       OVER0120
543                   ICYC=CNTROL(20)-1                                 OVER0130
544                   RETURN                                            OVER0140
545     2             DO 3 J=1,JJ                                       OVER0150
546                   A(J,J)=S(J,5)                                     OVER0160
547     3             B(J,1)=P(J)                                       OVER0170
548                   NOR=NOR-1                                         OVER0180
549                   IF (BIN(L,2).EQ.1.) PRINT 5, NOR,ICYC             OVER0190
550                   RETURN                                            OVER0200
551     C                                                               OVER0210
552        4 FORMAT ('0 NO CONVERGENCE IN SCALING INTERVAL. OVERRIDE MECHANISM OVER0220
553         INOW OPERABLE - OVERRIDE ',I3,' SUM OF SQUARES MINIMUM IS',E14.6) OVER0230
554        5 FORMAT ('0 AFTER ',I3,' OVERRIDES    REFERENCE ITERATION ',I4) BIN00000
555                   END                                               BIN00010
556                   FUNCTION BIN (N,N)                                BIN00020
557                   BIN=0.5-0.5*(-1.)**(N/2**(N-1))                   BIN00030
558                   RETURN                                            WRNG0000
559                   END                                               WRNG0010
560                   SUBROUTINE WRNGWY (L,NWW,SOMIN,JJ,A,S,B,NWFLAG,BDIM) WRNG0020
561                   COMMON CNTROL                                     WRNG0030
562                   INTEGER CNTROL(25),BDIM                           WRNG0040
563                   DIMENSION A(BDIM,BDIM), S(24,6), P(24), B(BDIM,4) WRNG0050
564                   IF (BIN(L,2).EQ.1.) PRINT 7, SOMIN                WRNG0060
565                   IF (NWW.NE.0) GO TO 2                             WRNG0070
566                   DO 1 J=1,JJ                                       WRNG0080
567                   S(J,5)=A(J,J)                                     WRNG0090
568     1             P(J)=S(J,1)                                       WRNG0100
569                   ICYC=CNTROL(20)-1                                 WRNG0110
570     2             SMAX=ABS(S(1,3)/S(1,1))                           WRNG0120
571                   DO 3 J=2,JJ                                       WRNG0130
572                   IF (ABS(S(J,3)/S(J,1)).GT.SMAX) SMAX=ABS(S(J,3)/S(J,1)) WRNG0140
573     3             CONTINUE                                          WRNG0150
574                   DO 4 J=1,JJ                                       WRNG0160
575                   SFRAC=ABS(S(J,3)/(SMAX*S(J,1)))                   WRNG0170
576     4             B(J,1)=S(J,1)-0.05*S(J,6)*S(J,1)*SFRAC            WRNG0180
577                   CNTROL(10)=-1                                     WRNG0190
578                   IF (NWW.GE.IABS(CNTROL(12))) GO TO 5              WRNG0200
579                   NWW=NWW+1                                         WRNG0210
580     C..NWFLAG LIMITS WRONGWAY PARTITION TO ONE RESTRAINED CHANGE CYCLE WRNG0220
581                   NWFLAG=1                                          WRNG0230
582                   RETURN                                            WRNG0240
583     5             DO 6 J=1,JJ                                       WRNG0250
584                   A(J,J)=S(J,5)                                     WRNG0260
585     6             B(J,1)=P(J)                                       WRNG0270
586                   IF (BIN(L,2).EQ.1.) PRINT 8, ICYC                 WRNG0280
587                   RETURN                                            WRNG0290
588     C                                                               WRNG0300
589        7 FORMAT ('0 NO CONVERGENCE IN SCALING INTERVAL. WRONGWAY MECHANISM WRNG0310
590         INOW OPERABLE   SUM OF SQUARES MINIMUM IS ',E14.6)          WRNG0320
591        8 FORMAT ('0 WRONGWAY LIMIT EXCEEDED     REFERENCE ITERATION',I4) PCHA0000
592                   END                                               PCHA0010
593                   SUBROUTINE PCHANG (NVSTOR,SOMIN,PCHGSS,JJ,S,A,B,BDIM,NN,*,*) PCHA0020
594                   COMMON CNTROL                                     PCHA0030
595                   INTEGER CNTROL(25),BDIM                           PCHA0040
596                   DIMENSION S(24,6), P(24), B(BDIM,1), A(BDIM,BDIM) PCHA0050
597                   IF (CNTROL(21).EQ.1.OR.CNTROL(21).EQ.2) CNTROL(21)=09 PCHA0060
598                   IF (NVSTOR.NE.0.AND.SOMIN.GE.PCHGSS) GO TO 2      PCHA0065
599                   DO 1 J=1,JJ                                       PCHA0070
600                   CON13=CNTROL(13)
601
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      NUMBEE=JJ                                                          PCHA0080
      S(J,5)=A(J,J)                                                      PCHA0090
      IF (CNTROL(12).GE.0.0R.CNTROL(21).EQ.69) P(J)=S(J,1)               PCHA0100
      CONTINUE                                                           PCHA0110
      IF (SOMIN.NE.PCHGSS) GO TO 5                                       PCHA0120
    1 PRINT 8                                                            PCHA0130
    2 CNTROL(1)=IABS(CNTROL(2))                                          PCHA0140
      NVSTOR=ISTORE                                                      PCHA0150
      IF (NVSTOR.NE.0) GO TO 4                                           PCHA0160
      DO 3 J=1,NUMBEE                                                    PCHA0170
      CNTROL(13)=CON13                                                   PCHA0180
      CNTROL(1)=NUMBEE                                                   PCHA0190
      JJ=NUMBEE                                                          PCHA0200
      A(J,J)=S(J,5)                                                      PCHA0210
      B(J,1)=P(J)                                                        PCHA0220
    3 RETURN 1                                                           PCHA0230
      NVSTOR=0                                                           PCHA0240
    4 JJ=CNTROL(1)                                                       PCHA0250
      CNTROL(11)=0                                                       PCHA0260
      CNTROL(12)=0                                                       PCHA0270
      RETURN 2                                                           PCHA0280
      PCHGSS=SOMIN                                                       PCHA0290
    5 ISTORE=CNTROL(1)                                                   PCHA0300
      IF (CNTROL(1).NE.IABS(CNTROL(2))) GO TO 6                          PCHA0310
      CNTROL(1)=IABS(CNTROL(2))-1                                        PCHA0320
      GO TO 7                                                            PCHA0330
    6 CNTROL(1)=IABS(CNTROL(2))                                          PCHA0340
      NVSTOR=ISTORE                                                      PCHA0350
    7 NVAB=IABS(NVSTOR)                                                  PCHA0360
      NE=NV=IABS(CNTROL(1))                                              PCHA0370
      PRINT 9, NVAB,NE=NV                                                PCHA0380
      NN=CNTROL(20)                                                      PCHA0390
      JJ=CNTROL(1)                                                       PCHA0400
      RETURN 2                                                           PCHA0410
C                                                                        PCHA0420
    8 FORMAT ('0 PARAMETER CHANGE OPTION UNAVAILING')                    PCHA0430
    9 FORMAT ('0THE NUMBER OF LSTSQ PARAMETERS HAS BEEN CHANGED FROM',I3PCHA0440
     1,' TO',I3)                                                         PCHA0450
      END                                                                PCHA0460
      SUBROUTINE MATINV (A,N,B,M,DETERM,IDIM)                            MATI0000
C                                                                        MATI0010
C     **  THE DIMENSIONS OF IPIVOT,PIVOT AND INDEX MUST BE EQUAL TO BDIMMATI0020
C                                                                        MATI0030
      DIMENSION A(IDIM,IDIM), B(IDIM,IDIM)                               MATI0040
      DIMENSION IPIVOT(24), INDEX(24,2), PIVOT(24)                       MATI0050
      EQUIVALENCE (IROW,JROW), (ICOLUM,JCOLUM), (AMAX,T,SWAP)            MATI0060
C                                                                        MATI0070
C     INITIALIZATION                                                     MATI0080
C                                                                        MATI0090
      DETERM=1.0                                                         MATI0100
    1 DO 2 J=1,N                                                         MATI0110
      IPIVOT(J)=0                                                        MATI0120
    2 DO 22 I=1,N                                                        MATI0130
C                                                                        MATI0140
C     SEARCH FOR PIVOT ELEMENT                                           MATI0150
C                                                                        MATI0160
      AMAX=0.0                                                           MATI0170
      DO 7 J=1,N                                                         MATI0180
      IF (IPIVOT(J)-1) 3,7,3                                             MATI0190
    3 DO 6 K=1,N                                                         MATI0200
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      IF ((IPIVOT(K)-1) 4,6,26                                          MAT10210
      IF (ABS(AMAX)-ABS(A(J,K))) 5,6,6                                  MAT10220
    4 IROW=J                                                            MAT10230
    5 ICOLUM=K                                                          MAT10240
      AMAX=A(J,K)                                                       MAT10250
    6 CONTINUE                                                          MAT10260
    7 CONTINUE                                                          MAT10270
      IF (AMAX) 8,27,8                                                  MAT10280
    8 IPIVOT(ICOLUM)=IPIVOT(ICOLUM)+1                                   MAT10290
C                                                                       MAT10300
C     INTERCHANGE ROWS TO PUT PIVOT ELEMENT ON DIAGONAL                 MAT10310
C                                                                       MAT10320
      IF (IROW-ICOLUM) 9,13,9                                           MAT10330
    9 DETERM=-DETERM                                                    MAT10340
      DO 10 L=1,N                                                       MAT10350
      SWAP=A(IROW,L)                                                    MAT10360
      A(IROW,L)=A(ICOLUM,L)                                             MAT10370
   10 A(ICOLUM,L)=SWAP                                                  MAT10380
      IF (M) 13,13,11                                                   MAT10390
   11 DO 12 L=1,M                                                       MAT10400
      SWAP=B(IROW,L)                                                    MAT10410
      B(IROW,L)=B(ICOLUM,L)                                             MAT10420
   12 B(ICOLUM,L)=SWAP                                                  MAT10430
   13 INDEX(I,1)=IROW                                                   MAT10440
      INDEX(I,2)=ICOLUM                                                 MAT10450
      PIVOT(I)=A(ICOLUM,ICOLUM)                                         MAT10460
      DETERM=DETERM*PIVOT(I)                                            MAT10470
      A(ICOLUM,ICOLUM)=1.0                                              MAT10480
      DO 14 L=1,N                                                       MAT10490
   14 A(ICOLUM,L)=A(ICOLUM,L)/PIVOT(I)                                  MAT10500
      IF (M) 17,17,15                                                   MAT10510
   15 DO 16 L=1,M                                                       MAT10520
   16 B(ICOLUM,L)=B(ICOLUM,L)/PIVOT(I)                                  MAT10530
C                                                                       MAT10540
C     REDUCE NON-PIVOT ROWS                                             MAT10550
C                                                                       MAT10560
   17 DO 22 L1=1,N                                                      MAT10570
      IF (L1-ICOLUM) 18,22,18                                           MAT10580
   18 T=A(L1,ICOLUM)                                                    MAT10590
      A(L1,ICOLUM)=0.0                                                  MAT10600
      DO 19 L=1,N                                                       MAT10610
   19 A(L1,L)=A(L1,L)-A(ICOLUM,L)*T                                     MAT10620
      IF (M) 22,22,20                                                   MAT10630
   20 DO 21 L=1,M                                                       MAT10640
   21 B(L1,L)=B(L1,L)-B(ICOLUM,L)*T                                     MAT10650
   22 CONTINUE                                                          MAT10660
C                                                                       MAT10670
C     INTERCHANGE COLUMNS                                               MAT10680
C                                                                       MAT10690
      DO 25 I=1,N                                                       MAT10700
      L=N+1-I                                                           MAT10710
      IF (INDEX(L,1)-INDEX(L,2)) 23,25,23                               MAT10720
   23 JROW=INDEX(L,1)                                                   MAT10730
      JCOLUM=INDEX(L,2)                                                 MAT10740
      DO 24 K=1,N                                                       MAT10750
      SWAP=A(K,JROW)                                                    MAT10760
      A(K,JROW)=A(K,JCOLUM)                                             MAT10770
                                                                        MAT10780
                                                                        MAT10790
                                                                        MAT10800
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      A(K,JCOLUM)=SWAP                                                  MATI0810
                                                                        MATI0820
  24  CONTINUE                                                          MATI0830
  25  CONTINUE                                                          MATI0840
  26  RETURN                                                            MATI0850
  27  DETERM=0.                                                         MATI0860
      RETURN                                                            MATI0870
      END                                                               PRIN0000
      SUBROUTINE PRINDA (IC,Z,M,N)                                      PRIN0010
      DIMENSION IC(25), Z(M,N)                                          PRIN0020
      PRINT 2                                                           PRIN0030
      NPTS=IABS(IC(3))                                                  PRIN0040
      NVAR=IC(5)                                                        PRIN0050
      DO 1 I=1,NPTS                                                     PRIN0060
      PRINT 3, I,(Z(J,I),J=1,NVAR)                                      PRIN0070
    1 RETURN                                                            PRIN0080
                                                                        PRIN0090
C                                                                       PRIN0100
    2 FORMAT( '0  INPUT DATA'//)                                        PRIN0110
    3 FORMAT (I4,8E15.5)                                                FINA0000
      END                                                               FINA0010
      SUBROUTINE FINALE (B,Z,MDIM,NDIM,IDIM,A)                          FINA0020
      COMMON CNTROL                                                     FINA0030
      DIMENSION Z(MDIM,NDIM), B(IDIM), A(IDIM,IDIM), VAR(24)            FINA0040
      INTEGER CNTROL(25)                                                FINA0050
      DIMENSION FRCTDV(9)                                               FINA0060
      JJ=IABS(CNTROL(1))                                                FINA0070
      NUMBER=IABS(CNTROL(3))                                            FINA0080
      AV=0.0                                                            FINA0090
      AV1=0.0                                                           FINA0100
      AV2=0.0                                                           FINA0110
      YMAX=0.0                                                          FINA0120
      ZMAX=0.0                                                          FINA0130
      ZZMAX=0.0                                                         FINA0140
      SUMSQ=0.                                                          FINA0150
      CNTROL(10)=-2                                                     FINA0160
CNTROL(10)=-2 DIRECTS SETUP TO CALCULATE VARIANCE RATHER THAN SUMSQ     FINA0170
      CALL SETUP (B,Z,A,SUMSQ,MDIM,NDIM,IDIM)                           FINA0180
      DO 1 I=1,JJ                                                       FINA0190
    1 VAR(I)=SQRT(A(I,I)*SUMSQ)                                         FINA0200
      SS=SUMSQ*(NUMBER-JJ)                                              FINA0210
      STDEV=SQRT(SUMSQ)                                                 FINA0220
      PRINT 5, CNTROL(20),(B(J),J=1,JJ)                                 FINA0230
      PRINT 6, (VAR(I),I=1,JJ)                                          FINA0240
      DO 2 I=1,JJ                                                       FINA0250
    2 FRCTDV(I)=VAR(I)/B(I)                                             FINA0260
      PRINT 7, (FRCTDV(I),I=1,JJ)                                       FINA0270
      PRINT 8, SS,SUMSQ,STDEV                                           FINA0280
      PRINT 9                                                           FINA0290
      IF (CNTROL(5).EQ.2) PRINT 10                                      FINA0300
      IF (CNTROL(5).NE.2) PRINT 11                                      FINA0310
      DO 4 N=1,NUMBER                                                   FINA0320
      YC=YCOMP(N,B,Z,MDIM,NDIM,IDIM)                                    FINA0330
      DELY=Z(1,N)-YC                                                    FINA0340
      IF (Z(1,N).NE.0.0) RATIO=DELY/Z(1,N)                              FINA0350
      IF (Z(1,N).EQ.0.0) RATIO=1.0E30                                   FINA0360
      ABSRAT=ABS(RATIO)                                                 FINA0370
      AV=AV+DELY                                                        FINA0380
      AV1=AV1+RATIO                                                     FINA0390
      AV2=AV2+ABSRAT                                                    FINA0400
      IF (CNTROL(5).EQ.2) PRINT 13, N,Z(2,N),Z(1,N),YC,DELY,RATIO
      IF (CNTROL(5).NE.2) PRINT 12, N,Z(1,N),YC,DELY,RATIO
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      ABSVAL=ABS(DELY)                                                  FINA0410
      IF (YMAX.GT.ABSVAL) GO TO 3                                       FINA0420
      YMAX=ABSVAL                                                       FINA0430
      YYMAX=DELY                                                        FINA0440
      MARK=N                                                            FINA0450
      IF (ZMAX.GT.ABSRAT) GO TO 4                                       FINA0460
    3 ZMAX=ABSRAT                                                       FINA0470
      ZZMAX=RATIO                                                       FINA0480
      MARK1=N                                                           FINA0490
    4 CONTINUE                                                          FINA0500
      D=NUMBER                                                          FINA0510
      AV=AV/D                                                           FINA0520
      AV1=AV1/D                                                         FINA0530
      AV2=AV2/D                                                         FINA0540
      RTMNSQ=SQRT(SUMSQ)                                                FINA0550
      PRINT 14, AV,AV1,AV2,YYMAX,MARK,ZMAX,MARK1,RTMNSQ                 FINA0560
      CALL MANIP (A,B,Z,MDIM,NDIM,IDIM)                                 FINA0570
      RETURN                                                            FINA0580
C                                                                       FINA0590
    5 FORMAT (8H- AFTER I3,12H  ITERATIONS/45H0 THE MINIMIZING VALUES OFFINA0600
     1 THE PARAMETERS ARE//(1X,5E15.7))                                 FINA0610
     6 FORMAT ('0 WITH THE FOLLOWING STD. DEVIATIONS '//                FINA0620
     1(1X,5E15.7))                                                      FINA0630
     7 FORMAT('0 WITH THE FOLLOWING FRACTIONAL DEVIATIONS'//   1X,5F15.3)FINA0640
     8 FORMAT ('0  THE SUM OF SQUARES,VARIANCE AND SAMPLE STD. DEVIATION FINA0650
     1 FOR THIS FIT ARE'3E16.5)                                         FINA0660
C                                                                       FINA0670
    9 FORMAT (118H0**********************************************************FINA0680
     1**********************************/)                              FINA0690
   10 FORMAT (1H0,'NUMBER      X OBSERVED     Y OBSERVED      Y CFINA0690
     1ALCULATED      OBS - CALC     (OBS - CALC) / OBS   '/)           FINA0700
   11 FORMAT (94H0NUMBER      Y OBSERVED     Y CALCULATED      OFINA0710
     1BS - CALC     (OBS - CALC) / OBS        /)                      FINA0720
   12 FORMAT (I5,E23.5,E17.5,2E19.5)                                    FINA0730
   13 FORMAT (I5,E23.5,2E17.5,2E19.5)                                   FINA0740
   14 FORMAT (30H0  AVERAGE DEVIATION          E14.5/30H   AVERAGE RELFINA0750
     1 DEV          E14.5/30H  AVE ABS REL DEV          E14.5/30H   MFINA0760
     2AXIMUM DEVIATION    E14.5,6X,10H AT POINT I4/30H   MAXIMUM RFINA0770
     3EL DEV          E14.5,6X,10H AT POINT I4/30H   ROOT MEAN SQUARE FINA0780
     4DEVIATION E14.5)                                                  FINA0790
      END                                                               FINA0800
      SUBROUTINE MANIP (M,B,Z,MDIM,NDIM,IDIM)                           MANI0000
C     CALLS TO THIS SUBROUTINE ALLOW MODIFICATION OF THE DATA.          MANI0010
C     M = 1 AFTER B'S ARE READ.                                         MANI0020
C     M = 2 IF CNTROL(3) IS LESS THAN 0. AND DATA ARE READ BY THIS SUB- MANI0030
C           ROUTINE.                                                    MANI0040
C     M = 3 AFTER DATA ARE READ IN BY INDATA.                           MANI0050
C     M = 4 AFTER STATISTICS HAVE BEEN COMPUTED (CALL OUTDAT)           MANI0060
      COMMON CNTROL                                                     MANI0070
      INTEGER CNTROL(25)                                                MANI0080
      DIMENSION B(IDIM), Z(MDIM,NDIM)                                   MANI0090
      GO TO (1,2,3,12), M                                               MANI0100
    1 RETURN                                                            MANI0110
    2 RETURN                                                            MANI0120
    3 NPTS=IABS(CNTROL(3))                                              MANI0130
      NVARS=IABS(CNTROL(5))                                             MANI0140
      DO 4 I=1,NPTS                                                     MANI0150
      STOR=Z(1,I)                                                       MANI0160
      Z(1,I)=Z(2,I)                                                     MANI0170
    4 Z(2,I)=STOR                                                       MANI0180
      MID=NPTS/2                                                        MANI0190
```

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                                                                                MANI0200
                                                                                MANI0210
                                                                                MANI0220
                                                                                MANI0230
842          5      Z(2,1)=Z(2,1)/(B(4)/B(5))                                   MANI0240
843                 IF (B(2)) 7,6,7                                             MANI0250
844                 B(2)=Z(1,1)+(Z(1,1)-Z(1,2))*(Z(2,1)/(Z(2,2)-Z(2,1)))        MANI0260
845          6      IF (B(3)) 9,8,9                                             MANI0270
846          7      B(3)=Z(1,NPTS)                                              MANI0280
847          8      IF (B(1)) 11,10,11                                          MANI0290
848          9      B(1)=-(ALOG((Z(1,MID)-B(3))/(Z(1,1)-B(3)))/(Z(2,MID)-Z(2,1))MANI0300
849         10      PRINT 13, (B(I),I=1,3)                                      MANI0310
850         11      RETURN                                                      MANI0320
851                 CALL OUTDAT (B,Z,MDIM,NDIM,IDIM)                            MANI0330
852         12      RETURN                                                      MANI0340
853                                                                             MANI0350
854          C                                                                  YCOM0000
855            13 FORMAT (1H0,'THE ESTIMATED FIRST ORDER RATE PARAMETERS ARE:  ',YCOM0010
856              13G12.4)                                                       YCOM0020
857                END                                                          YCOM0030
858                FUNCTION YCOMP (N,B,Z,MDIM,NDIM,BDIM)                        YCOM0040
859                INTEGER CNTROL(25)                                           YCOM0050
860                COMMON CNTROL                                                YCOM0060
861                INTEGER BDIM                                                 YCOM0070
862                DIMENSION B(BDIM), Z(MDIM,NDIM)                              OUTD0000
863           1    YCOMP=(B(2)-B(3))*EXP(-B(1)*Z(2,N))+B(3)                     OUTD0010
864                RETURN                                                       OUTD0020
865                END                                                          OUTD0030
866                SUBROUTINE OUTDAT (B,Z,MDIM,NDIM,IDIM)                       OUTD0040
867                DIMENSION B(IDIM), Z(MDIM,NDIM)                              OUTD0050
868                COMMON CNTROL                                                OUTD0060
869                REAL K2LOG                                                   OUTD0070
870                INTEGER CNTROL(25)                                           OUTD0080
871                IF (CNTROL(2).NE.6.OR.B(6).EQ.0.0) RETURN                    OUTD0090
872                K2LOG=ALOG10(B(1)/B(6))                                      OUTD0100
873                PRINT 1, K2LOG                                               OUTD0110
874                RETURN                                                       OUTD0120
875          C                                                                  
876             1 FORMAT(1H0,' THE BASE 10 LOGARITHM OF THE SECOND ORDER RATE CONSTA
877              INT IS:  ',F6.3)                                               
878                END                                                          
END OF FILE
```