Rialdi, G., and Hermans, J. (1966), *J. Amer. Chem. Soc. 88*, 5719.
Scarpa, J., Mueller, D., and Klotz, I. (1967), *J. Amer. Chem. Soc. 89*, 6024.
Schwarz, G., and Seelig, J. (1968), *Biopolymers 6*, 1263.
Segal, D., and Harrington, W. (1967), *Biochemistry 6*, 768.
Snipp, R., Miller, W., and Nylund, R. (1965), *J. Amer. Chem. Soc. 87*, 3547.
Woodward, C., and Rosenberg, A. (1970), *Proc. Nat. Acad. Sci. U. S., 66*, 1067.
Zimm, B., and Bragg, J. (1959), *J. Chem. Phys. 31*, 526.
Zimm, B., and Rice, S. (1960), *Mol. Phys. 3*, 391.

Kinetics of the Oxidation of Ferrocyanide by Horseradish Peroxidase Compounds I and II*

Brian B. Hasinoff and H. B. Dunford†

ABSTRACT: The kinetics of the oxidation of ferrocyanide by both horseradish peroxidase compounds I and II (HRP-I and HRP-II) have been studied as a function of pH at 25° and an ionic strength of 0.11. The apparent second-order rate constant, $k_{2,app}$, for the reaction of HRP-I with ferrocyanide varied from 4.1×10^7 to 5.5×10^5 M^{-1} sec^{-1} over the pH range 3.7–11.3. The value of $k_{3,app}$ for the reaction of HRP-II with ferrocyanide varied from 1.3×10^7 to 2.4×10^2 M^{-1} sec^{-1} over the pH range 2.8–10.3. The pH dependence of the HRP-I–ferrocyanide reaction has been interpreted in terms of a single ionization of pK = 5.3 at the active site of HRP-I. The pH dependence of the HRP-II–ferrocyanide reaction has been interpreted in terms of three ionizations with pK's of 3.4, 5.2, and 8.6 at the active site of HRP-II. The steady-state kinetics of the HRP-catalyzed oxidation of ferrocyanide by H_2O_2 were studied by the measurement of initial reaction velocities. Rate constants obtained by the steady-state method were in agreement with those obtained from studies of isolated reactions on the stopped-flow apparatus. Our kinetic results can be correlated with some of the current ideas on the structures of HRP-I and HRP-II. The rate constants for oxidation of ferrocyanide by HRP-I and HRP-II are compared to rate constants for other substrates available in the literature.

Horseradish peroxidase (EC 1.11.1.7, donor–H_2O_2 oxidoreductase) was first observed to form a spectroscopically distinct compound, HRP-II, with H_2O_2 over 30 years ago (Keilin and Mann, 1937).[1] Theorell (1941) found another compound, HRP-I, was formed prior to HRP-II when HRP was treated with H_2O_2.

The generally accepted reaction sequences and stoichiometries for the reaction of HRP and H_2O_2 with a substrate such as ferrocyanide are

$$\text{HRP} + \text{H}_2\text{O}_2 \xrightarrow{k_{1,app}} \text{HRP-I} \quad (1)$$

$$\text{HRP-I} + \text{Fe(CN)}_6^{4-} \xrightarrow{k_{2,app}} \text{HRP-II} + \text{Fe(CN)}_6^{3-} \quad (2)$$

$$\text{HRP-II} + \text{Fe(CN)}_6^{4-} \xrightarrow{k_{3,app}} \text{HRP} + \text{Fe(CN)}_6^{3-} \quad (3)$$

(George, 1952, 1953a; Chance, 1952a). No particular states of ionization of the reactants are implied. The rate constants are labeled apparent rate constants as $k_{2,app}$ and $k_{3,app}$ at least show a marked dependence on pH. Ferrocyanide was chosen as a substrate because it undergoes a simple one-electron oxidation to ferricyanide without the production of free-radical intermediates. A recent study on the HRP-catalyzed oxidation of luminol by H_2O_2 at pH 8.0 has shown that the free radicals produced from the substrate are oxidized at kinetically comparable rates by HRP-I (Cormier and Prichard, 1968). Even though ferrocyanide is not a substrate of physiological significance for HRP, the analyses of the pH dependence of the $k_{2,app}$ and $k_{3,app}$ rate data can reveal information about possible ionizations at the active site of HRP-I and HRP-II which affect the kinetics of the ferrocyanide oxidation. Whether the ionizations are of importance

* From the Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada. *Received October 17, 1969.* Supported financially by the National Research Council of Canada. B. B. H. is indebted to the National Research Council of Canada for a scholarship.

† To whom to address correspondence.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: HRP, horseradish peroxidase; HRP-I, HRP-II, HRP-CN, and HRP-F: compounds I and II, cyanide and fluoride complexes of HRP; RZ, purity number; P-II, HP-II, H_2P-II, and H_3P-II: states of ionization of HRP-II; P-I and HP-I: states of ionization of HRP-I; ferrocyanide, hexacyanoferrate(II) without regard to state of protonation; ferricyanide, hexacyanoferrate(III); K_A, the acid dissociation constant of $HFe(CN)_6^{3-}$; K_1, K_2, etc., single acid dissociation constants either on HRP-I or HRP-II as indicated; $k_{2,obsd}$ and $k_{3,obsd}$: pseudo-first-order rate constants for the reactions of HRP-I and HRP-II with ferrocyanide; $k_{2,app}$, $k_{3,app}$: apparent second-order rate constants of the reactions of HRP-I and HRP-II with ferrocyanide; k_1, k_2, etc., second-order rate constants; v, initial reaction velocity; [ ] and [ ]$_0$: molar concentration and initial molar concentration without regard to state of protonation; V_t, V_∞, A_t, A_∞: voltage and absorbance at time t and time infinity; ΔV, ΔA: $|V_t - V_\infty|$ and $|A_t - A_\infty|$; $t_{1/2}$, reaction half-time; μ, ionic strength.

in other HRP oxidation reactions can only be determined by experiment.

Experimental Section

Materials. Purified, lyophilized HRP obtained from Boehringer-Mannheim had an initial RZ of 0.8, measured by taking the ratio of absorbance at 403 and 280 nm. A further purification of the HRP was achieved by gel chromatography. A sample of 0.5 g of HRP was dissolved in 5 ml of phosphate buffer of pH 6.2 and ionic strength of 0.1. This was added to a 2.5 × 45 cm column (Sephadex laboratory column type K25145) of Sephadex C50-(CM) which had also been equilibrated with pH 6.2 phosphate buffer of ionic strength 0.1. When the ionic strength increased linearly from 0.1 to 0.2 the major peak was observed to come off the column centered at an effluent volume of 90 ml. The major peak was collected in 5-ml fractions and those of RZ 2.8 or larger were combined and stored as an ammonium sulfate precipitate at 5°. The precipitate was dissolved, then dialyzed, centrifuged, and filtered through a Millipore filter prior to use. The activity of the HRP was measured by the *o*-dianisidine test described by the Worthington Biochemical Corp. (1967) and increased from 550 to 2400 units per mg upon purification. The concentration of HRP was determined spectrophotometrically at 403 nm using a molar absorptivity of 9.1×10^4 M^{-1} cm^{-1} (Keilin and Hartree, 1951).

Doubly distilled water was used in the preparation of all solutions. The ionic strength of all reaction mixtures was kept constant at 0.11 with 0.01 contributed by the buffer and the remainder by KNO_3 and ferrocyanide. Solutions of ferrocyanide were prepared less than 1 hr before use from Baker reagent grade $K_4Fe(CN)_6 \cdot 3H_2O$ under diffuse lighting and were protected from light during storage and use. These precautions were taken to prevent either thermal or photodissociation. The molar absorptivity of ferricyanide was measured to be 1060 M^{-1} cm^{-1} at 420 nm in agreement with the published value of 1050 M^{-1} cm^{-1} (Birk, 1969). The molar absorptivity of ferrocyanide is much less than ferricyanide and can be neglected. The concentrations of the H_2O_2 solutions were determined spectrophotometrically after the molybdate catalyzed oxidation of iodide to iodine (Ovenston and Rees, 1950; Ramette and Sandford, 1965).

Stopped-Flow Kinetic Experiments. Kinetic measurements were made on a pneumatically driven stopped-flow apparatus. The driving syringes and reaction chambers were thermostatted at 25.0°. The reactions were followed spectrophotometrically at 425 nm using a detection system which has been described elsewhere (Ellis and Dunford, 1968). The total absorbance change was kept small ($\Delta A < 0.020$) so that the relative voltage changes observed on the oscilloscope, ΔV, were proportional to ΔA.

The buffer and HRP solutions were not mixed prior to commencement of the reaction in the stopped-flow apparatus to ensure that the HRP did not decompose at either high or low pH. The rate of the reaction being studied was always much faster than the rate of decomposition of HRP at these pH extremes (Maehly, 1953).

Steady-state kinetic measurements were conducted spectrophotometrically on a Cary 14 recording spectrophotometer equipped with a slide-wire for the absorbance range 0–0.1. The cell compartments were thermostatted at 25.0°. The reaction was followed by measuring the increase in absorbance at 420 nm due to the production of ferricyanide. Ferrocyanide, HRP, H_2O_2, and sufficient water to obtain a constant total volume of 2.08 ml were added with Hamilton microliter syringes to a cuvet containing 2 ml of buffer–KNO_3 solution. The final addition, which initiated the reaction, was made by stirring the solution with a glass rod on which microliter amounts of H_2O_2 solution had been deposited. The initial velocity, v, was computed at the extrapolated zero time by measuring the slope of the absorbance–time trace. Blank reaction rate runs were conducted without HRP present and in no case was appreciable reaction rate measured for the uncatalyzed H_2O_2–ferrocyanide reaction.

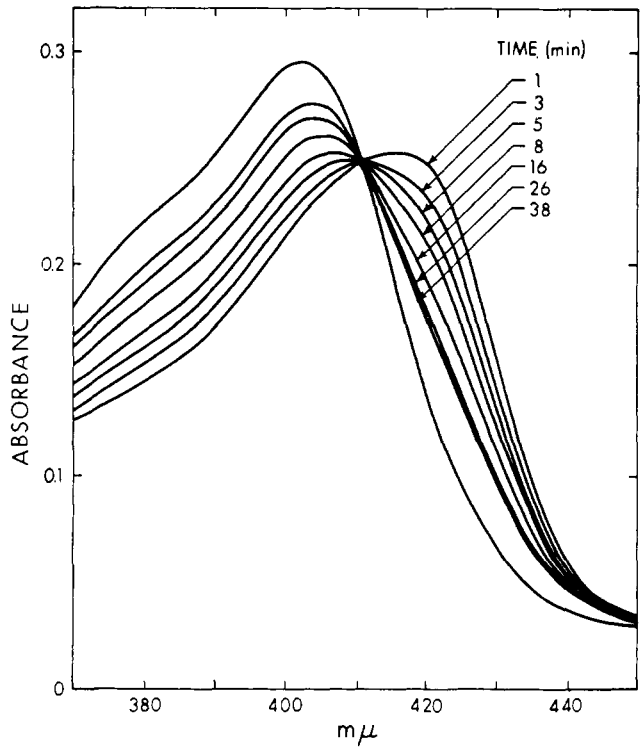


FIGURE 1: Plot of absorbance *vs.* wavelength at the times indicated showing the conversion of HRP-II into HRP. The isosbestic point at 411 nm indicated the presence of only two absorbing species, HRP and HRP-II.

Results

Neither ferrocyanide nor ferricyanide in solution with HRP was observed to change the absorption spectrum of HRP at least over a period of 1 hr. This experiment indicates that no detectable complex is formed between either of these compounds and HRP. It also indicates that dissociation of cyanide is not occurring from either ferro- or ferricyanide. The buffer solutions were chosen so that their effective pH ranges overlapped. From the continuous nature of the rate data as a function of pH it was concluded that there were no anomalous effects caused by buffers.

The ionization constant, K_A, of $HFe(CN)_6^{3-}$, was obtained by interpolation of data from an ionic strength dependence study (Jordan and Ewing, 1962). The value of K_A at an ionic strength of 0.11 and 25° is 7.24×10^{-4} M. Similarly, the

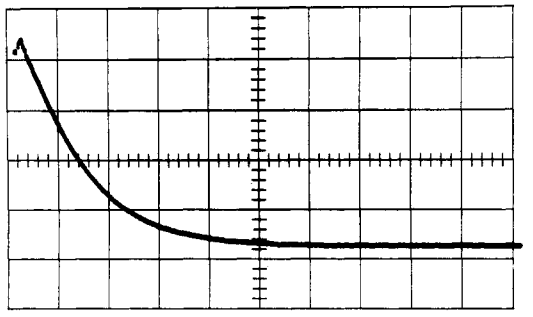


FIGURE 2: Oscilloscope trace of voltage *vs.* time for the reaction of HRP-II with ferrocyanide at pH 8.81. The reaction was observed at a monochromator setting of 425 nm. Each large division on the horizontal axis corresponds to 200 msec. The initial concentrations of ferrocyanide and HRP-II were 6.1×10^{-4} and 1×10^{-6} M, respectively. Data points from this trace are plotted in Figure 3.

ionization constant for $H_2Fe(CN)_6^{2-}$ is 6.00×10^{-2} M. Other ionization constants for $H_4Fe(CN)_6$ are larger than 0.1 M.

Kinetics of the HRP-II–Ferrocyanide Reaction. It was found experimentally that HRP-II could be prepared by adding approximately 1.1 molar equiv of H_2O_2 and 0.5 molar equiv of *p*-cresol to a dilute unbuffered solution of HRP

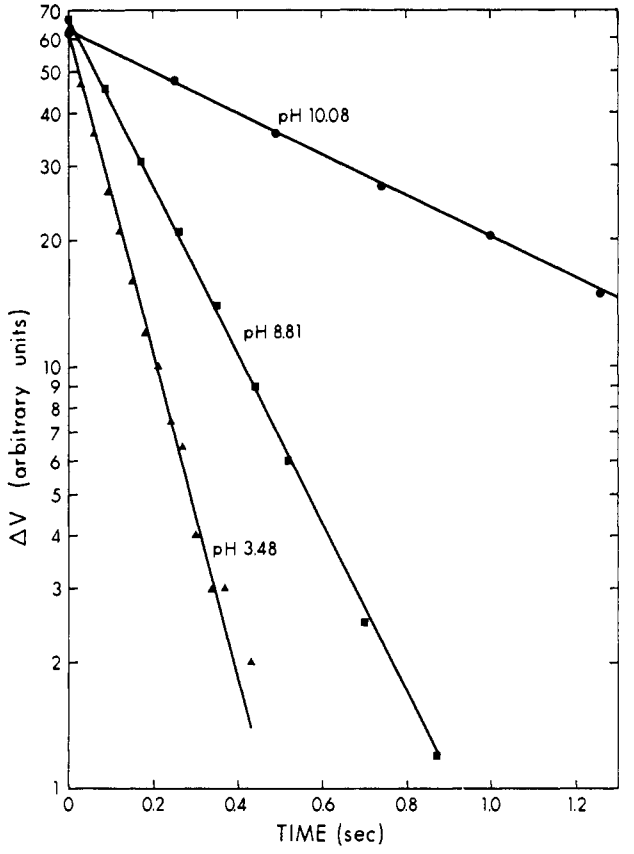


FIGURE 3: Semilogarithmic plots of ΔV *vs.* time for the reaction of HRP-II with ferrocyanide. At each pH at which the reaction was studied the initial concentration of HRP-II was about 1×10^{-6} M. At pH 10.08, $[Fe(CN)_4^{4-}]_0 = 3.7 \times 10^{-3}$ M, at pH 8.81, 6.1×10^{-4} M, and at pH 3.48, 2.9×10^{-5} M. The pseudo-first-order rate constants, $k_{3,obsd}$, were obtained from the slopes of the lines.

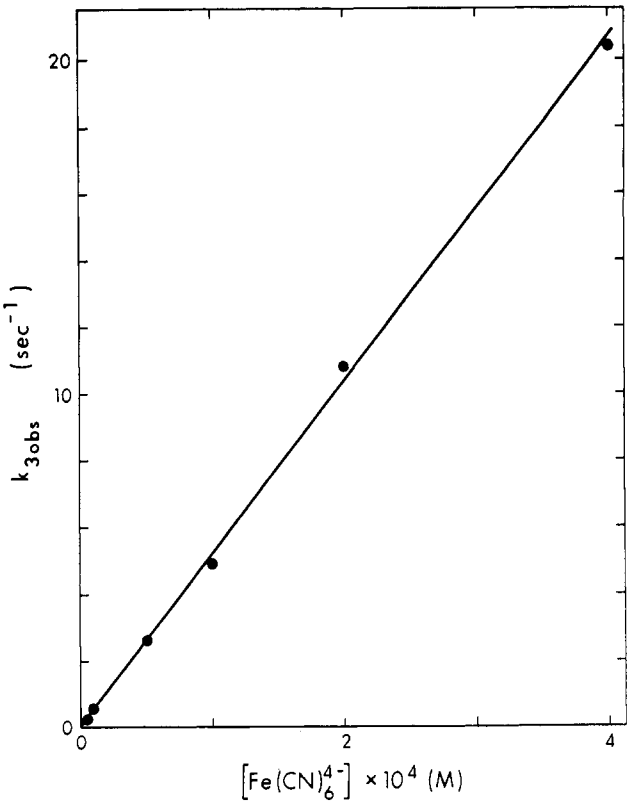


FIGURE 4: Plot of $k_{3,obsd}$ *vs.* $[Fe(CN)_6^{4-}]$ at pH 5.90 for the HRP-II–ferrocyanide reaction. The straight line is the weighted least-squares best-fit line. The slope, $k_{3,app}$, with its standard error is $(5.1 \pm 0.1) \times 10^5$ M^{-1} sec^{-1}. The intercept with its standard error is $(3.0 \pm 1.5) \times 10^{-2}$ sec^{-1} which is zero within the interval of the 95% confidence limit.

of pH approximately 6. About 70% HRP-II and 30% HRP were formed initially as measured from their molar absorptivities (George, 1953a). The HRP-II was converted directly into HRP over a period of minutes as shown by the return of the original HRP spectrum (Figure 1). An isosbestic point at 411 nm was maintained, indicating the presence of only two absorbing species, HRP and HRP-II. The original HRP spectrum was obtained when the reaction was allowed to go to completion over a long period of time or upon the addition of ferrocyanide. Sufficient quantities of HRP-II were present up to 0.5 hr after preparation to study the oxidation of ferrocyanide.

The kinetics of the HRP-II–ferrocyanide reaction at a given pH and for a large excess of ferrocyanide are consistent with the differential rate expression

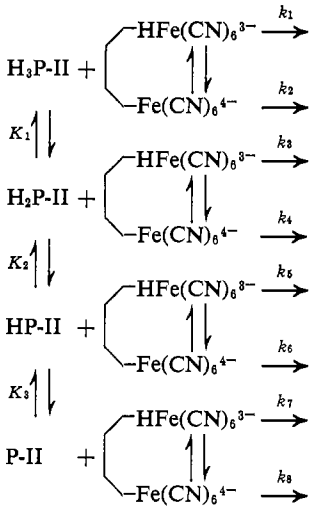
$$-\frac{d[\text{HRP-II}]}{dt} = k_{3,obsd}[\text{HRP-II}] \quad (4)$$

Linear semilogarithmic plots of ΔV *vs.* time proved the validity of eq 4 and facilitated the determination of $k_{3,obsd}$. An example of an experimental trace is shown in Figure 2 and of semilogarithmic plots in Figure 3. Values of $k_{3,obsd}$ as a function of ferrocyanide concentration at a constant pH of 5.90, shown in Figure 4, prove the relation

$$k_{3,obsd} = k_{3,app}[Fe(CN)_6^{4-}] \quad (5)$$

from which values of $k_{3,app}$ were calculated. The total concentration of HRP in the reaction mixture was 1.5×10^{-6} M. The $[Fe(CN)_6^{4-}]$ was at least 10 times larger than [HRP-II] at low pH and for pH values greater than 4, larger excesses of ferrocyanide were used. The apparent rate constants, $k_{3,app}$, are plotted semilogarithmically *vs.* pH in Figure 5 and listed in Table I.

The simplest reaction that accounts satisfactorily for the $k_{3,app}$ data within the estimated experimental error is



(I)

The vertical arrows denote fast proton-transfer reactions. In a reaction which involves two or more ionizations, molecular rather than group ionization constants are necessarily implied (Dixon and Webb, 1964). Group ionization constants, of which the molecular ionization constants consist, cannot be determined from the analysis of the rate data alone.

From eq 1 it follows that $k_{3,app} =$

$$\left\{\frac{k_1[H^+]}{K_A} + \left(k_2 + \frac{k_3K_1}{K_A}\right) + \left(k_4 + \frac{k_5K_2}{K_A}\right)\frac{K_1}{[H^+]} + \left(k_6 + \frac{k_7K_3}{K_A}\right)\frac{K_1K_2}{[H^+]^2} + \frac{k_8K_1K_2K_3}{[H^+]^3}\right\} \Big/ \left\{\left(1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2} + \frac{K_1K_2K_3}{[H^+]^3}\right)\left(1 + \frac{[H^+]}{K_A}\right)\right\} \quad (6)$$

Two pathways such as

$$HP\text{-}II + Fe(CN)_6^{4-} \xrightarrow{k_6}$$

and

$$P\text{-}II + HFe(CN)_6^{3-} \xrightarrow{k_7}$$

are kinetically indistinguishable and are combined together into one term in eq 6. The slope of the line in Figure 5 is -1 for pH values greater than 8.5, indicating that k_8 is insignificant (Dixon and Webb, 1964). After a preliminary graphical analysis of the semilogarithmic plot of $k_{3,app}$ *vs.* pH to obtain approximate values for the ionization and rate constants, a nonlinear least-squares computer analysis[2] was carried out on the $k_{3,app}$–pH data to obtain the best-fit parameters for eq 6. It is necessary to use a nonlinear least-squares analysis as eq 6 contains more than two adjustable parameters.[3] It was found that $(k_2 + k_3K_1/K_A)$ did not contribute significantly to the computed values of $k_{3,app}$. Consequently in the final analysis of the data, k_8 and $(k_2 + k_3K_1/K_A)$ were set equal to zero. The values of the remaining parameters, listed in Table

TABLE I: Apparent Rate Constants for the HRP-II–Ferrocyanide Reaction at 25.0° and $\mu = 0.11$.[a]

pH	$k_{3,app}$ (M^{-1} sec^{-1})	Buffer[b]	pH	$k_{3,app}$ (M^{-1} sec^{-1})	Buffer[b]
2.80	1.3×10^7	GH	6.18	3.0×10^4	M
3.12	7.6×10^6	GH	6.18	3.6×10^4	M
3.38	4.7×10^6	GH	6.47	3.0×10^4	M
3.39	5.0×10^6	GH	6.57	2.9×10^4	P
3.48	3.3×10^6	GH	6.78	2.3×10^4	M
3.87	1.1×10^6	A	6.83	1.8×10^4	P
4.11	5.6×10^5	A	7.39	1.5×10^4	P
4.28	3.1×10^5	A	7.43	1.4×10^4	T
4.41	2.8×10^5	A	7.38	3.6×10^4	P
4.71	1.4×10^5	A	7.79	1.4×10^4	T
4.83	2.1×10^5	A	7.86	1.3×10^4	T
4.83	1.9×10^5	M	8.41	9.5×10^3	T
5.02	2.0×10^5	M	8.58	8.9×10^3	T
5.22	1.1×10^5	A	8.81	8.4×10^3	GN
5.22	1.1×10^5	A	8.83	6.4×10^3	T
5.25	1.3×10^5	A	8.98	5.5×10^3	T
5.41	1.3×10^5	A	9.11	4.0×10^3	GN
5.46	9.0×10^4	M	9.35	2.1×10^3	C
5.65	6.8×10^4	M	9.61	1.4×10^3	GN
5.67	6.6×10^4	M	9.79	9.3×10^2	C
5.71	5.8×10^4	M	9.92	5.4×10^2	GN
5.90	5.2×10^4	P	10.32	2.4×10^2	GN

[a] Standard errors on the experimental points are estimated at ±10%. [b] Buffer key: A, acetic acid–NaOH; GH, glycine–HNO_3; M, maleic acid–NaOH; T, Tris–HNO_3; C, $NaHCO_3$–NaOH; GN, glycine–NaOH; P, KH_2PO_4–NaOH.

[2] IBM Share Library (1964), SDA 3094, reprogrammed for use on a University of Alberta IBM 360 computer by L. L. Rines, J. A. Plambeck, and D. J. Francis.

[3] Given a model

$$\hat{Y}_i = f(X_{i1}, X_{i2}, \ldots, X_{im}; b_1, b_2, \ldots b_k)$$

which predicts the value, $\hat{Y}$, of a dependent variable Y, where the model f contains m independent variables, X_{im} and k parameters, b_k, and given n observations

$$Y_i, X_{i1}, X_{i2}, \ldots, X_{im} = 1, 2, \ldots, n$$

the nonlinear least-squares computer program will compute the least-squares estimates of the b's. That is, the program will adjust the b's to minimize

$$\phi = \sum_{i=1}^{n} (Y_i - \hat{Y}_i)^2$$

(Marquardt, 1963).[2]

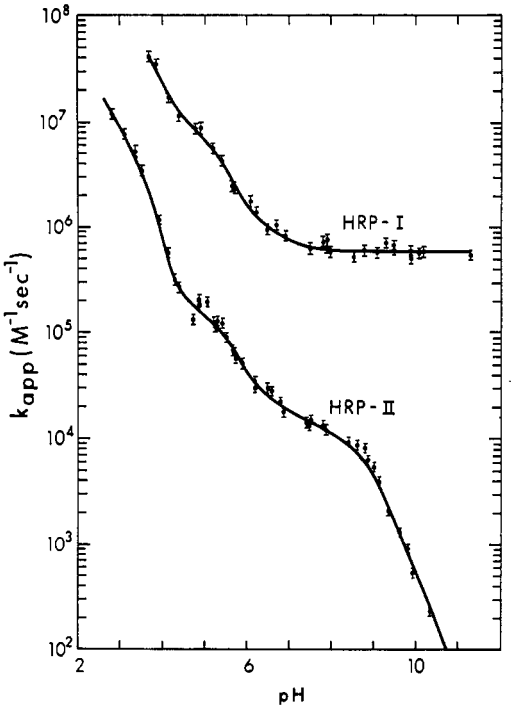


FIGURE 5: Semilogarithmic plot of $k_{2,app}$ and $k_{3,app}$ *vs.* pH for the reactions of HRP-I and HRP-II with ferrocyanide. The error limits drawn on the data points are based on an estimated standard deviation of ±10%. The solid lines were computed on the basis of best-fit parameters obtained for reactions I and II.

II, were used to calculate the lower solid line in Figure 5.[4] Upper limits for $(k_2 + k_3K_1/K_A)$ and k_8 of 1×10^6 and 25 M^{-1} sec^{-1} are obtained by assuming that they can contribute a maximum of 10% to $k_{3,app}$. An upper limit of 1×10^6 M^{-1} sec^{-1} for $(k_2 + k_3K_1/K_A)$ is still significantly larger than the value of either of two other parameters, $(k_4 + k_5K_2/K_A)$ and $(k_6 + k_7k_8/K_A)$, that contribute to the value for $k_{3,app}$. The large magnitude of k_1 has "swamped out" the effect of the $(k_2 + k_3K_1/K_A)$ term in determining the value of $k_{3,app}$.

Kinetics of the HRP-I–Ferrocyanide Reaction. A judicious choice of reactant concentrations was required so that the formation of HRP-I was complete in less than the dead time of the stopped-flow apparatus (5 msec) and so that the subsequent reaction of HRP-II with ferrocyanide did not interfere. This was accomplished by using a large excess of H_2O_2 (1×10^{-4} M) over HRP (5×10^{-7} M) and a smaller excess of ferrocyanide (1.5×10^{-6} M).

The reaction of HRP-I with ferrocyanide becomes faster at lower pH and could not be studied below pH 3.67 on our stopped-flow apparatus. At pH values greater than 6, the reaction is sufficiently slow that it could be studied in the presence of a large excess of ferrocyanide. Under these conditions pseudo-first-order kinetics were observed.

[4] The standard error limits listed in Table II were obtained from the nonlinear least-squares computer program and are analogous to those obtained for two parameters in a linear least-squares analysis. The magnitude of the standard error of a parameter estimate is taken to indicate the interval of values over which a particular parameter can vary without significantly affecting the fit of the data to the equation and is due to random error in the data.

TABLE II: Rate and Ionization Constants[a] Obtained by Nonlinear Least-Squares Analysis for the Reaction of HRP-II with Ferrocyanide (eq 6).

k_1	$(2.3 \pm 0.4) \times 10^7$ M^{-1} sec^{-1}
$\left(k_2 + \frac{k_3K_1}{K_A}\right)$	0
$\left(k_4 + \frac{k_5K_2}{K_A}\right)$	$(2.2 \pm 0.3) \times 10^5$ M^{-1} sec^{-1}
$\left(k_6 + \frac{k_7K_3}{K_A}\right)$	$(1.6 \pm 0.1) \times 10^4$ M^{-1} sec^{-1}
k_8	0
K_1	$(3.6 \pm 1.2) \times 10^{-4}$ M
K_2	$(6.1 \pm 1.5) \times 10^{-6}$ M
K_3	$(2.7 \pm 0.3) \times 10^{-9}$ M

[a] Errors given are the standard errors of the parameter estimates obtained from nonlinear least-squares analysis.

$$\frac{-d[HRP\text{-}I]}{dt} = k_{2,obsd}[HRP\text{-}I] \quad (7)$$

Nonlinear least-squares analysis of the ΔV *vs.* time data in the integrated equation proved the validity of eq 7. Values of $k_{2,obsd}$ at a constant pH of 7.86 are plotted in Figure 6 and show that

$$k_{2,obsd} = k_{2,app}[Fe(CN)_6^{4-}] \quad (8)$$

Thus the differential rate expression for the HRP-I–ferrocyanide reaction is

$$\frac{-d[HRP\text{-}I]}{dt} = k_{2,app}[Fe(CN)_6^{4-}][HRP\text{-}I] \quad (9)$$

For the pH range 3.7–6.0 an integrated form of eq 9 was used in the determination of $k_{2,app}$ (Frost and Pearson, 1961). In the analysis of the integrated equation it is not necessary to know the change in molar absorptivity in going from HRP-I to HRP-II as, under the experimental conditions described above, the concentration of HRP-I is, at zero time, effectively equal to the total concentration of HRP (5×10^{-7} M) in the reaction mixture which in turn is proportional to voltage change at zero time, ΔV_0. Hence the concentration of HRP-I at any time is proportional to ΔV at that time. In order to determine ΔV it is necessary to know the position of the base line at infinite time; it was found possible to obtain a better analysis by making this position an adjustable parameter within narrow limits. To estimate ΔV_0 the traces were extrapolated back to zero time using the dead time of the stopped-flow apparatus (5 msec). The value of ΔV_0 was also refined within narrow limits. Values of the concentration of ferrocyanide as a function of time were obtained from the initial concentration of ferrocyanide minus the amount of HRP-I which had reacted. The actual computer analysis of the second-order rate equation thus involved three adjustable parameters: the rate constant, $k_{2,app}$, ΔV_0, and a term for the

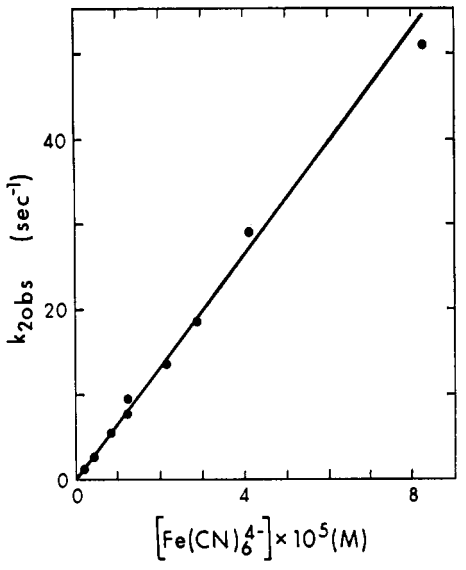


FIGURE 6: Plot of $k_{2,obsd}$ *vs.* $[Fe(CN)_6^{4-}]$ at pH 7.86 for the HRP-I–ferrocyanide reaction. The straight line is the weighted least-squares best-fit line. The slope, $k_{2,app}$, with its standard error is $(6.6 \pm 0.2) \times 10^5$ M^{-1} sec^{-1}. The intercept, (-0.20 ± 0.08) sec^{-1}, is zero within the interval of the 99% confidence limits.

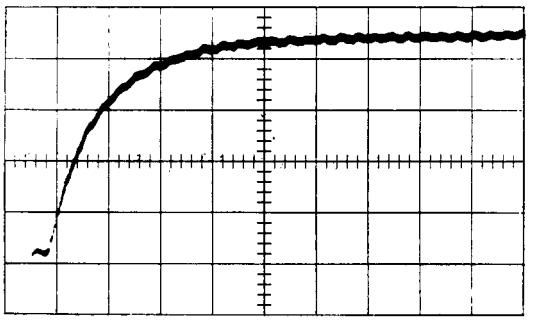


FIGURE 7: Oscilloscope trace of voltage *vs.* time for the reaction of HRP-I with ferrocyanide at pH 4.91. The reaction was followed at a monochromator setting of 425 nm. The large divisions on the horizontal axis correspond to 50 msec each. The initial concentrations of ferrocyanide and HRP-I were 2.5×10^{-6} and 4.8×10^{-7} M, respectively. Data points from this trace are plotted in Figure 8.

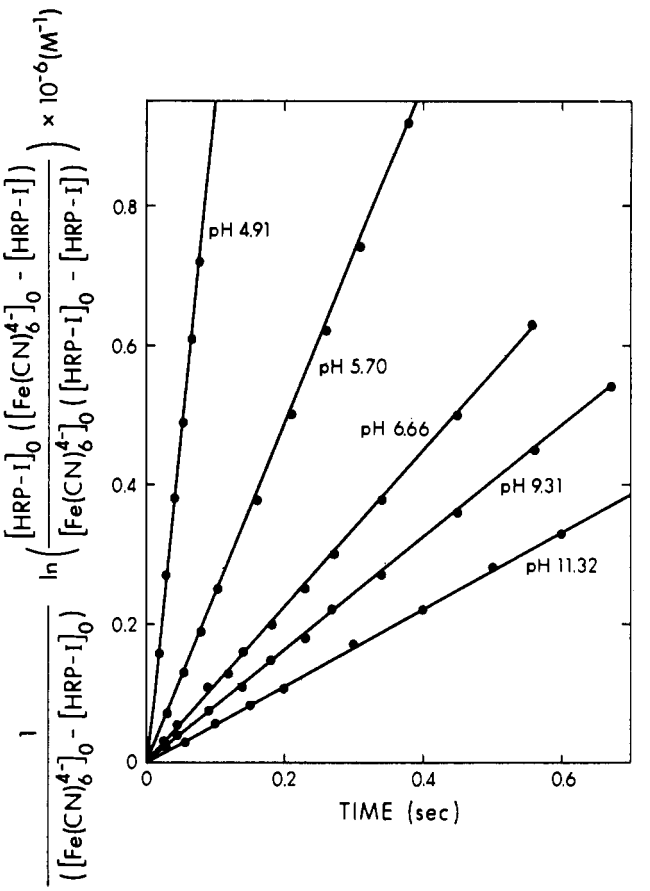


FIGURE 8: Plot of

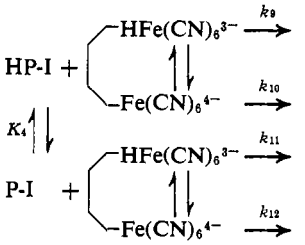
$$\frac{1}{(Fe(CN)_6^{4-}]_0 - [HRP\text{-}I]_0} \ln\left(\frac{[HRP\text{-}I]_0([Fe(CN)_6^{4-}]_0 - [HRP\text{-}I])}{[Fe(CN)_6^{4-}]_0([HRP\text{-}I]_0 - [HRP\text{-}I])}\right)$$

vs. time for the reaction of HRP-I with ferrocyanide. The straight lines were calculated from parameters obtained from a nonlinear least-squares analysis of ΔV time data in the integrated second-order rate equation and were plotted in this manner to give linear plots. The slopes of the solid lines give the apparent second-order rate constants, $k_{2,app}$. The initial concentration of HRP-I was 4.8×10^{-7} M at each pH the reaction was studied. At pH 4.91, $[Fe(CN)_6^{4-}]_0 = 2.6 \times 10^{-6}$ M, at pH 5.70, 2.6×10^{-6} M, at pH 6.66, 7.9×10^{-6} M, at pH 9.31, 7.9×10^{-6} M, and at pH 11.32, 5.4×10^{-6} M.

placement of the base line at infinite time. Typically the adjustments in the latter two parameters were less than one per cent of the magnitude of ΔV_0 (Hasinoff, 1970). To ensure a valid analysis 10–15 points were taken from each oscilloscope trace. An example of a trace is shown in Figure 7. At each pH three or four traces were used to determine an average $k_{2,app}$. Linear second-order plots of the computer results can be made, and examples are shown in Figure 8.

In experiments conducted at a constant pH but at two different wavelengths (411 and 425 nm) values of $k_{2,app}$ were found to be in agreement within experimental error which indicates that only a single reaction is being observed. In other experiments, conducted also at constant pH with $[H_2O_2]$ varied from 1×10^{-5} to 1×10^{-4} M, values of $k_{2,app}$ agreed within experimental error. The validity of the procedure is verified and as well it can be concluded that H_2O_2 does not react at an appreciable rate with HRP-II. Although H_2O_2 is known to react with HRP-II to give HRP-III (Chance, 1952b; George, 1953b) the latter cannot be formed to any significant extent under experimental conditions used in this work. The $k_{2,app}$ results are plotted *vs.* pH in Figure 5 and are listed in Table III.

The simplest reaction consistent with the $k_{2,app}$ data has a single ionization on HRP-I



(II)

TABLE III: Apparent Rate Constants for the HRP-I–Ferrocyanide Reaction at 25.0° and $\mu = 0.11$.[a]

pH	$k_{2,app}$ (M^{-1} sec^{-1})	Buffer[b]	pH	$k_{2,app}$ (M^{-1} sec^{-1})	Buffer[b]
3.67	4.1×10^7	A	7.81	7.3×10^5	P
3.85	3.6×10^7	A	7.87	6.6×10^5	P
4.15	1.9×10^7	M	7.87	8.3×10^5	P
4.40	1.2×10^7	A	7.99	5.8×10^5	T
4.80	8.8×10^6	A	8.54	5.1×10^5	T
4.91	8.9×10^6	M	8.80	6.1×10^5	GN
5.21	5.6×10^6	A	9.09	5.9×10^5	GN
5.40	4.2×10^6	M	9.31	7.1×10^5	C
5.65	2.5×10^6	M	9.49	6.8×10^5	GN
5.70	2.5×10^6	M	9.52	6.1×10^5	C
6.10	1.8×10^6	P	9.88	5.3×10^5	C
6.22	1.5×10^6	M	9.90	5.9×10^5	GN
6.45	9.6×10^5	M	9.92	5.8×10^5	C
6.66	1.0×10^6	P	10.19	5.8×10^5	C
6.93	8.5×10^5	M	10.20	6.0×10^5	GN
7.47	6.3×10^5	T	11.32	5.5×10^5	C

[a] Standard errors on the experimental points are estimated at ±10%. [b] Key as in Table I.

from which

$$k_{2,app} = \frac{\frac{k_9[H^+]}{K_A} + \left(k_{10} + \frac{k_{11}K_4}{K_A}\right) + \frac{k_{12}K_4}{[H^+]}}{\left(1 + \frac{K_4}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_A}\right)} \quad (10)$$

From nonlinear least-squares analysis of the $k_{2,app}$ data using eq 10 the values of the parameters listed in Table IV were obtained. The upper solid line in the semilogarithmic plot of $k_{2,app}$ *vs.* pH in Figure 5 was computed with these values obtained from eq 10.

Steady-State Kinetics of the HRP-Catalyzed H_2O_2–Ferrocyanide Reaction. The stoichiometry of the H_2O_2–ferrocyanide reaction was checked at each pH value as both the concentration of the H_2O_2 added and the ferricyanide produced in the reaction mixture were known. Within experimental error, 2 moles of ferricyanide was always produced per mole of H_2O_2 added (George, 1953a; Chance, 1952c). This check showed that no detectable amount of exogenous or endogenous donor reacted or less than the stoichiometric amount of ferricyanide would have been produced.

The initial velocity expression under steady-state conditions has been derived for eq 1–3 (Cormier and Prichard, 1968)

$$v = \frac{2k_{1,app}k_{2,app}k_{3,app}[HRP]_0[Fe(CN)_6^{4-}]_0[H_2O_2]_2}{(k_{1,app}k_{2,app} + k_{1,app}k_{3,app})[H_2O_2]_0 + k_{2,app}k_{3,app}[Fe(CN)_6^{4-}]_0} \quad (11)$$

where v is $d[Fe(CN)_6^{3-}]_0/dt$. The proportionality of v and $[HRP]_0$ was checked at pH 4.79. The values of $[Fe(CN)_6^{4-}]_0$ and $[H_2O_2]_0$ were kept constant at 1.00×10^{-4} and 3.55×10^{-5} M and v was measured as a function of $[HRP]_0$. The results are plotted in Figure 9. The intercept is zero within the 99% confidence interval, which indicates that no additional constant term is required for eq 11.

TABLE IV: Rate and Ionization Constants[a] Obtained by Nonlinear Least-Squares Analysis for the Reaction of HRP-I with Ferrocyanide (eq 10).

k_9	$(1.5 \pm 0.2) \times 10^8$ M^{-1} sec^{-1}
$\left(k_{10} + \frac{k_{11}K_4}{K_A}\right)$	$(7.5 \pm 1.6) \times 10^6$ M^{-1} sec^{-1}
k_{12}	$(6.0 \pm 0.2) \times 10^5$ M^{-1} sec^{-1}
K_4	$(4.9 \pm 1.6) \times 10^{-6}$ M

[a] Errors given are the standard error of the parameter estimates obtained from nonlinear least-squares analysis.

From our previous results it was found that $k_{2,app}$ is at least 40 times larger than $k_{3,app}$. With the assumption that $k_{2,app} \gg k_{3,app}$ and rearrangement of eq 11, one obtains

$$\frac{[HRP]_0}{v} = \frac{1}{2k_{1,app}[H_2O_2]_0} + \frac{1}{2k_{3,app}[Fe(CN)_6^{4-}]_0} \quad (12)$$

From eq 12 it is predicted that a plot of $[HRP]_0/v$ *vs.* $1/[Fe(CN)_6^{4-}]_0$ at a constant $[H_2O_2]_0$ should be linear with the slope equal to $1/2k_{3,app}$ and an intercept equal to $1/2k_{1,app}[H_2O_2]_0$. Data obtained at three values of pH are plotted in Figure 10. Weighted linear least squares gave the rate constants and the error limits listed in Table V.

It can also be shown from eq 12 that a plot of $[HRP]_0/v$ *vs.* $1/[H_2O_2]_0$ at constant pH and $[Fe(CN)_6^{4-}]_0$ should be linear with a slope of $1/2k_{1,app}$ and an intercept of $1/2k_{3,app}[Fe(CN)_6^{4-}]_0$. Data obtained at pH 4.82 are plotted in Figure 11. Values of $k_{1,app}$ and $k_{3,app}$ obtained from a weighted linear least-squares analysis of the data are also listed in Table V.

Values of $k_{3,app}$ obtained from steady-state kinetics and from stopped-flow kinetics agree within the error limits as shown in Table V. The error in the determination of $k_{1,app}$ by the steady-state method is necessarily large because of the small value of the intercept in Figure 10 and the uncertainty in drawing an accurate slope through the data points in Figure 11. This arises becasue the term $1/2k_{3,app}[Fe(CN)_6^{4-}]_0$ in eq 12 is, under typical experimental conditions, approximately 100 times larger than the $1/2k_{1,app}[H_2O_2]_0$ term, effectively "swamping out" the smaller term. The value of $k_{1,app}$ has been measured to be 1×10^7 M^{-1} sec^{-1} (Chance, 1949; Chance *et al.*, 1967). Within the large error limits of our steady-state kinetic determination, the order-of-magnitude agreement on the value of $k_{1,app}$ is as good as could be expected.

Discussion

The steady-state kinetic study has shown that eq 1–3 represents the minimum reaction sequence for the HRP-

TABLE V: Rate Constants for the HRP-Catalyzed Oxidation of Ferrocyanide by H_2O_2 at 25.0° and $\mu = 0.11$.

$k_{3,app}$[a] (M^{-1} sec^{-1}) (Stopped Flow)	$k_{3,app}$[b] (M^{-1} sec^{-1}) (Steady State)	$k_{1,app}$[b] (M^{-1} sec^{-1}) (Steady State)	pH	Buffer[c]
$(1.7 \pm 0.5) \times 10^5$	$(2.5 \pm 0.3) \times 10^{5}$[d]	$(5.2 \pm 2.2) \times 10^{6}$[d]	4.82	M
$(1.7 \pm 0.5) \times 10^5$	$(2.0 \pm 0.2) \times 10^{5}$[e]	$(8.0 \pm 15.5) \times 10^{6}$[e]	4.82	M
$(3.2 \pm 0.9) \times 10^4$	$(2.3 \pm 0.3) \times 10^{4}$[d]	*f*	6.24	M
$(6.6 \pm 1.9) \times 10^3$	$(5.1 \pm 0.2) \times 10^{3}$[d]	$(6.3 \pm 5.3) \times 10^{5}$[d]	8.73	GN

[a] Rate constants calculated using eq 6 from parameters in Table II. Error limits calculated from 95% confidence limits of the computer fit. [b] Error limits are 95% confidence limits from weighted linear least-squares analysis of data in Figures 10 and 11. [c] Buffer key as in Table I. [d] From weighted linear least-squares analysis of data in Figure 10 obtained at constant $[H_2O_2]_0$. [e] From weighted linear least-squares analysis of data in Figure 11 obtained at constant $[Fe(CN)_6^{4-}]_0$. [f] Negative intercept found.

catalyzed oxidation of ferrocyanide by H_2O_2 at a fixed pH. The inclusion of faster nonrate-determining steps would also lead to an equation of the same form as eq 11. Since the values of $k_{3,app}$ obtained from the steady-state method agree with those obtained from the stopped-flow study, no detectable loss of activity of HRP occurs in the steady-state reaction cycle. The second-order kinetics observed in the stopped-flow experiments and the validity of eq 12 for the steady-state results both provide evidence that complexing does not occur between either HRP-I or HRP-II and ferrocyanide under the experimental conditions used in these studies.

The catalytic effect of cations on the ferrocyanide–ferricyanide-exchange reaction has been well documented (Shporer *et al.*, 1965; Campion *et al.*, 1967; Sykes, 1967). The cations appear to facilitate the approach of the ions of high negative charge (Campion *et al.*, 1967), an effect which might be expected to occur at high pH in the ferrocyanide reactions with the compounds of HRP. It is in this region that the ferrocyanide reaction rates are particularly simple to explain in terms of acid–base equilibria. Furthermore any possible effect of cations is common to the reactions of ferrocyanide with both HRP-I and HRP-II and so does not detract from a comparison of the two reactions.

Two striking features of our stopped-flow kinetic results are: (1) the absence of a pH dependence in the HRP-I–ferrocyanide reaction above pH 8 contrasted to the slope of −1 above pH 9 when the log of the rate of the HRP-II–ferrocyanide reaction is plotted *vs.* pH. This is accounted for by

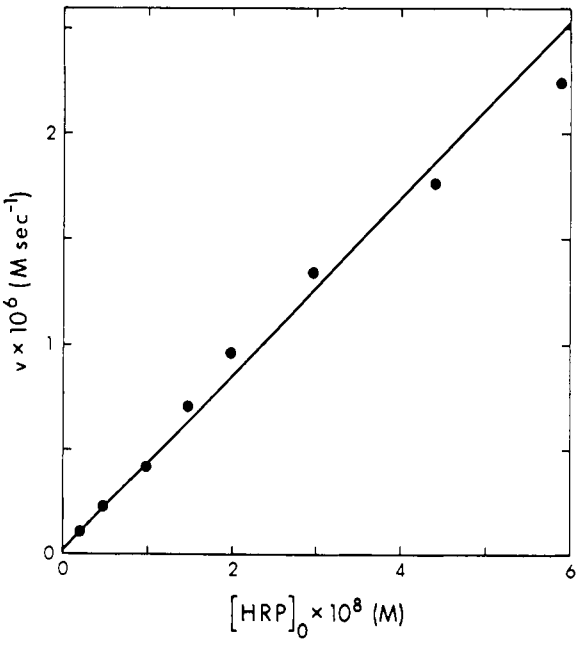


FIGURE 9: Plot of initial steady-state velocity, *v*, *vs.* $[HRP]_0$ at pH 4.79 and constant initial concentrations of ferrocyanide and H_2O_2 of 1.00×10^{-4} and 3.55×10^{-5} M, respectively. The slope and intercept of the weighted linear least-squares best-fit line, with their 95% confidence limits are (42 ± 4) sec^{-1} and $(3 \pm 2) \times 10^{-8}$ M sec^{-1}.

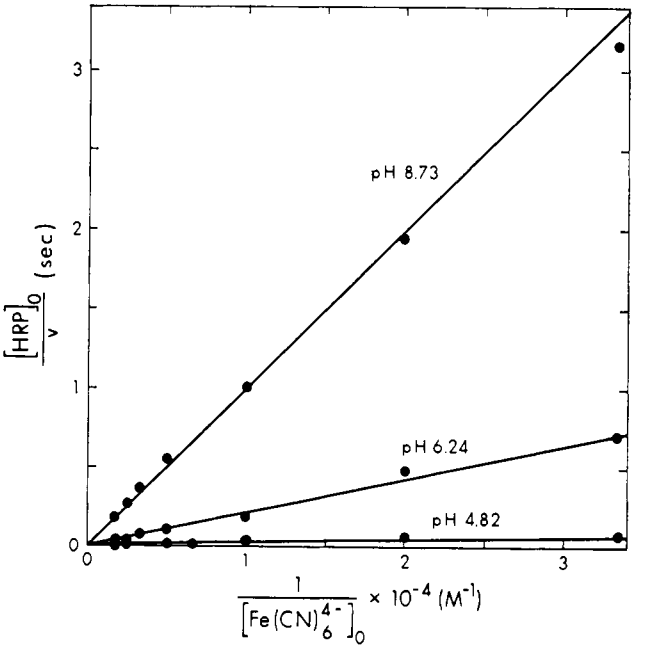


FIGURE 10: Plot of $[HRP]_0/v$ *vs.* $1/[Fe(CN)_6^{4-}]_0$ at pH values shown. For each plot, pH, $[H_2O_2]_0$, and $[HRP]_0$ are constant and their respective values are: 4.82, 3.55×10^{-5} M, and 1.96×10^{-8} M; 6.24, 3.79×10^{-5} M, and 9.00×10^{-8} M; 8.73, 4.03×10^{-5} M, and 4.50×10^{-7} M. The solid lines have been calculated from weighted linear least-squares analysis.

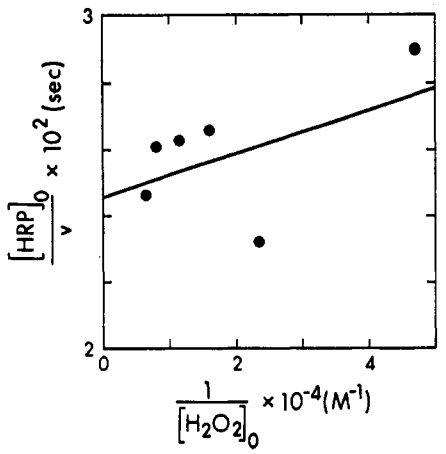


FIGURE 11: Plot of $[HRP]_0/v$ *vs.* $1/[H_2O_2]_0$ at a pH of 4.82 and at constant initial concentrations of ferrocyanide and HRP of 1.00×10^{-4} and 2.55×10^{-8} M, respectively. The solid line was calculated from a weighted linear least-squares analysis. The large errors are caused because the term $1/2k_{1,app}$, $[H_2O_2]_0$ is small (eq 13).

TABLE VI: Apparent Rate Constants for the Reaction of HRP-I and HRP-II with Substrates.[a]

Substrate	HRP-I $k_{2,app}$ (M^{-1} sec^{-1})	HRP-II $k_{3,app}$ (M^{-1} sec^{-1})	pH
Ferrocyanide[b]	8×10^5	2×10^4	7
Ferrocyanide[b]	9×10^6	2×10^5	4.7
Nitrous acid[c]	2×10^7	2×10^5	Free acid
Guaiacol	9×10^6	3×10^5	7
Ascorbic acid		2×10^4	4.7
Aniline		7×10^4	7
p-Aminobenzoic acid	9×10^6	2×10^3	
Luminol[d]	2×10^6	7×10^4	8

[a] Except as marked data obtained primarily by Chance and coworkers at 25–30° and compiled by Brill (1966). [b] Interpolated from Figure 3. [c] Qualitative arguments were used to deduce that the free acid is the reactive species. [d] Cormier and Prichard (1968).

the presence of an acid group in the active site of HRP-II with a pK of 8.6. This group does not influence the kinetics of the HRP-I reaction. (2) The similar shoulders near pH 5 found in the rate data plotted in Figure 5. These are accounted for by acid groups with pK's of 5.3 and 5.2 in the active sites of HRP-I and HRP-II.

The presence of an acid group of pK = 3.4 in the active site of HRP-II is not quite so well established since this pK value is similar to the pH values at one extreme of the experimental pH range. Similarly the absence of a comparable group in HRP-I cannot be ruled out with certainty since the study of the HRP-I reaction with ferrocyanide could not be studied below pH 3.67.

The structures proposed for HRP-I and HRP-II have been reviewed (Saunders *et al.*, 1964; Brill, 1966). Among the most recent contributions are those of Peisach *et al.* (1968), Blumberg *et al.* (1968), Brill and Sandberg (1968), and the Mossbauer study of Moss *et al.* (1969). The latter study is compatible with an oxidation state of IV for both HRP-I and HRP-II. Possible correlations of the pH dependence of the ferrocyanide reactions with available data on structures of HRP-I and HRP-II and with earlier studies on pH dependences of ligand binding by native HRP are discussed elsewhere (Dunford and Hasinoff, 1970).

Brill (1966) in a recent review has compiled rate constants obtained mainly by Chance and coworkers for the reaction of a number of organic substrates with HRP-I and HRP-II most of which were obtained at only a single pH value. These results are listed in Table VI along with the apparent rate constants for the oxidation by HRP-I and HRP-II of ferrocyanide at the same pH. The constants quoted in Table VI are all apparent rate constants, as illustrated in eq 1–3, defined in terms of total concentrations of reactants. The value of such a comparison of rate constants depends partly on whether the reactants are partitioned among various species differing in their extent of ionization, which in turn may be reflected in the presence or absence of a pH dependence for the reactions. Work is progressing in this laboratory on the reaction of HRP-I and HRP-II with other substrates, both organic and inorganic, to determine the pH profile of their reaction rates.

Acknowledgment

The technical assistance of George S. Cembrowski is gratefully acknowledged.

References

Birk, J. P. (1969), *J. Amer. Chem. Soc. 91*, 3189.
Blumberg, W. E., Peisach, J., Wittenberg, B. A., and Wittenberg, J. B. (1968), *J. Biol. Chem. 243*, 1854.
Brill, A. S. (1966), *Comp. Biochem. 14*, 447.
Brill, A. S., and Sandberg, H. E. (1968), *Biochemistry 7*, 4254.
Campion, R. J., Deck, C. F., King, P., and Wahl, A. C. (1967), *Inorg. Chem. 6*, 672.
Chance, B. (1949), *Arch. Biochem. 22*, 224.
Chance, B. (1952a), *Arch. Biochem. Biophys. 41*, 416.
Chance, B. (1952b), *Arch. Biochem. Biophys. 41*, 404.
Chance, B. (1952c), *Arch. Biochem. Biophys. 37*, 235.
Chance, B., De Vault, D., Legallais, U., Mela, L., and Yonetani, T. (1967), Fast Reactions and Primary Processes in Chemical Kinetics, Stockholm, New York, N. Y., Interscience, p 437.
Cormier, M. J., and Prichard, P. M. (1968), *J. Biol. Chem. 243*, 4706.
Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, London, Longmans, Green, pp 116–145.
Dunford, H. B., and Hasinoff, B. B. (1970), Proceedings of American Chemical Society–Chemical Institute of Canada Bioinorganic Symposium, Blacksburg, Va.
Ellis, W. D., and Dunford, H. B. (1968), *Biochemistry 7*, 2054.
Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley, p 16.
George, P. (1952), *Nature (London) 169*, 612.
George, P. (1953a), *Biochem. J. 54*, 267.

George, P. (1953b), *J. Biol. Chem. 201*, 427.
Hasinoff, B. B. (1970), Ph.D. Thesis, University of Alberta.
Jordan, J., and Ewing, G. J. (1962), *Inorg. Chem. 1*, 587.
Keilin, D. and Hartree, E. F. (1951), *Biochem. J. 49*, 88.
Keilin, D. and Mann, T. (1937), *Proc. Roy. Soc., Ser. B 122*, 119.
Maehly, A. C. (1953), *Arch. Biochem. Biophys. 44*, 430.
Marquardt, D. W. (1963), *J. Soc. Ind. Appl. Math. 11, No. 2.*
Moss, T. H., Ehrenberg, A., and Bearden, A. J. (1969), *Biochemistry 8*, 4159.
Ovenston, T. C. J., and Rees, W. T. (1950), *Analyst (London) 75*, 204.
Peisach, J., Blumberg, W. E., Wittenberg, B. A., and Wittenberg, J. B. (1968), *J. Biol. Chem. 243*, 1871.
Ramette, R. W., and Sandford, R. W. (1965), *J. Amer. Chem. Soc. 87*, 5001.
Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. (1964), Peroxidase, Washington, D. C., Butterworths.
Shporer, M., Ron, G., Loewenstein, A., and Varon, G. (1965), *Inorg. Chem. 4*, 361.
Sykes, A. G. (1967), *Advan. Inorg. Chem. Radiochem. 10*, 153.
Theorell, H. (1941), *Enzymologia 10*, 250.
Worthington Biochemical Corp. (1967), Freehold, N. J., Catalogue.

Enzymic and Immunochemical Properties of Lysozyme. Evaluation of Several Amino Group Reversible Blocking Reagents*

A. F. S. A. Habeeb† and M. Z. Atassi

ABSTRACT: Reactions of lysozyme with several reversible blocking reagents for amino groups have been studied. Derivatives obtained by complete modification of the amino groups by reaction with tetrafluorosuccinic, maleic, or citraconic anhydride or by reaction with diketene were electrophoretically heterogeneous. In each case modification of some hydroxyl groups was also obtained. Complete loss of enzymic activity accompanied the complete modification of the amino groups by any of these reagents. Removal of the maleyl blocking groups in ML_7Lys resulted in a grossly electrophoretically heterogeneous preparation, with recovery of 90% of free amino groups, 83% of enzymatic activity, and almost all the ability to react with antisera to lysozyme. Similarly removal of blocking groups in derivatives prepared by reaction with tetrafluorosuccinic anhydride or with diketene was not complete yielding, in each case, heterogeneous preparations with incomplete recovery of enzymic and immunochemical properties. With CT_7Lys, complete deblocking of amino groups was obtained at pH 4.2 in 3 hr. Deblocked derivatives could be prepared which showed little or no electrophoretic heterogeneity, and absolutely complete restoration of enzymic activity and antigenic reactivity. Conformational changes associated with complete blocking of amino groups were not large and complete reversion to native conformation was obtained on deblocking with CT_7Lys, but not with ML_7Lys. The results show that, of the reversible amino group blocking reagents studied here, citraconic anhydride is the most satisfactory.

Reversible blocking of amino groups is a valuable tool for protecting these groups from side reactions which might, in certain cases, take place during modification of some functional groups. Also, such protecting groups are useful for rendering hydrolysis with trypsin specific for cleavage at arginine residues. Many such reversible blocking reagents have been reported. However, a careful investigation of their applicability and comparison of their specificity, ease of removal, homogeneity of the blocked and deblocked derivatives, and changes in their conformation and their biological activities has not been carried out.

In the present work, lysozyme has been chosen as the protein model for such investigation. Thus the reactions of lysozyme with maleic anhydride (Butler *et al.*, 1967), citraconic anhydride (Dixon and Perham, 1968), tetrafluorosuccinic anhydride (Braunitzer *et al.*, 1968), or diketene (Marzotto *et al.*, 1967, 1968) have been studied. The specificity and reversibility of the reactions and the homogeneity of all reaction products were examined. Enzymic and immunochemical properties of the lysozyme derivatives and of lysozyme preparations obtained by removal of blocking groups are reported together with accompanying conformational changes.

* From the Division of Clinical Immunology and Rheumatology, Department of Medicine and Departments of Biochemistry and Microbiology, The Medical Center, University of Alabama, Birmingham, Alabama 35233, and from the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received November 14, 1969.* The work was done during the tenure to M. Z. A. of an Established Investigatorship of the American Heart Association. The research was supported by Grant AM-13389 from the National Institutes of Health, and by the Office of Naval Research, Biochemistry Branch, Contract No. N00014-C-0137. This represents the third paper in the series entitled: Enzymic and Immunochemical Properties of Lysozyme; for the first and second papers in the series, see Atassi and Habeeb (1969) and Habeeb and Atassi (1969).

† To whom to address correspondence.