Effect of Mixed Solvents on the Formation of Horseradish Peroxidase Compound I. The Importance of Diffusion-Controlled Reactions†

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ABSTRACT: In aqueous glycerol solutions, the rate constant for the formation of compound I of horseradish peroxidase from hydrogen peroxide is independent of the viscosity. However, when *m*-chloroperbenzoic acid is used to form compound I, the rate is viscosity dependent, indicating that this reaction is diffusion controlled. The problems in obtaining accurate information about diffusion-controlled reactions are discussed as is the possible significance of diffusion control. Ethanol has been used in previous studies to decrease the dielectric constant of the medium but it was found that its dominant effect on horseradish peroxidase was to bind to the sixth coordination position of the heme ion. The stability of the native enzyme in different environments was tested by difference absorption and circular dichroic spectroscopy and new circular dichroic data for compound I are reported.

The rate constant for the formation of compound I of horseradish peroxidase in the reaction of the native enzyme with hydrogen peroxide, studied over a wide range of pHs (Dolman et al., 1975; Job and Dunford, submitted for publication), is independent of pH between 5 and 9 and has an activation energy of 3.5 kcal/mol. It has been suggested that this reaction may be diffusion controlled (Hewson and Dunford, 1975). Aqueous glycerol solutions have been used previously to study the effect of viscosity on the rate of bimolecular reactions (Strother and Ackerman, 1961; Warrick et al., 1972; Cerjan and Barnett, 1972). Glycerol is particularly suitable for this application due to its high viscosity, high dielectric constant, and because it forms nearly ideal solutions with water (Robinson and Stokes, 1959; Stokes and Robinson, 1966). A viscosity dependence provides the ultimate criterion for a diffusion-controlled reaction and tests for such a dependence are undertaken in this study.

Aqueous solutions of other organic solvents also have been used to study the effect of dielectric constant upon reaction rates (Laidler and Eyring, 1940; Hiromi, 1960a), and this theory has been applied to enzyme reactions (Barnard and Laidler, 1952; Laidler and Ethier, 1953; Hiromi, 1960b). The reaction rate between catalase and hydrogen peroxide has been shown to decrease with increasing concentrations of ethanol (Ogura et al., 1950; Kremer, 1970). Ethanol slows the peroxidation of guaiacol by peroxidase (from Japanese radish) and hydrogen peroxide (Tonomura, 1953). The rate of this same reaction, but catalyzed by three isozymes of turnip peroxidase, is decreased by ethanol (Hosoya, 1960). An expression for the reaction rate between dipolar molecules was used to interpret these results. The steady-state rate of oxidation of guaiacol by horseradish peroxidase and hydrogen peroxide is inhibited by methanol (Maurel and Travers, 1973) and was explained in terms of competitive inhibition where 2 mol of methanol bind to 1 mol of horseradish peroxidase. It has been noted previously that the rate of compound I formation for horseradish peroxidase isozymes A and C is affected by ethanol, and this was interpreted in terms of the hydrophobicity of the heme pocket (Marklund et al., 1974). Studies in aqueous ethanol were undertaken to evaluate the effect of this solvent system on horseradish peroxidase reactions. CD[1] and electronic absorption spectroscopy were used to monitor the influence of the mixed solvents on the enzyme and some new CD results for horseradish peroxidase compound I are reported.

Experimental Procedure

Materials

Horseradish peroxidase (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) was purchased from Sigma as a lyophilized salt-free powder (type VI, lot 25C-9570), and also from Boehringer Mannheim GmbH as a purified ammonium sulfate suspension (lot 7315529). The Sigma preparation after vacuum dialysis against multiply distilled water (Hewson and Dunford, 1976) had a PN of 3.1. (PN is the ratio of absorbance at 403 to that at 280 nm.) The Boehringer Mannheim preparation had a PN of 3.3 after extensive dialysis. Isozyme C (Shannon et al., 1966; Paul and Stigbrand, 1970) has been determined to be the major component of many commercial preparations of horseradish peroxidase (Delincée and Radola, 1970; Hollenberg et al., 1974; Santimone, 1973). Glycerol from Fisher was vacuum distilled (140 °C, 1.5 Torr) to remove trace impurities which caused a reaction with native horseradish peroxidase. All other materials were reagent grade.

Methods

Absorbance spectra and measurements were obtained with a Cary 14 spectrophotometer equipped with a 0-1-2 and 0-0.1-0.2 slide wire. The cell compartment temperature was maintained by circulating thermostated water. Difference spectra were obtained by first recording the baseline with 2 mL of the aqueous–organic solution in the sample cell and 2 mL of aqueous solution in the reference cell. Both cells contained buffer. An identical amount of enzyme was pipetted into each cell, and the solutions were thoroughly mixed with a Teflon plumper before recording the spectrum. CD spectra were recorded with a Jasco Model ORD/UV-5 spectrometer which had been specially modified for sensitive CD measurements.

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[1] Abbreviations used: CD, circular dichroic; *I*, ionic strength.

TABLE I: Second-Order Rate Constants for the Formation of Compound I from H_2O_2 as a Function of the Concentration of Glycerol in Phosphate Buffer near pH 7 at 40 °C.

$10^{-7} \times k_{1,app}$ (M^{-1} s^{-1})	[Glycerol] (M)	$\eta_{rel}^{40°C}$	$\epsilon_{corr}^{40°C}$ [a]
1.88 ± 0.06	0.0	1.00	71.4
1.92 ± 0.07	0.342	1.07	70.6
1.93 ± 0.06	0.685	1.13	69.7
1.98 ± 0.08	1.03	1.20	68.9
1.99 ± 0.09	1.37	1.27	68.1
1.85 ± 0.08	1.57	1.32	67.6
1.94 ± 0.07	1.71	1.37	67.3
1.84 ± 0.07	2.05	1.46	66.6
1.92 ± 0.07	2.40	1.58	65.7
1.89 ± 0.07	2.74	1.71	64.9
1.81 ± 0.06	2.88	1.78	64.6
1.82 ± 0.07	3.08	1.86	64.1
1.86 ± 0.08	3.42	1.95	63.3
2.05 ± 0.09	3.77	2.19	62.5
1.80 ± 0.06	4.11	2.42	61.7

[a] Dielectric data from Åkerlöf (1932) is corrected for the presence of electrolyte by the method of Hasted et al. (1948).

TABLE II: Second-Order Rate Constants for the Formation of Compound I from *m*-Chloroperbenzoic Acid as a Function of Viscosity in Phosphate Buffer near pH 5.8 at 25 °C.

$10^{-7} \times k_{d,app}$ (M^{-1} s^{-1})	[Glycerol] (M)	$\eta_{rel}^{25°C}$
8.2 ± 0.5	0.0	1.00
8.5 ± 0.5	0.685	1.14
6.4 ± 0.3	1.37	1.26
6.3 ± 0.3	1.71	1.38
6.2 ± 0.3	2.40	1.57
4.6 ± 0.3	2.74	1.64
4.9 ± 0.2	3.08	1.89
3.8 ± 0.2	3.77	2.10
3.4 ± 0.2	4.11	2.23
3.1 ± 0.1	1.16 M sucrose	3.01

The jacketed cells used to obtain CD spectra could be thermostated with circulating water for precise temperature control. A rapid flow of anhydrous nitrogen through the cell compartment prevented condensation of water on the cell windows during the low-temperature measurements. From 600 to 250 nm in a 2-cm cell the concentration of horseradish peroxidase or compound I was 9 μM in phosphate buffer (pH 7.04) with $I = 0.005$. Below 250 nm, a 1-mm cell was used with a 1.93 μM concentration of compound I or horseradish peroxidase in the same buffer.

The rate constant measurements were made using a Gibson-Durrum stopped-flow apparatus Model D-110 using at least a tenfold excess of hydrogen peroxide, *m*-chloroperbenzoic acid, or cyanide compared with the enzyme to ensure pseudo-first-order conditions. The enzyme concentration was 0.6 μM. The solutions were prepared in 10-mL volumetric flasks. Unlike hydrogen peroxide, a tenfold excess of *m*-chloroperbenzoic acid resulted in the slow destruction of the enzyme in a reaction which followed compound I formation. The rate of compound I formation or cyanide binding was followed at 403 or 420 nm, respectively. Both drive syringes contained 0.1 M potassium nitrate and buffer, $I = 0.01$, for a total ionic strength of 0.11. For the stopped-flow experiments in aqueous–organic solutions, equal amounts of glycerol or ethanol were added to the enzyme and substrate solutions. For each set of conditions, eight individual determinations of the rate constant were averaged for the best value. The nonlinear least-squares method of computing first-order rate constants has been described (Roman et al., 1971). The kinetic curves obeyed eq 1 showing the reaction is first-order with respect to the concentration of native horseradish peroxidase

$$\frac{-d[HRP]}{dt} = k_{obsd}[HRP] \qquad (1)$$

where k_{obsd} is a pseudo-first-order rate constant. Errors given for the rate constants are the larger of either the average standard deviations or the average deviation from the mean value.

The concentration of horseradish peroxidase was determined spectrophotometrically at 403 nm using a molar absorptivity of 1.02×10^5 M^{-1} cm^{-1} (Schonbaum and Lo, 1972). The concentration of hydrogen peroxide was checked weekly and the concentration of *m*-chloroperbenzoic acid was checked daily using the horseradish peroxidase assay (Cotton and Dunford, 1973). Potassium cyanide solutions were prepared immediately prior to use and were not used for longer than a few hours. An Orion 801 pH meter equipped with a Fisher combination electrode calibrated to ±0.03 pH unit with commercial standard buffers was used to measure pH. The pH of the phosphate buffer was 7.04 at 25 °C in the absence of organic solvent. The addition of glycerol or ethanol (Bates et al., 1963) alters the pH significantly. However, the rates of compound I formation (Dolman et al., 1975) and cyanide binding (Ellis and Dunford, 1968) are pH independent near pH 7. For the reaction between horseradish peroxidase and *m*-chloroperbenzoic acid, the pH-independent region occurs between 5 and 7, and this reaction was studied near pH 5.8. For the same reason the change in pH as a function of temperature does not affect the rate of compound I formation or cyanide binding. (The ethanol concentrations were corrected to account for using 95% v/v stock solutions.)

An Ostwald viscometer thermostated at 40 °C was used to determine relative viscosities for studies using hydrogen peroxide. The flow time of an aqueous solution of 0.6 μM horseradish peroxidase in 0.1 M potassium nitrate and phosphate buffer (pH 7.04), $I = 0.01$, at 40 °C, was assigned unit relative viscosity and all aqueous glycerol viscosities were assigned relative values, $\eta_{rel}^{40°C}$. For the experiments with *m*-chloroperbenzoic acid, all the viscosities were measured at 25 °C in similar fashion. Flow times were determined in triplicate and were reproducible within 0.2 s with times ranging from 72 to 313 s.

The dielectric constants of the aqueous–organic solutions at 40 °C were interpolated from published data (Åkerlöf, 1932) and were then corrected for the effect of 0.11 M electrolyte (Hasted et al., 1948). The electrolyte correction required that the dielectric constant be diminished by 2 units.

Compound I Formation in Glycerol. The rate constant for compound I formation from hydrogen peroxide, $k_{1,app}$, studied in the presence of glycerol to determine the effect of viscosity, was measured in phosphate buffer near pH 7 at 40 °C with $[H_2O_2] = 15$ μM. The dielectric constant of the solutions, as well as the viscosity, is changed by the glycerol. The values of $k_{1,app}$, the concentration of glycerol, the relative viscosity, and the dielectric constant (corrected for the presence of electrolyte), recorded in Table I, show that the rate constant is independent of the viscosity and dielectric constant of the solvent. Therefore compound I formation from hydrogen peroxide is not a diffusion-controlled reaction. However, the rate constant

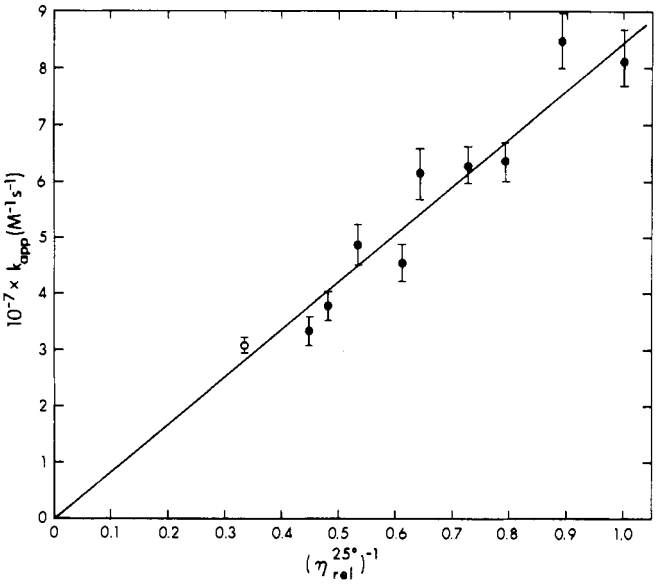

FIGURE 1: Linear plot of $k_{d,app}$ versus the reciprocal of $\eta_{rel}^{25°C}$ for the reaction of *m*-chloroperbenzoic acid with native horseradish peroxidase. The best straight line was calculated using a weighted linear least-squares analysis. The slope of $(8.5 \pm 0.9) \times 10^7$ M^{-1} s^{-1} is the value of $k_{d,app}$ at unit relative viscosity, and the intercept at infinite viscosity is zero within its standard deviation. Experimental conditions are described in Table II. The open symbol corresponds to the solution of sucrose.

for compound I formation from *m*-chloroperbenzoic acid, $k_{d,app}$, is viscosity dependent, as shown by the data in Table II and Figure 1. The viscosity of one solution was controlled using sucrose, which helps to demonstrate that the decrease in the rate constant is not solely due to a specific interaction of glycerol with the enzyme or enzyme-peroxy acid complex. As expected for a diffusion-controlled rate constant, the plot of $k_{d,app}$ versus $(\eta_{rel}^{25°C})^{-1}$ in Figure 1 is linear and has a zero intercept (at infinite viscosity the rate constant is zero). The slope of the plot, $(8.5 \pm 0.9) \times 10^7$ M^{-1} s^{-1}, is equal to the rate constant at unit relative viscosity.

Compound I Formation in Ethanol. The rate of compound I formation was studied in the presence of ethanol in phosphate buffer near pH 7 at 40 °C with $[H_2O_2] = 15$ μM. The values of $k_{1,app}$, the concentration of ethanol, and the dielectric constant are recorded in Table III. A significant decrease in $k_{1,app}$ occurs with increasing ethanol concentration. The result is most simply interpreted in terms of competitive inhibition where the

$$\begin{array}{ccc} \text{HRP} + \text{H}_2\text{O}_2 & \xrightarrow{k_a} & \text{compound I} \\ K \Big\downarrow & & \\ \text{HRP–EtOH} & & \end{array} \qquad (2)$$

enzyme is in equilibrium with an enzyme-ethanol complex. The complex is considered to be unreactive or at least not significantly reactive compared with uncomplexed horseradish peroxidase. Defining K as the dissociation constant for the HRP-EtOH complex, and using the expression for the reaction velocity, eq 3 can be derived

$$\frac{1}{k_{1,app}} = \frac{[EtOH]}{k_a K} + \frac{1}{k_a} \qquad (3)$$

A linear plot of the reciprocal of $k_{1,app}$ versus [EtOH] is shown in Figure 2 from which $K = 6.8 \pm 0.4$ M can be calculated.

Cyanide Binding to Horseradish Peroxidase in Ethanol. These measurements were undertaken because of the great similarity between the mechanisms of cyanide binding and compound I formation. The rate of formation of the cyanide complex of native horseradish peroxidase is also inhibited by ethanol, as shown by rate measurements obtained in phosphate buffer near pH 7 at 40 °C with [KCN] = 50 μM. The values of $k_{2,app}$ (the second-order apparent rate constant for cyanide binding) as a function of ethanol concentration are recorded in Table III. An analogous inhibition scheme to that used for compound I formation is applicable.

$$\begin{array}{ccc} \text{HRP} + \text{cyanide} & \xrightarrow{k_b} & \text{HRP–CN} \\ K \Big\downarrow & & \\ \text{HRP–EtOH} & & \end{array} \qquad (4)$$

A linear plot of the reciprocal of $k_{2,app}$ versus [EtOH] yields nearly the same value of K within its standard deviation (8.3 ± 0.8 M) as did the results for the ethanol-inhibited compound I formation.

TABLE III: Second-Order Rate Constants for the Formation of Compound I from H_2O_2 and Cyanide Binding as a Function of the Concentration of Ethanol in Phosphate Buffer near pH 7 at 40 °C.[a]

$10^{-7} \times k_{1,app}$ (M^{-1} s^{-1})	$10^{-5} \times k_{2,app}$ (M^{-1} s^{-1})	[EtOH] (M)	$\epsilon_{corr}^{40°C}$ [b]
2.03 ± 0.1	1.81 ± 0.06	0.0	71.4
1.83 ± 0.09		0.0	71.4
	1.76 ± 0.04	0.406	70.2
1.72 ± 0.07	1.72 ± 0.02	0.811	69.5
1.62 ± 0.06	1.56 ± 0.04	1.22	68.7
	1.59 ± 0.05	1.62	67.6
1.48 ± 0.1	1.49 ± 0.1	2.03	66.5
1.42 ± 0.04	1.46 ± 0.04	2.43	65.4
1.36 ± 0.05	1.47 ± 0.03	2.84	64.2
1.28 ± 0.05	1.36 ± 0.09	3.25	63.2
1.22 ± 0.03	1.31 ± 0.06	3.65	62.4
1.21 ± 0.04	1.32 ± 0.03	4.06	61.4
1.19 ± 0.04	1.14 ± 0.08	4.46	60.1
1.14 ± 0.04	1.07 ± 0.1	4.87	58.8

[a] The pH was measured at 25 °C. [b] See footnote *a* of Table I.

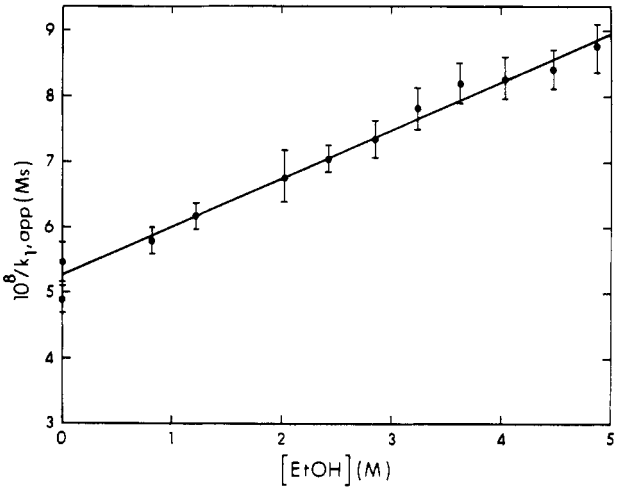

FIGURE 2: Linear plot according to eq 3 of the reciprocal of $k_{1,app}$ for the reaction of H_2O_2 with horseradish peroxidase versus the concentration of ethanol in phosphate buffer near pH 7 at 40 °C. $k_{1,app}$ was determined from k_{obsd} at $[H_2O_2] = 15$ μM. The values of $k_a = (1.92 \pm 0.01) \times 10^7$ M^{-1} s^{-1} and $K = 6.8 \pm 0.4$ were calculated from the ordinate intercept and slope which were determined by a linear least-squares analysis.

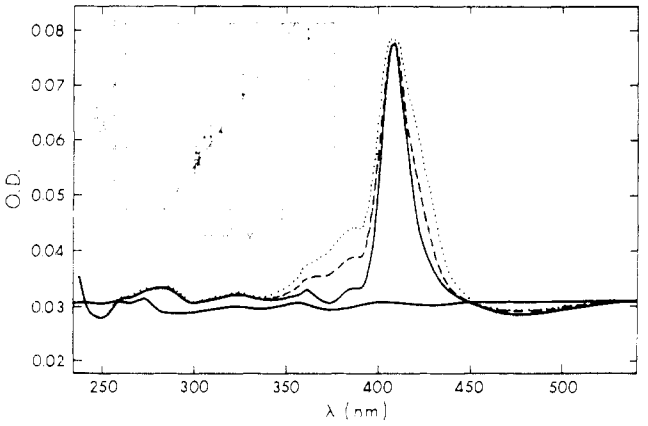

FIGURE 3: The difference absorption spectra in 1-cm cells of horseradish peroxidase (17.5 μM) in ethanol (2.6 M) and phosphate buffer near pH 7 (ionic strength, 0.005) at 40 °C. The reference cell contained equal quantities of everything in the sample cell but ethanol. The first spectrum (—) was recorded within 1 min of mixing, and the second (- - -) and third (· · ·) at 25 and 60 min. An isosbestic point occurs at 452 nm. Horseradish peroxidase (0.7 mL of 67.4 μM) was pipetted into the reference and then the sample cell, each containing 2 mL of solution. The 0–0.1 slide wire was used. Inset: Double-reciprocal plot of the absorbance change, ΔA, versus the concentration of ethanol in phosphate buffer near pH 7 (ionic strength, 0.005) at 40 °C. ΔA was determined by difference spectroscopy. The straight line was calculated using a linear least-squares analysis weighted by the reciprocal of the estimated percent error in each data point. The value $K = 4 \pm 1$ was obtained from the ordinate intercept and slope or from the negative abscissa intercept.

Spectroscopic Detection of the HRP-EtOH Complex. A difference absorption spectrum of the ethanol complex of native horseradish peroxidase, recorded as a function of time, is shown in Figure 3. Positive extrema are located at 409 and 386 nm and negative extrema at 480 and 255 nm. An isosbestic wavelength on the baseline at 452 nm indicates the interconversion of two species. After 60 min the spectrum ceased to change. The maximum difference in absorbance at 409 nm represents less than 3% of the total absorbance based on an estimated molar absorptivity of 9.4×10^4 M^{-1} cm^{-1} for horseradish peroxidase at 409 nm. The difference spectral data were used to determine the dissociation constant, K, as defined in eq 2–4. The reciprocal of the absorbance change at 409 nm, ΔA^{409}, was plotted against the reciprocal of the concentration of ethanol according to eq 5

$$\frac{1}{\Delta A^{409}} = \frac{K}{\Delta\epsilon^{409}[\text{HRP}]_{\text{total}}[\text{EtOH}]} + \frac{1}{\Delta\epsilon^{409}[\text{HRP}]_{\text{total}}} \quad (5)$$

where $[\text{HRP}]_{\text{total}} = [\text{HRP}] + [\text{HRP-EtOH}]$ and $\Delta\epsilon^{409} = \epsilon_{\text{HRP EtOH}}{}^{409} - \epsilon_{\text{HRP}}{}^{409}$. The plot is shown as an insert in Figure 3. The linear slope and ordinate intercept or the negative abscissa intercept can be used to calculate $K = 4 \pm 1$ M.

Circular dichroic spectroscopy was also used to detect the ethanol complex of horseradish peroxidase. The CD spectrum of native horseradish peroxidase in Figure 4 is identical with that already published for isozyme C (Strickland, 1968; Strickland et al., 1968). In 3.5 M ethanol there are slight reproducible differences in the CD spectrum, presumably due to the enzyme–ethanol complex. The ellipticity of the positive band in the Soret region is increased slightly and is shifted from 407 to 410 nm. Also, the negative bands at 372 and 340 nm have slightly smaller ellipticities. Other regions of the CD spectrum, including the large negative band at 208 nm (not shown) which correlates with the α-helical content, remain unchanged upon ethanol addition.

Spectrophotometric Detection of Interactions between

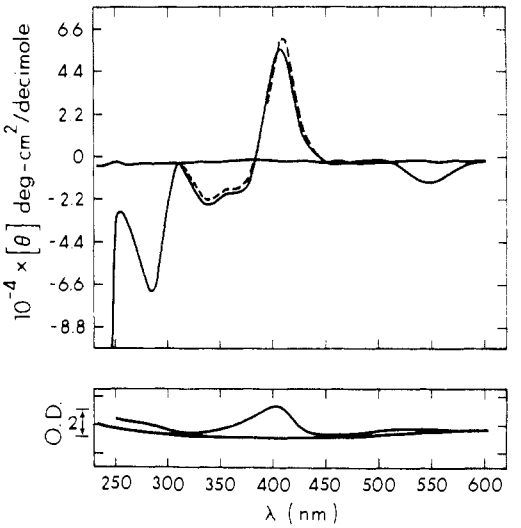

FIGURE 4: Circular dichroic spectra of horseradish peroxidase (—) and its ethanol complex (- - -). The enzyme concentration was 9 μM with phosphate buffer near pH 7 (ionic strength 0.005) at 40 °C in a 2-cm cell. The ethanol concentration was 3.5 M. The absorption spectra at the bottom of the figure were recorded simultaneously with the CD spectra. The baselines for each solution were coincident.

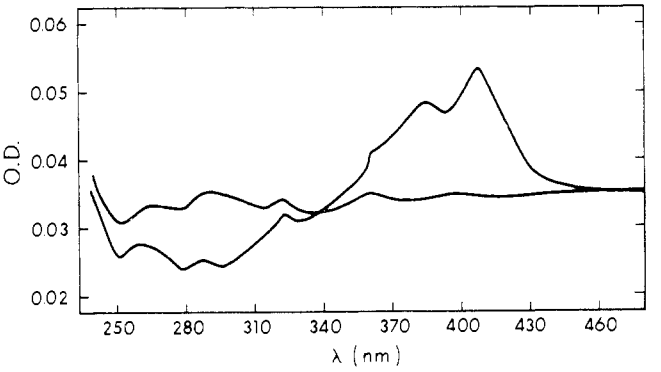

FIGURE 5: The difference absorption spectrum in 1-cm cells of horseradish peroxidase (13.5 μM) in glycerol (2.8 M) and phosphate buffer near pH 7 (ionic strength, 0.005) at 40 °C. The reference cell contained everything in the sample cell but glycerol. The spectrum was recorded within 1 min of mixing and did not change significantly with time. Horseradish peroxidase (0.5 mL of 67.4 μM) was pipetted into the reference and then the sample cell, each containing 2 mL of solution. The 0–0.1 slide wire was used.

Horseradish Peroxidase and Glycerol. Difference absorption spectroscopy was used to detect small changes in native horseradish peroxidase in the presence of glycerol. The spectrum is shown in Figure 5. Broad absorbance maxima occur at 407 and 385 nm, and a minimum occurs at about 290 nm. The absorbance change at 407 nm represents approximately 1.5% of the total absorbance. The CD spectra of horseradish peroxidase in water and in glycerol are identical within instrumental sensitivity from 600 to 190 nm.

Temperature Dependence of the CD Spectrum of Horseradish Peroxidase. The temperature dependence of the CD spectrum of horseradish peroxidase, Figure 6, was investigated to determine whether thermally induced structural changes occur. Increasing the temperature from 4 to 67 °C decreased the ellipticity of the positive Soret band. From 300 to 250 nm only the spectrum at 67 °C was detectably different. After the sample was held at 67 °C for 30 min (the time to record the spectrum), it was quickly cooled in ice water and then allowed to return to 26 °C. Its spectrum was identical with that of the

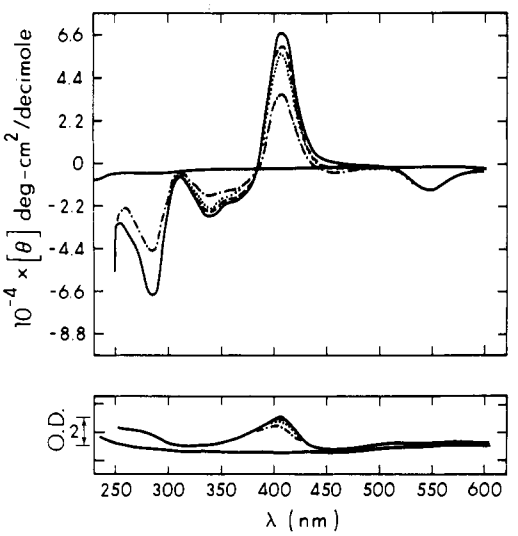

FIGURE 6: Circular dichroic spectra of horseradish peroxidase at several temperatures: (—) 4 °C; (- - -) 27 °C; (· · ·) 48 °C; and (- · · · ·) 67 °C. The concentration of horseradish peroxidase was 9 μM with phosphate buffer (pH 7.04) (ionic strength, 0.005) in a 2 cm cell. The same solution was used for all temperatures. The absorption spectra at the bottom of the figure were recorded simultaneously with the CD spectra. When the sample at 67 °C had been cooled to 26 °C, it produced CD and absorption spectra identical with those at 27 °C, indicating reversibility. Both baselines were temperature independent.

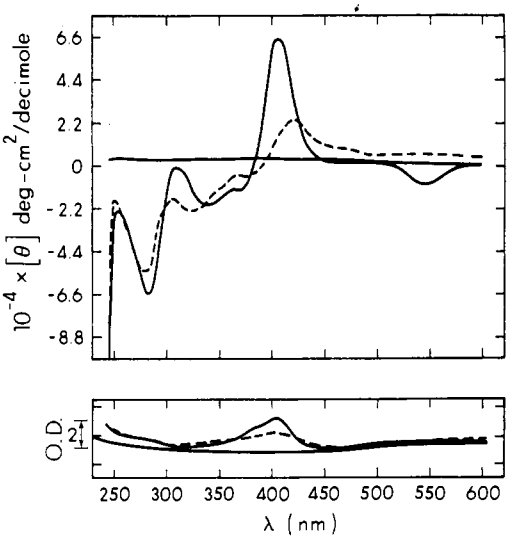

FIGURE 7: Circular dichroic spectra of horseradish peroxidase (—) and compound I (- - -). The enzyme concentration was 9 μM with phosphate buffer (pH 7.04) (ionic strength, 0.005) at 20 °C in a 2-cm cell. Compound I was prepared by adding 1 molar equiv of H_2O_2 contained in 20 mL of solution. The absorption spectra at the bottom of the figure were recorded simultaneously with the CD spectra. After recording the spectrum of compound I, a check of the absorbance at 403 nm showed that virtually none of compound I spontaneously decayed.

unheated sample at 27 °C. Therefore, these thermally induced changes are reversible. The changes in the absorption spectrum are also reversible.

CD Spectrum of Compound I. The CD spectrum of compound I, Figure 7, was recorded at 20 °C and is very different from that of the native enzyme (Strickland et al., 1968). The negative band at 544 nm of the native enzyme has disappeared in compound I, whereas the negative band at 208 nm (not shown) remained unchanged. In contrast, the ellipticity of the positive Soret band has decreased and shifted from 407 to 423 nm. The stability of the compound I preparation was verified by observing that there was no detectable change in its absorption spectrum in the Soret region after the time required to record the spectrum.

Discussion

It was reported recently that several substituted perbenzoic acids form compound I at a significantly faster rate than hydrogen peroxide (Davies et al., 1976). As with hydrogen peroxide (Dolman et al., 1975), it is the un-ionized form of the perbenzoic acid which is reactive. *m*-Chloroperbenzoic acid was among the fastest reacting perbenzoic acids with a reported rate constant of about 10^8 M^{-1} s^{-1} at pH 5. From pH 5 to 6.5 the rate of reaction between horseradish peroxidase and the perbenzoic acids was pH independent. Our study of the viscosity dependence of the rate of compound I formation with *m*-chloroperbenzoic acid indicates that this reaction is diffusion controlled. The plot in Figure 1 is linear within the experimental error and has a zero intercept when extrapolated to infinite viscosity. The slope, which is the diffusion-controlled rate constant at unit relative viscosity, has a value of $(8.5 \pm 0.9) \times 10^7$ M^{-1} s^{-1}, in fair agreement with the previously reported rate constant (Davies et al., 1976). On the other hand, the rate of compound I formation from hydrogen peroxide is independent of the concentration of glycerol and therefore is independent of viscosity (Table I). The concentration range of glycerol used at 40 °C simulates the viscosity changes of water from 40 to 2 °C.

Even when saturation kinetics are not observed, elementary reactions of native peroxidase, as well as of compounds I and II, may be formulated as

$$E + S \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} ES \xrightarrow{k_d} \text{products} \qquad (6)$$

so that the second-order rate constant, k_{app}, is

$$k_{app} = \frac{k_d}{K_M} = \frac{k_s k_d}{k_d + k_{-s}} \qquad (7)$$

(Critchlow and Dunford, 1972; Job and Dunford, submitted for publication). These equations are the analogue of Michaelis–Menten steady-state kinetics but it should be emphasized that they have been applied to the transient state kinetics of peroxidase reactions. (In the steady state, the kinetics are zero order, whereas in the transient state the much more informative pseudo-first-order kinetics of individual elementary reactions are observed.) It can be shown that the Arrhenius activation energy is partly a function of the enthalpy of formation of the precursor complex (Marcus and Sutin, 1975; Jones and Dunford, submitted for publication) so that the measured activation energy for compound I formation from hydrogen peroxide (Hewson and Dunford, 1975) does not correspond to the bimolecular diffusion step. Thus a low activation energy is a necessary but not a sufficient condition for a diffusion-controlled reaction.

The results of Figures 3 to 6 show that the weak binding of ethanol has little effect on the CD and optical spectra of native horseradish peroxidase and the effect of glycerol is even smaller. The small effect of glycerol may be caused by the binding of glycerol to the protein through the formation of stable hydrophobic bonds between glycerol and hydrophobic amino acid side chains (Kaminsky and Davidson, 1969; Herskovits et al., 1970; Jacobson and Krueger, 1975). Since sucrose also decreases the rate of compound I formation, it is evident that the decrease in aqueous glycerol is not solely due to an interaction between the glycerol and the enzyme. The possibility remains, however, that the hydroxyl groups of su-

crose and of glycerol affect the binding process in a similar manner.

It would appear reasonable to proceed on the assumption that the effect of the glycerol (and sucrose) on the rate of formation of compound I from *m*-chloroperbenzoic acid is predominantly due to change in viscosity of the solvent, hence, change in the diffusion coefficients of reactants. An interesting model for diffusion-controlled enzymatic reactions has been presented by Schmitz and Schurr (1972). They found that the mathematics was simplified by regarding the enzyme as a stationary planar surface with a hemispherical target area above the plane. If the substrate molecule is large and has a reactive center, as opposed to uniform reactivity over its spherical surface, then there are severe angular orientational constraints on reaction, which are offset somewhat by rotational diffusion. The constraints, nevertheless, can reduce the diffusion-controlled rate by several orders of magnitude. In the case of *m*-chloroperbenzoic acid, the reactive site is at one end of the molecule, but the substrate is small enough that the angular restraints may affect the rate by a factor of less than two and will therefore be ignored in the following crude calculation.

A substrate molecule has a 50% chance of being on the side of the planar surface containing the target area, so that the rate constant for a collision with this surface would be close to 2×10^9 M^{-1} s^{-1} (Schmitz and Schurr, 1972). Since the observed rate constant for *m*-chloroperbenzoic acid is 8×10^7 M^{-1} s^{-1}, the difference, a factor of $1/25$, could be regarded as the fraction of the planar surface which is the target area. The target area might represent the active site on the surface of the enzyme; alternatively it could represent the opening into a cleft containing the active site. The correct representation may have to await the x-ray structural determination of horseradish peroxidase, which unfortunately is stymied by lack of suitable crystals (Braithwaite, 1976). In any event, if the enzyme has a radius of 25 Å, its surface area is 7800 $Å^2$ and so the target area can be estimated to have a size of 300 $Å^2$ which appears to be a reasonable estimate of the area of the porphyrin ring or perhaps of the entrance into a cleft containing the porphyrin ring. Electrostatic effects are ignored since the diffusing substrate is electrically neutral (Davies et al., 1976).

Whether enzymatic reactions occur at the diffusion-controlled limit has potential far-reaching implications. Albery and Knowles (1976) state that an enzyme which reacts at close to the diffusion-controlled limit has reached the end of its evolutionary development. Many enzymatic reactions appear to occur at a slower rate (Hammes and Schimmel, 1970) and one explanation is that there is a fundamental compromise between specificity and rate (Eigen, 1974). Another is that the rate-determining step is often product release (Cleland, 1975). There are, of course, thermodynamic restrictions on the rate of the reverse reaction (Cleland, 1975).

Peroxidases have a specificity toward oxidizing substrates of the type H-O-O-R, a category to which the peroxybenzoic acids belong. One explanation for the rapid reaction rate of the latter substrates is that the assumption of a fundamental compromise between specificity and rate is wrong. On the other hand, an alternative explanation is favored. The peroxybenzoic acids not only react with peroxidase more rapidly than other molecules of the type H-O-O-R (Davies et al., 1976) but an excess of *m*-chloroperbenzoic acid slowly destroys the active site after compound I formation. Thus if, during the course of their biochemical evolution, the peroxidases had been exposed to the perbenzoic acids, they might have developed a compromise between specificity and rate which would protect them from the harmful side reactions.

The reactivity of oxidizing substrates of the type H-O-O-R toward native peroxidases increases in the order R = C_2H_5, H, and ClC_6H_5CO (Dunford and Stillman, 1976). This can be explained on the basis that the best leaving groups fall in the order $C_2H_5OH < H_2O < ClC_6H_5COOH$. Alternatively, the perbenzoic acids may make use of a hydrophobic binding site (Dunford and Stillman, 1976). Whatever the explanation of the enhanced reactivity of the perbenzoic acids, it is extremely unlikely that they are the natural oxidizing substrates for the peroxidases.

The peroxidases from a wide variety of sources appear to react with hydrogen peroxide at about the same (nondiffusion-controlled) rate (Dunford and Stillman, 1976) which is evidence that the ultimate chemical efficiency of forming compound I from hydrogen peroxide has been reached. The pattern may emerge that it is not only reaction rate and specificity, but restraints imposed by the chemistry of the reaction being catalyzed, which must be considered in deciding upon the state of evolutionary development. An important philosophical point is whether nature can ultimately devise the perfect catalyst for every reaction of interest or whether the restraints of chemistry must sometimes prevail.

With increased attention to the importance of diffusion-controlled reactions, there are certain aspects of the interpretation of the experimental results which deserve special attention. Firstly, if the reaction is controlled by the phenomenological process of diffusion of reactants through the solvent to the enzyme, then by definition the rate of reaction will be controlled by the viscosity of the solvent. Therefore the appropriate experiments should be performed to determine the onset of the diffusion control. In the case of horseradish peroxidase, the experiments are relatively easy to perform, because the enzyme is robust enough (within limits) to withstand rather severe changes in its environment. Furthermore, for many of its reactions there is a broad pH-independent region in the rate profile, so that one does not need to be concerned about the influence of solvent composition upon pH. However, for more fragile enzymes, glycerol-water or sucrose-water mixtures may cause too large a change in dielectric constant. For such enzymes the elegant technique developed by Douzou and collaborators might be extended (Bielski and Freed, 1964; Douzou et al., 1970; Douzou, 1973). In this technique enzyme reaction rates are slowed by lowering the temperature, hence, increasing the viscosity, while maintaining a fixed dielectric constant with appropriate additions of a nonaqueous solvent. It is suggested here that it should be possible to design two different solvent systems which have widely different viscosities but the same dielectric constant at the same temperature. Hence, with various mixtures of the two solvent systems, a definitive test can be made as to whether a given enzymatic reaction is diffusion controlled.

Secondly, the size of the target area on the enzyme is of crucial importance. If surface diffusion is important, then the entire enzyme surface might be the target area (Koenig and Brown, 1972). However, in the case of carbonic anhydrase, surface diffusion was postulated because it was assumed that the substrate is carbonic acid, rather than the predominant form of hydrated carbon dioxide, the bicarbonate ion. If the reaction is written as

$$EH^+ + HCO_3^- \rightleftarrows E + H_2O + CO_2$$

then the principle of microscopic reversibility is not violated and, in a buffered solution, the interconversion of EH^+ and E is rapid enough to permit optimum turnover efficiency

(Khalifah, 1973; Lindskog and Coleman, 1973). Special attention has been paid to the carbonic anhydrase reaction by Chou and Jiang (1974). The latter workers assume that the Koenig and Brown (1972) analysis is correct and apply a large term for van der Waals attractive forces between carbonic acid and the enzyme. It would appear to be a moot point, however, whether solute–solute interactions would be significantly greater than solvent–solute van der Waals forces. At the present time, although strong evidence for surface diffusion in other biological processes does exist (Adam and Delbrück, 1968; Richter and Eigen, 1974), it would appear to be lacking for enzymatic reactions. In the latter case, appropriate grouping of enzymes on a membrane surface would appear to be nature's way of improving upon the diffusion-controlled limit (Ginsburg and Stadtman, 1970; Richter and Eigen, 1974).

Thirdly, electrostatic interactions between charged reactants can affect a diffusion-controlled reaction rate (Debye, 1942). In the case of enzyme reactions, total charge on the protein and charge on the active site are two factors to be taken into account. The latter has been assumed to be the dominant effect (Alberty and Hammes, 1958) with the treatment extended to allow correction for the former (Hammes and Alberty, 1959). It was pointed out that total charge detected by electrophoretic mobility was more accurate in principle than charge from titration data (or calculation based on pH and primary sequential data). Unfortunately, electrophoretic data cannot be obtained at low ionic strength where the Debye–Hückel limiting law is valid. In an attempt to apply Marcus theory to redox reactions of cytochrome *c* and inorganic reagents, Wherland and Gray (1976) made corrections for effective target radius and charge. If the entire protein molecule was taken as the target area, then the total charge on the protein gave a good fit to the data, but, if a much smaller target area was chosen, then a much smaller total charge gave an equally good fit. Perhaps the best qualitative tests of the relative significance of total charge and active site charge are those made under conditions where the two charges are opposite in sign. Such a test, using the secondary salt effect was made on alkaline horseradish peroxidase (Ellis and Dunford, 1969). Assuming the titration with base converts aquoferriperoxidase to the hydroxide form, a charge of $+2.0 \pm 0.5$ was obtained for the active site of the aquoenzyme. (The ferric ion in protoporphyrin IX has a formal charge of +1.) Over the pH range of 10–11, horseradish peroxidase has a large negative total charge (Phelps et al., 1971). Whether the hydroxide is the alkaline ligand has recently been questioned (Lanir and Schejter, 1975) but the presence of hydroxide remains the simplest explanation of the observed effects (Job et al., 1977; Job and Dunford, submitted for publication). Unfortunately, the Debye–Hückel limiting law is valid only for point charges in solutions of ionic strengths in the 0 to 0.01 range and the extended form which allows for ionic size is restricted to much the same range of ionic strengths. All extended forms for higher ionic strengths are semiempirical in nature.

In summary, diffusion control is now of such importance that much more experimental data are required. The existence of diffusion control can be tested unambiguously in aqueous glycerol and sucrose solutions, provided the integrity of the enzyme is maintained. If the integrity is not maintained, it should be possible to design more exotic solvent systems with variable viscosity but fixed dielectric constant at a fixed temperature. Both theory and experiment indicate diffusion control for enzyme reactions with rate constants of approximately 10^8 M^{-1} s^{-1} and perhaps considerably slower (Schmitz and Schurr, 1972). The present state of evidence appears to favor a target area the size of the active site rather than the total enzyme surface; there appears to be no solid evidence to favor surface diffusion effects in enzyme reactions. If the target area is the active site, then the effective charge of the enzyme should also be predominantly that of the active site.

The constant rate data in Table I provides good evidence that horseradish peroxidase maintains its structural integrity in the mixed solvent systems. The data in Table I would also indicate that the rate of formation of compound I from hydrogen peroxide in aqueous glycerol is independent of the dielectric constant, although the change in dielectric constant is considerably smaller than the corresponding change in viscosity. A cancellation of viscosity and dielectric constant effects would be fortuitous. Furthermore it would not be expected for a polar transition state in a diffusion-controlled reaction since in this case both a decrease in dielectric constant and an increase in viscosity would decrease the rate.

Ethanol has a much greater effect than glycerol on the dielectric constant of aqueous media. It soon became apparent, however, that the predominant effect of ethanol on peroxidase is to act as a ligand. The presence of ethanol decreases the rates of both compound I formation with hydrogen peroxide and the rate of cyanide binding. The inhibition of both cyanide binding and compound I formation implies that ethanol is bound to the sixth coordination position of the heme. Quantitative evidence is supplied by the agreement of the two kinetically determined enzyme–ethanol dissociation constants in which one ethanol is observed to bind per molecule of horseradish peroxidase. The agreement between the kinetically and spectrophotometrically determined dissociation constants seems only fair, but the errors in the spectrophotometric determination are large due to the very small absorbance changes. The kinetically and spectrophotometrically determined dissociation constants almost certainly refer to the same equilibrium. The binding of ethanol is also indicated by comparing the CD spectrum of horseradish peroxidase in the presence and absence of ethanol. The indication of ethanol binding to hemoproteins is a significant result since hemoproteins are often studied in the presence of ethanol (Kaminsky et al., 1972).

Evidence is accumulating that ethanol can act as a ligand for both ferrihemes (Angerman et al., 1969) and for ferrihemoproteins. Indications of ethanol binding to methemoglobin and methemoglobin hydroxide have been obtained by magnetic susceptibility measurements (Coryell and Stitt, 1940). The methemoglobin–EtOH complex formation leads to an increase in high spin character at pH 6.5. The predominantly low spin methemoglobin–hydroxide is converted into a high spin complex in 20% v/v of ethanol. These authors also tentatively concluded that ethanol binds to the heme iron atom, and they estimate a dissociation constant for the ethanol complex of about 0.4 M. Their interpretation is also based on the binding of only one molecule of ethanol per heme. Very recently the binding of ethanol to methemoglobin at pH 6.3 has been demonstrated with difference absorption and electron paramagnetic resonance spectroscopy (Brill et al., 1976). The binding stoichiometry was one ethanol molecule per heme group, and the dissociation constant of methemoglobin–EtOH was 0.2 M at pH 6.3 Ethanol also produced spectral changes in the hemoproteins metmyoglobin and catalase, but these absorbance changes were deemed too small for quantification.

The spectral changes observed in the presence of ethanol, displayed in Figure 4, did not occur at the same rate. The sharp maximum at 409 nm is fully developed and can be recorded

within the 60 s required to mix the samples and scan the spectrum. However, the maximum at 386 nm requires about 60 min to develop its full absorbance. This slow step may represent a sluggish conformational change which occurs subsequent to ethanol binding. Following the formation of the alkaline form of horseradish peroxidase and turnip peroxidase P_1, two slow absorbance changes have been noted (Job and Dunford, submitted for publication), which also may be a manifestation of conformational changes.

Many results concerning the decrease in rate of peroxidase reactions in the presence of ethanol and methanol have been interpreted in terms of the dielectric constant of the medium, although the possibility of other important factors was realized (Ogura et al., 1950; Tonomura, 1953; Hosoya, 1960). The theory of reaction rates as a function of dielectric constant used by Hiromi (1960b) is based on a model involving ions or dipolar molecules. An enzyme in solution may have catalytically important ionizable groups; and thus the dielectric constant may alter the reaction rate. However, in view of the mounting evidence for the binding of ethanol and methanol to hemoproteins, these kinetic studies might be best interpreted in terms of competitive inhibition.

The small thermally induced reversible changes in the CD spectrum of horseradish peroxidase may indicate that the asymmetric environment of the heme changes slightly due to a temperature-dependent enzyme conformation or that the heme itself is changing. The CD bands may also have changed as a consequence of the thermal equilibrium between the high and low spin states of ferric horseradish peroxidase (Tamura, 1971) since at pH 7 and 40 °C the enzyme is only about 80% high spin (Hartree, 1946).

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Mechanistic Analysis of the (Na^+,K^+)ATPase Using New Pseudosubstrates†

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ABSTRACT: 2,4-Dinitrophenyl phosphate and β-(2-furyl)-acryloyl phosphate are shown to be excellent substrates for the (Na^+,K^+)ATPase of axonal membranes. With K^+ and Na^+ both present, the maximal activity of the enzyme on dinitrophenyl phosphate and furylacryloyl phosphate is, respectively, 1.7 and 3.9 times greater than that observed with ATP. In the presence of K^+ only, the advantage of using the synthetic substrates rather than ATP is even greater, since 2,4-dinitrophenyl phosphate and furylacryloyl phosphate are hydrolyzed by the ATPase at maximal rates that are 100 and 200 times greater, respectively, than that of ATP. In the presence of K^+ only, the two substrates are hydrolyzed 6 and 13 times faster than *p*-nitrophenyl phosphate, the more usual pseudosubstrate. The dependence of substrate concentration, pH, and Na^+ and K^+ concentration on the ATPase activity is reported for these two substrates. Detailed results concerning the Na^+ and K^+ dependence of the rates of hydrolysis of 2,4-dinitrophenyl phosphate and β-(2-furyl)acryloyl phosphate are used to propose a mechanism of interaction between Na^+ and K^+ sites essential for enzyme activity. A good fit to experimental data was obtained with a model involving four Na^+ and two K^+ sites per mole of ATPase. Na^+ binding to its sites in this model is characterized by positive and negative cooperativity. The proposed model suggests a mechanism by which Na^+ modulates the cooperativity for K^+ binding.

Most of the kinetic data which have lead to the present knowledge of the mechanism of the (Na^+,K^+)ATPase[1] of eucaryotic cells have been obtained with ATP itself or with ATP analogues (Schwartz et al., 1972; Dahl and Hokin, 1974; Askari, 1974; Skou, 1975; Whittam and Chipperfield, 1975; Glynn and Karlish, 1975; Karlish et al., 1976). It has been shown that the (Na^+,K^+)ATPase hydrolyzes ATP in a stepwise fashion involving a Na^+-dependent phosphorylation of the enzyme followed by a K^+-dependent hydrolysis of the phosphoenzyme intermediate. It is also well known that (Na^+,K^+)ATPase preparations invariably exhibit an ouabain-inhibitable K^+-dependent phosphatase activity (Judah et al., 1962; Yoshida et al., 1969; Koyal et al., 1971). *p*-Nitrophenyl phosphate and acetyl phosphate which have free energies of hydrolysis higher than typical phosphate esters will also serve as substrates for the ATPase. The (Na^+,K^+)ATPase activity on these substrates occurring in the presence of K^+ only has often been compared to the final step in the reaction sequence of ATP hydrolysis, i.e., the K^+-dependent hydrolysis of the phosphoenzyme intermediate.

The purpose of this paper is to provide detailed information concerning the steady-state kinetic properties of the (Na^+,K^+)ATPase on two new synthetic substrates, 2,4-dinitrophenyl phosphate and β-(2-furyl)acryloyl phosphate. These substrates which have high free energies of hydrolysis are much better substrates than *p*-nitrophenyl phosphate and even ATP itself. The work presented in this paper is carried out with a pure membrane preparation (Balerna et al., 1975) containing

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[1] Abbreviations used are: (Na^+,K^+)ATPase, sodium, potassium activated adenosine triphosphatase (EC 3.6.1.3); Tris, tris(hydroxymethyl)-aminomethane; NADH, reduced nicotinamide adenine dinucleotide.