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Effect of Conformation on Isotopic Exchange in Synthetic Polypeptides*

Wilmer G. Miller

ABSTRACT: Calculated exchange rates are presented for amide isotopic exchange in synthetic polypeptide homopolymers and copolymers. The effects of pH, temperature, and ionic strength are considered. It is assumed that exchange does not occur from intramolecularly hydrogen-bonded (helical) amides and that the kinetics of the helix to random-coil conformation change is rapid compared with the isotopic exchange. The exchange rate law is considered to be a sum of independent parallel first-order terms with the apparent rate constants dependent on conformation as well as other factors. In homopolymers deviation from first-order behavior results from the high probability of residues near the end of a chain being in nonhelical conformation under all environmental conditions.

The calculated rates are shown to be consistent with known experimental data with respect to apparent reaction order, ionic strength effects, and conformational contribution to the exchange activation energy. The effects of composition and sequence on the exchange kinetics are investigated in copolymers where one of the monomers has an ionizable side chain. Decreasing the amount of ionizable monomer increases the deviation from simple first-order behavior. Comparison with experimental data indicates that only the conformational thermodynamics, and not the conformational kinetics, need to be considered to understand the exchange kinetics in copolymers.

Amide hydrogen–deuterium or hydrogen–tritium exchange in simple amides follows a first-order rate law although the reaction mechanism is complex, involving both acid and base catalysis (Berger *et al.*, 1959; Nielsen, 1960; Klotz and Frank, 1965). The pseudo-first-order rate constant at any pH varies somewhat with the amide. When amides are incorporated into a random-coil homopolymer the rate law is still simple first order (Ikegami *et al.*, 1965; Scarpa *et al.*, 1967; Englander and Poulsen, 1969). However, there may be differences between monomer and polymer exchange kinetics, one of the more striking examples being that of poly-*N*-isopropylacrylamide (Scarpa *et al.*, 1967; Klotz and Mueller, 1969).

Other than a simple first-order rate law might be expected in a polymer containing internal structure as a result of intramolecular amide hydrogen bonding, or in a copolymer if the exchange rate constant is different for each monomer. The rate law would be expected to be a sum of parallel first-order terms, or more complicated if the kinetics of conformational change must be included. It is thus interesting that Leichtling and Klotz (1966) and Ikegami and Kono (1967) found simple first-order behavior for polyglutamic acid irrespective of the degree of intramolecular hydrogen bonding. Deviation from first-order behavior has been observed, however, in copolymers of alanine and glutamic acid (Ikegami and Kono, 1967), which appears to depend in a complicated manner on the alanine content.

In many naturally occurring polymers the exchange kinetics are quite complicated. Exchange in proteins (Hvidt and Nielsen, 1966) and in nucleic acids (Printz and von Hippel, 1968) must be described frequently by a sum of first-order terms. Separation of copolymeric effects from effects of internal polymer structure are of importance but difficult.

In this communication calculated exchange curves are presented for polypeptide polymers whose residues exist either in disordered or in intramolecularly hydrogen-bonded helical conformations. The effects of pH, ionic strength, temperature, and monomer composition are considered. Comparison is made with the experimental values for polyglutamic acid and alanine–glutamic acid copolymers.

Basic Assumptions and Equations. An amide proton in a collection of polypeptide chains each containing $N + 1$ residues is classified as being in one of two states, according to whether or not it is intramolecularly hydrogen bonded. According to conformation, an intramolecularly H-bonded amide is assigned to a helical conformation, otherwise the amide is in a disordered or random-coil conformation.

The rate of isotopic exchange of a H-bonded or helical amide is taken to be negligible compared to the exchange rate

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when nonbonded. The kinetics of the helix–random-coil conformation change is considered to be very rapid relative to the exchange kinetics. The exchange is irreversible as a result of an infinite solvent reservoir. Under these assumptions the integrated rate equation for exchange out of the ith amide is

$$(n_i) = (n_i)_0 \exp(-f_{c,i}k_{c,i}t) \tag{1}$$

where $(n_i)/(n_i)_0$ is the fraction not exchanged out at time t, $f_{c,i}$ is the equilibrium probability of finding the ith amide in a random-coil conformation, and $k_{c,i}$ is the pseudo-first-order rate constant characterizing the exchange when nonbonded. The exchange rate constant from a nonbonded or random-coil unit has a complex pH, temperature, and solvent dependence. Inasmuch as our interest here is in conformational effects it is convenient to replace $k_{c,i}t$ by the generalized time τ. Thus

$$(n_i) = (n_i)_0 \exp(-f_{c,i}\tau) \tag{1'}$$

Experimentally the exchange of each amide is not followed separately. Assuming each amide was labeled initially to the same extent the overall fraction of protons not exchanged (f_{nex}) is given by a sum of parallel pseudo-first-order terms

$$f_{\text{nex}} = (1/N)\sum_{i=1}^{N} \exp\,(-f_{c,i}k_{c,i}t) \tag{2}$$

or by

$$f_{\text{nex}} = (1/N)\sum_{i=1}^{N} \exp\,(-f_{c,i}\tau) \tag{2'}$$

if the exchange rate constant for each amide proton in a disordered conformation is the same.

Experimentally $f_{c,i}$ cannot be measured. However, $f_{c,i}$ averaged over all positions in the chain, $\langle f_c\rangle$, can be measured by optical rotation or ultraviolet absorption measurements, and is given by

$$\langle f_c\rangle = (1/N)\sum_{i=1}^{N} f_{c,i} \tag{3}$$

Thus only the left-hand side of eq 2 and 3 can be determined experimentally. Theoretical values may be calculated after deriving an expression for $f_{c,i}$.

As a consequence of the cooperative nature of the thermodynamic stability of the helical conformation the value of $f_{c,i}$ is a function of chain position. Considering first an uncharged polymer, the conformational stability may be described by the Zimm–Bragg parameters σ and s related to helix initiation and growth, respectively (Zimm and Bragg, 1959). Following the usual approach for a chain with first-neighbor interactions a matrix of statistical weights, U_i, is constructed for the ith amide,

$$U_i = \begin{bmatrix} 1 & \sigma s \\ 1 & s \end{bmatrix}_i \tag{4}$$

whose elements depend on the conformation of amides $i - 1$ and i. The probability that the ith amide is in a random-coil conformation is

$$f_{c,i} = \frac{[10]U_1U_2 \cdots U_{i-1}U_i'U_{i+1} \cdots U_N[11]^T}{[10]U_1U_2 \cdots\cdots\cdots U_N[11]^T} \tag{5}$$

where

$$U_i' = \begin{bmatrix} 1 & 0 \\ 1 & 0 \end{bmatrix}_i \tag{6}$$

The average fraction of units in random-coil conformations can then be obtained from eq 3 or in the limit of large N from

$$\langle f_c\rangle = 0.5\{1 + (1 - s)/[(1 - s)^2 + 4\sigma s]^{1/2}\} \tag{7}$$

If some or all of the amino acid side chains have ionizable groups additional parameters describing side-chain charge and charge interactions must be included (Zimm and Rice, 1960; Miller and Monroe, 1968). Considering, for example, polyglutamic acid in the approximation of first-neighbor charge and conformational interactions

$$U_i = \begin{bmatrix} 1 & \lambda_c & \sigma s & \sigma s\lambda_h \\ 1 & W_{cc}\lambda_c & \sigma s & \sigma sW_{ch}\lambda_h \\ 1 & \lambda_c & s & s\lambda_h \\ 1 & W_{hc}\lambda_c & s & sW_{hh}\lambda_h \end{bmatrix} \tag{8}$$

$$U_i' = \begin{bmatrix} 1 & \lambda_c & 0 & 0 \\ 1 & W_{cc}\lambda_c & 0 & 0 \\ 1 & \lambda_c & 0 & 0 \\ 1 & W_{hc}\lambda_c & 0 & 0 \end{bmatrix} \tag{9}$$

and

$$f_{c,i} = \frac{[1000]U_1 \cdots U_{i-1}U_i'U_{i+1} \cdots U_N[1111]^T}{[1000]U_1 \cdots\cdots\cdots U_N[1111]^T} \tag{10}$$

where $\lambda_c = K_c/(\mathrm{H}^+)$, $\lambda_h = K_h/(\mathrm{H}^+)$, and W_{ab} are Boltzmann factors given by $W_{ab} = \exp(-w_{ab}/kT)$. Here K_c and K_h are carboxyl ionization constants and w_{ab} is an electrostatic interaction parameter. At pH values where the polymer is un-ionized $\langle f_c\rangle$ is given by eq 3 or 7. At other pH values an effective s may be defined which approaches s in the limit of no ionization (Zimm and Rice, 1960).

In a binary copolymer composed of monomers X and Y with only X having an ionizable side chain, four types of U and U' matrices are required, U_{xx}, U_{xy}, U_{yx}, and U_{yy} and similarly for U'. Here the second subscript refers to the side chain associated with amide proton i, and the first to $i - 1$. U_{xx} and U_{xx}' are given by eq 8 and 9, respectively. U_{yx} and U_{yx}' are obtained by setting $w_{ab} = 0$, and the remaining matrices are obtained by setting $w_{ab} = 0$, and $\lambda_c = \lambda_h = 1$.

These methods may be extended to include both longer range conformational as well as electrostatic interactions, the latter being particularly important at low ionic strengths. To do so increases the computational time considerably giving rise to some alteration in the exchange curves but to no change in the major conclusions (*cf. seq.*).

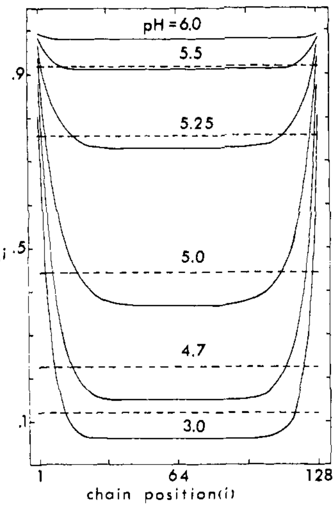

FIGURE 1: Random-coil probabilities as a function of chain position. The parameters used in making the calculations are appropriate for polyglutamic acid in 0.2 M NaCl. The discrete points have been fused into solid lines for clarity. Dashed curves indicate $\langle f_c \rangle$ at each pH.

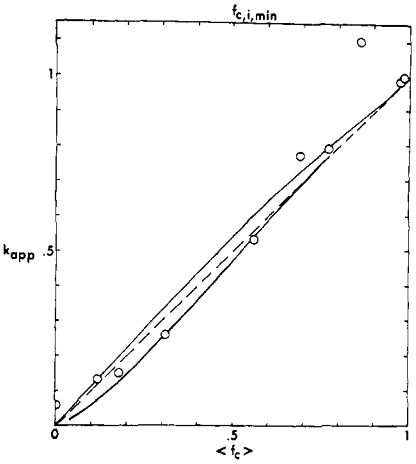

FIGURE 3: Conformational reduction of first-order rate constant as a function of $\langle f_c \rangle$ (lower curve) and the minimum value of $f_{c,i}$ at each pH (upper curve). Rate constants were determined from the slopes in Figures 2 and 4. Dashed line is the result expected for a one-to-one correspondence. Experimental values of Ikegami and Kono (1967) are also shown (circles).

Homopolymer Exchange. EFFECT OF pH AND IONIC STRENGTH. Calculations relevant to polyglutamic acid are presented inasmuch as it is the only homopolymer polypeptide whose exchange has been investigated through the region of the conformational transition. With $\sigma = 0.003$ and $\Delta G_{s,}{}^{\circ}{}_{298}$ ($= -RT \ln s$) $= -120$ cal/mole (Snipp *et al.*, 1965), and assigning $w_{hh}/kT = 2.0$, $w_{hc}/kT = w_{ch}/kT = 0.8$, $w_{cc}/kT = 0.7$, and $pK_h = pK_c = 4.45$, calculated titration and helix–coil transition curves are in agreement with experiment for polyglutamic acid in 0.2 M NaCl (Miller and Monroe, 1968). Using these parameters in eq 8–10 $f_{c,i}$ as a function of chain position was calculated for a chain of 128 amides, as shown in Figure 1. As expected the $f_{c,i}$ values are symmetric about the midpoint of the chain. The $f_{c,i}$ values were used in eq 2′ to calculate the exchange rate curves. The results are shown in Figure 2. Although the exchange curves appear to be nearly simple first order, particularly when $\langle f_c \rangle$ is >0.5, it is obvious from Figure 1 that a distribution of $f_{c,i}$ values exist at any pH. The apparent first-order rate constants were determined, ignoring the very early period of exchange in cases where there was detectable curvature. They are plotted in Figure 3 as a function of $\langle f_c \rangle$, and as a function of the minimum value of $f_{c,i}$ at each pH ($f_{c,i,min}$).

Changing the ionic strength effects the electrostatic interactions, and to a lesser degree s and σ. Most exchange experiments are performed in the absence of added electrolyte. Although long-range electrostatic interaction should be taken into account at low ionic strengths a first-neighbor calculation semiquantitatively fits the experimental titration and conformational data if the w_{ab} are adjusted properly. Figure 4 shows the exchange curves resulting from calcula-

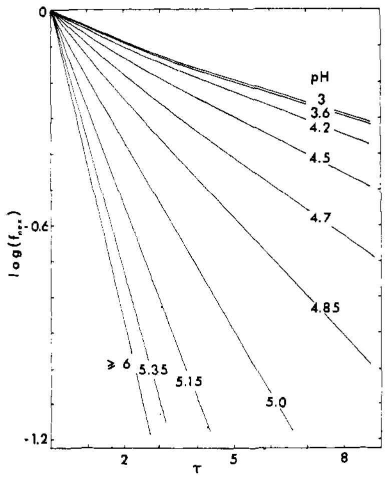

FIGURE 2: Log (f_{nex}) as a function of generalized time using the $f_{c,i}$ values from Figure 1.

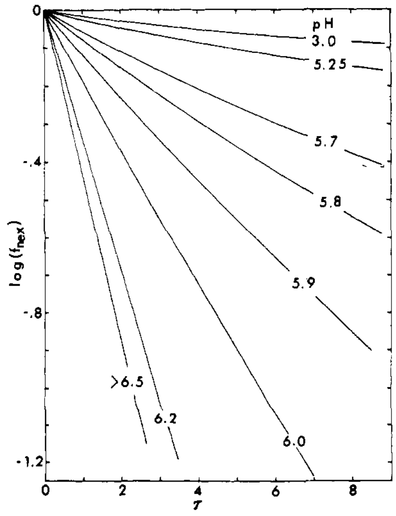

FIGURE 4: Exchange curves based on a set of parameters appropriate to polyglutamic acid at low ionic strength.

tions with $\Delta G_{s,}{}^{\circ}{}_{298} = -270$ cal/mole, $\sigma = 0.003$, $pK_h = pK_c = 4.75$, $w_{hh}/kT = 8.0$, $w_{hc}/kT = w_{ch}/kT = 1.5$, and $w_{cc}/kT = 1.4$. Compared at equal $\langle f_c \rangle$ the probability curves are nearly superimposable with the corresponding one in Figure 1, and the $k_{app} - \langle f_c \rangle$ curve is superimposable (Figure 3). This suggests that expanding the interaction matrix to include long-range electrostatic interactions will affect the exchange curves negligibly.

Increasing the chain length results in $\langle f_c \rangle$ being nearer $f_{c,i,min}$ as end effects which give rise to large values of $f_{c,i}$ become increasingly less important. Consequently the exchange curves show even less deviation from simple first order than those in Figures 2 and 4.

EFFECT OF TEMPERATURE. In addition to the temperature dependence of the $k_{c,i}$ the $f_{c,i}$ will be temperature dependent and thus make a contribution to the apparent activation energy, $E_{a,app}$. All polypeptide–aqueous solvent systems so far studied show a "normal" conformational temperature dependence, *i.e.*, increasing the temperature shifts the equilibrium towards the random coil. There are three distinct conformational regions to consider: high random-coil content, high helical content, and the transition region.

In the first region, all $f_{c,i}$ values are essentially unity and will be negligibly effected by increasing the temperature. Under conditions of high random-coil content there is no conformational contribution to $E_{a,app}$.

In the region of high helical content a distribution of $f_{c,i}$ values exists, although the majority lie close to the average value, as in Figure 1. The conformational contribution may conveniently be estimated from the temperature dependence of $\langle f_c \rangle$ using eq 7. As the helix is favored, $s > 1$ and eq 7 may be rearranged so that

$$\langle f_c \rangle = 0.5[1 - (1 - x)^{-1/2}] \qquad (11)$$

where

$$x = 4\sigma s(1 - s)^{-2} \qquad (12)$$

The conformational contribution to $E_{a,app}$ is

$$E_{a,conf} = d \ln \langle f_c \rangle / d(-1/RT) = [d \ln \langle f_c \rangle / d \ln x] \times [d \ln x / d(-1/RT)] \qquad (13)$$

An expression for each of the derivatives on the right-hand side of eq 13 may be obtained from eq 11 and 12 resulting n

$$d \ln \langle f_c \rangle / d \ln x = (x/2)(1 + x)^{-1}[(1 + x)^{1/2} - 1]^{-1} \qquad (14)$$

and

$$d \ln x / d(-1/RT) = \Delta H_{\sigma}^{\circ} + \Delta H_s^{\circ} + 2\Delta H_s^{\circ}/(e^{\Delta G_s^{\circ}/RT} - 1) \qquad (15)$$

The enthalpies and entropies associated with σ and s have been assumed to be temperature independent. The expression for d ln $\langle f_c \rangle$/d ln x is unity ln the limit of $x = 0$, greater than 0.9 if $x < 0.15$, and > 0.6 if $x < 1$. Unless the cooperativity is very slight (σ large) conditions only slightly on the helical side of the transition midpoint will correspond to σ and s values such that $x \ll 1$. Therefore the right-hand side of eq 14 is to a good approximation unity. The temperature dependence of $\langle f_c \rangle$ comes then from the d ln x/d($-1/RT$) factor. The magnitude of the quantity $\Delta H_{\sigma}^{\circ}$ is unknown. It has generally been assumed that cooperativity was entropic in origin, hence $\Delta H_{\sigma}^{\circ} = 0$ (Zimm and Bragg, 1959; Applequist, 1963). Calculations do indicate that it could be enthalpic (Brandt, 1968). If so, $\Delta H_{\sigma}^{\circ}$ would be positive. Experimentally the temperature dependence of σ has not been determined with certainty. Therefore the best limits which can be set are $0 \leqslant \Delta H_{\sigma}^{\circ} \leqslant -RT \ln \sigma$. The quantity ΔH_s° is negative for all known aqueous or aqueous–organic systems. In aqueous or aqueous–organic systems $0 < -\Delta G_s^{\circ}/RT < 1$ in all cases which have been studied. In order to stay under conditions of high helical content we set an arbitrary lower limit of $-\Delta G_s^{\circ}/RT > 0.2$. Using these limits for ΔG_s° the contribution from the second and third terms in eq 15 is 2–10 times $|\Delta H_s^{\circ}|$. It should be noted that even under conditions of high helical content the conformational contribution to $E_{a,app}$ is temperature dependent, *i.e.*, a nonlinear Arrhenius plot is expected. It is doubtful that this curvature can be detected experimentally.

It should be kept in mind that in aqueous or partially aqueous solvents most polypeptides which are soluble have at least some ionizable side chains. Typically they must be predominantly uncharged for the helix to be favored. The effective σ and s are close to the values for the unchanged transition and the previous discussion is valid. In the random-coil region there is a negligible conformational contribution making the presence of charged groups unimportant. In a given solvent system the transition may be reached by a change of temperature or pH. If by a change in temperature while keeping the polymer uncharged, the contribution to $E_{a,app}$ is given by eq 14 and 15. At the transition midpoint the contribution is $-\Delta H_s^{\circ}/2(\sigma)^{1/2}$ (Applequist, 1963). A nonlinear Arrhenius plot is expected but may go undetected unless a broad range of temperatures are studied. If in the transition region the polymer is partially charged the temperature dependence of the effective s is difficult to express.

Copolymers. Unlike a protein synthesis most nonbiological polymer synthesis lead to a wide variety of monomer sequences. Polypeptides polymerized from *N*-carboxyanhydrides are no exception. Experimentally polypeptides prepared in this manner are the only ones in which the amide proton exchange has been studied and are of the type alanine–glutamic acid (Ikegami and Kono, 1967). The approach of Lehman and McTague (1968) to copolymer conformation calculations could in principle be used here. Instead we have chosen to use a Monte Carlo type approach (Flory, 1969). A specific sequence is generated according to some prescription, f_{nex} at various times is computed, another chain generated, f_{nex} is computed and averaged with the first chain, and the procedure continued until the exchange curve is unchanged when additional chains are added. In practice only a few chains are needed for adequate convergence.

Only binary copolymers are considered. Monomer X has an ionizable side chain, whereas monomer Y is uncharged at any pH.

Exchange with $\sigma(Y) = \sigma(X)$, $s(Y) = s(X)$, *and* $k_c(Y) = k_c(X) = 1$. Numerical values for σ and s, and the electrostatic parameters necessary for X were assigned as in the first set of homopolymer calculations. Specific sequence chains were

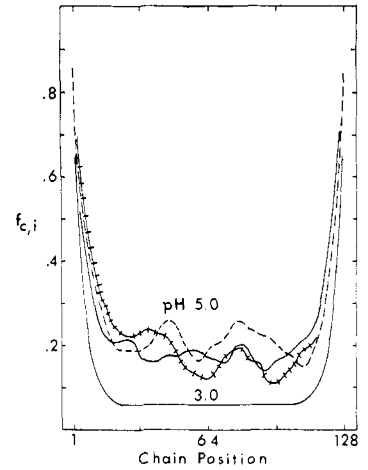

FIGURE 5: Random-coil probabilities in three randomly generated copolymer chains. Random generator set to give mole fraction monomer X of 0.75. Actual composition of chains generated is 0.750 (---), 0.727 (---), and 0.734 (+++++). Electrostatic parameters for monomer X are as in Figure 1, and σ and s for both X and Y are as in Figure 1. Lower curve—probability curve for each of the chains at pH 3.00; upper curves—probability curves when pH 5.00.

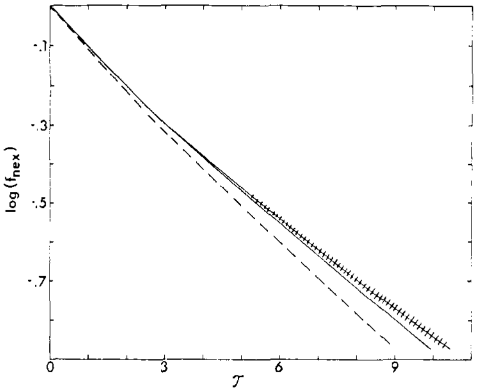

FIGURE 6: Exchange curves at pH 5.00 for each of the chains whose random-coil probabilities are given in Figure 5.

enumerated using a random number generator. Using eq 8–10 the $f_{c,i}$ were calculated and the exchange curve determined using eq 2′. At a pH less than the pK of the side-chain ionization in monomer X the results were as expected. The $f_{c,i}$ probabilities as a function of chain position are effectively independent of monomer composition or sequence. Typical of the results is the lowest curve in Figure 5. Compared at equal $\langle f_c \rangle$ this distribution is nearly superimposable with that of the homopolymer $f_{c,i}$ distribution. Therefore the exchange rate will also be similar to that of the homopolymer.

As the pH is raised so that the preponderance of the side chains associated with monomer X are ionized both composition and sequence affect the $f_{c,i}$ distribution. When the content of monomer Y is low the $f_{c,i}$ distribution is similar to that in the homopolymers. As the Y content is raised the probability of long sequences of Y is increased. In such a chain the ionization of the X units will disrupt the helix in the X-rich regions but not in the Y-rich regions. Under these conditions both sequence and composition become important. This effect is illustrated in the upper curves in Figure 5, and in Figure 7. With the random number generator set to yield an average monomer Y composition of 0.25, the pH 5.00 results vary from chain to chain, in contrast to the pH 3.00 results. The variation is not large, and the exchange curves, Figure 6, do not vary greatly from chain to chain. Increasing the Y composition to 0.70 increases markedly the variation in the distribution curves from chain to chain, as is shown in Figure 7. If the $f_{c,i}$ are averaged at each chain position over 20 chains, the averaged distribution function, Figure 7a, is obtained. This looks similar to a homopolymer distribution in marked contrast to an individual chain distribution. If additional chains are included in the average, the distribution becomes indistinguishable from a homopolymer distribution compared at equal $\langle f_c \rangle$. This does not hold for the exchange curves. The 20-chain exchange curve in Figure 8 deviates much more from simple first-order behavior than any in Figures 2 or 4. Individual exchange curves also vary much more than in the lower Y content copolymer. Purely by chance the first chain generated had an exchange curve fairly close to the 20-chain average.

Exchange with $\sigma(Y) = \sigma(X)$, $s(Y) = s(X)$, and Variable k_c. The chain whose sequence of $f_{c,i}$ distribution is given in Figure 7d was investigated for effect of varying k_c. With $k_c(X) = 1$, $k_{c,i}f_{c,i}$ values were tabulated and exchange curves calculated with $k_c(Y)$ equal to 0.1, 1.0, or 10.0. The time for half-exchange was $\tau_{1/2} = 0.63$, 3.9, or 24.5 and the average value of $f_{c,i}k_{c,i}$ 0.0858, 0.207, or 1.420, respectively. The distribution of $f_{c,i}k_{c,i}$ values for each case is shown in Figure 9 and the corresponding exchange curves in Figure 10 where τ has been divided by $\tau_{1/2}$ in order to approximately normalize the time scales. By choosing equal intervals on a logarithmic

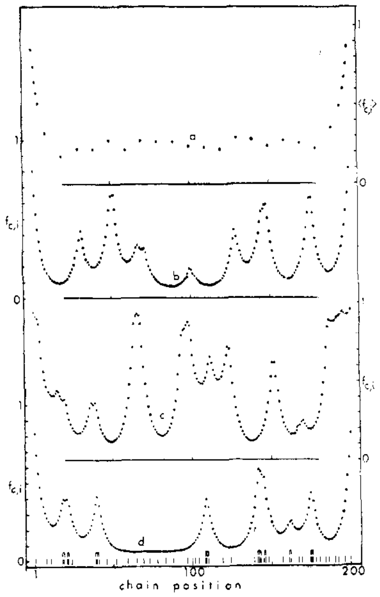

FIGURE 7: Random-coil probabilities with $N = 200$, pH 7.00, random number generator set for 0.30 mole fraction X, and with other parameters as in Figure 5. Upon generating 20 chains, (a) is the composite curve, (b) is the first chain generated, and (c) and (d) give exchange rates deviating most severely from the average. The vertical lines in d indicate the chain positions occupied by monomer X, and are connected (II) when X units are adjacent.

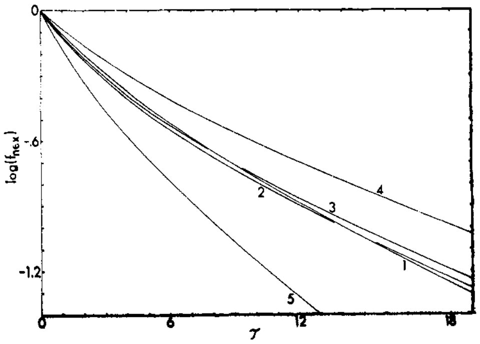

FIGURE 8: Exchange curves based on the probability curves in Figure 7: (1) first chain generated; (2) average of first 10 chains; (3) average of 20 chains; (4) and (5) the extreme exchange curves of the 20-chain sample.

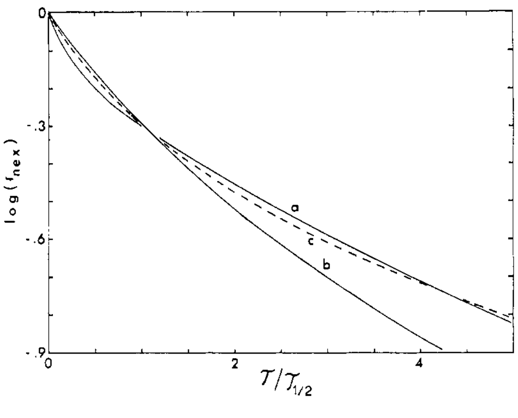

FIGURE 10: The exchange curves for the chain and under the conditions leading to the $f_{c,i}k_{c,i}$ values shown in Figure 9.

scale $f_{c,i}k_{c,i}$ distributions superimposable by an abscissa coordinate shift will lead to superimposable exchange curves when plotted as in Figure 10. The exchange curves are remarkably similar considering the variation in the distribution of the $f_{c,i}k_{c,i}$ values. Averaging over a number of chains leads to exchange curves which differ quantitatively but not qualitatively from the single-chain results.

Exchange with Variable σ and s. The $f_{c,i}$ distribution will be effected if monomers X and Y have different values of σ or s. At low pH where X is uncharged a large difference in either σ or s leads to an uneven distribution function resembling more the upper curves in Figure 5 than the lower. Increasing the pH to the point where a considerable fraction of the side chains of monomer X is ionized results in very irregular distribution functions similar to those in Figure 7. However, they appear at a lower mole fraction of monomer Y than when the σ and s values are equal in the two monomers.

Discussion

Calculated Exchange Curves. The calculations are based on the assumption that isotopic exchange is slow compared to conformational change, allowing the use of equilibrium conformational properties. Several relaxation methods indicate that the half-time for changing the length of a homopolymer helical sequence by one residue ranges from 0.01 to 1 μsec (Lumry *et al.*, 1964; Hammes and Roberts, 1969; Schwarz and Seelig, 1968) compared to a half-time of seconds for exchange from a simple amide. The equilibrium constant for changing the length by one residue is always of the order of unity for water-soluble polypeptides. On the basis of a simple random walk it would appear that the lifetime of a residue in either conformation is short compared to the exchange lifetime. However, recent nuclear magnetic resonance data in mixed organic solvents indicate a conformational lifetime of greater than 0.1 sec (Ferretti and Paolillo, 1969). The apparent inconsistency between the nuclear magnetic resonance and other relaxation data has not been explained fully nor has this inconsistency been reported in aqueous systems. The effect of a conformational lifetime comparable to an exchange lifetime is to further increase the dispersion in the apparent simple first-order exchange behavior in contradiction to the experimental data (*cf. seq.*).

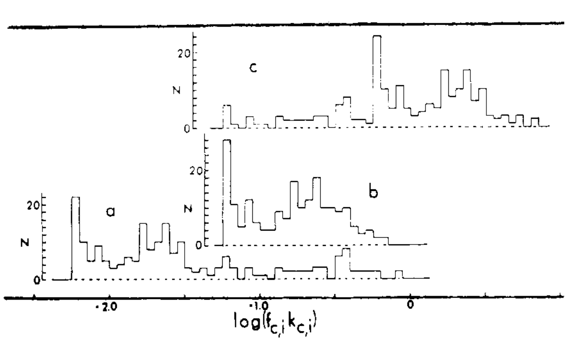

FIGURE 9: Distribution of $f_{c,i}k_{c,i}$ values at pH 7.00 for the chain whose sequence is shown in Figure 7d with $k_c(X)$ = 1.0, and with $k_c(Y)$ = 0.1(a), 1.0(b), or 10.0(c). The $f_{c,i}k_{c,i}$ values were grouped into equal logarithm intervals within each decade. The frequency (N) of values within each interval is plotted against the distribution interval on a logarithmic scale.

Using a first-neighbor interaction matrix is admittedly questionable, particularly at low ionic strengths where electrostatic interactions are significant over large distances. In homopolymers the shapes of the $f_{c,i}$ distribution is controlled primarily by the average length of a random-coil sequence, which in turn is dependent on the effective s and σ. Changes in the effective s and σ resulting from better accounting of the long-range interactions may be offset by slightly shifting the pH. Consequently, the homopolymer calculations will be little effected by more appropriate handling of the electrostatic interaction. The method of handling the electrostatic interactions will, however, strongly influence the shape of the $f_{c,i}$ distribution in copolymers. The strongly peaked $f_{c,i}$ distributions encountered in Figure 7 will be considerably smoothed when higher order electrostatic interactions are taken into account. The first-order exchange curves will be less curved in general and fewer chains needed to obtain the averaged exchange curve.

One of the striking features of the calculated exchange curves is the wide variety of apparent rate constant distributions giving rise to exchange curves which appear to be nearly simple first order. If the time scale in eq 2 is expanded, the largest value of $f_{c,i}k_{c,i}$ will be related to the initial slope in the first-order plot, and the smallest to the final slope at infinite time. The distribution, however, may be essentially continuous and highly irregular in frequency of occurrence. Also experimentally the exchange is rarely followed past 90 or 95% completion. One may inquire whether or not there is any distribution of $f_{c,i}k_{c,i}$ values which will lead to simple first-order exchange. If such a distribution did exist, then

$$f_{\text{nex}} = (1/N)\sum_{i=1}^{N}\exp(-k_it) \qquad (16)$$

could be replaced by

$$f_{\text{nex}} = \exp\left(-\langle k\rangle t\right) \qquad (17)$$

where $\langle k\rangle$ is an averaged rate constant. If eq 16 can be replaced by eq 17

$$\mathrm{d}\ln f_{\text{nex}}/\mathrm{d}t = -\langle k\rangle \qquad (18)$$

and

$$\mathrm{d}^2\ln f_{\text{nex}}/\mathrm{d}t^2 = 0 \qquad (19)$$

Expressions for these derivatives may be obtained from eq 16, and upon substitution become

$$\sum_{i=1}^{N}(\langle k\rangle - k_i)\exp(-k_it) = 0 \qquad (20)$$

and

$$\sum_{i=1}^{N-1}\sum_{j=2}^{N}(k_i - k_j)^2\exp[-(k_i + k_j)t] = 0 \qquad (21)$$

Equation 20 contains both positive and negative terms making it difficult to see whether or not it can be satisfied. Each term in eq 21, however, is positive. Hence, eq 21 cannot be satisfied unless $k_i = k_j$, *i.e.*, unless there is a single rate constant.

Irrespective of the method used to describe neighbor interaction a large number of chains gives a composite $f_{c,i}$ distribution curve very similar to the homopolymer curves. The composite exchange curve, however, is markedly different from the corresponding homopolymer curve, *e.g.*, Figure 2 and 8-3. The use of an averaged rate constant at each chain position will lead to erroneous exchange curves. The origin of this can be seen in eq 21 in that a distribution of rate constants can never be replaced by a single averaged value. This is analogous to the chain dimensions of copolymers where composition averaging at each chain position leads to erroneous conformational dimensions (Flory, 1969).

Comparison to Experimental Results on Homopolymers. The exchange kinetics for polyglutamic acid are reported by Ikegami *et al.* (1965) to be simple first order. Representative data from their paper are shown in Figure 11, where we have drawn straight lines through their data points to give a better indication of the linearity. The relevant calculated curves, Figures 2 and 4, indicate that linearity should not be vigorously correct. However the deviation is less than their experimental error. By comparison with model compounds Ikegami and Kono (1967) separated conformation from other effects on the rate constant. The conformation contributions a shown in Figure 3 for comparison with our calculated values.

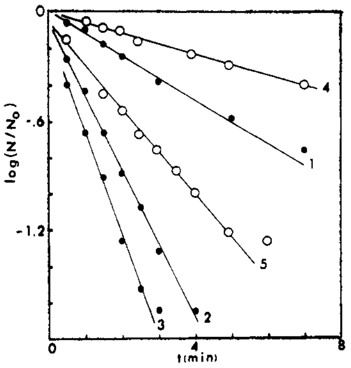

FIGURE 11: Exchange curves for polyglutamic acid at pH 5.69(1), 5.88(2), and 6.00(3) and for an alanine–glutamic acid copolymer (20:80) at pH 5.54(4) and 6.01(5) (Ikegami *et al.*, 1965; Ikegami and Kono, 1967).

Leichtling and Klotz (1966) have also studied exchange from polyglutamic acid. The rate law was reported to be simple first order. No exchange curves are presented so direct comparison cannot be made.

The Arrhenius activation energy is 17 kcal for random-coil polylysine or poly-D-L-alanine (Hvidt and Nielsen, 1966; Englander and Poulsen, 1969), and 20 kcal for random-coil though partially H-bonded poly-*N*-isopropylacrylamide (Scarpa *et al.*, 1967). At low pH where the helical content is a maximum, Leichtling and Klotz (1966) observed an activation energy of 27 ± 5 kcal for polyglutamic acid, 10 ± 5 kcal above the random-coil values. Under these conditions, the conformational contribution to the activation energy is given by eq 13–15. For polyglutamic acid $\Delta H_s° \approx -1$ kcal (Miller and Nylund, 1965; Rialdi and Hermans, 1965; Olander and Holtzer, 1968) and we estimate $-\Delta G_s/RT$ to be > 0.05 but <1. The predicted conformational contribution is 3–5 kcal plus $\Delta H_\sigma°$. As $\sigma \approx 10^{-3}$ the contribution from $\Delta H_\sigma°$ is 0–4 kcal. The several kilocalories increase in activation energy in exchange from the predominately helical polymer is easily explained as a conformation contribution, although this small increase could come from other sources. It furthermore strengthens the view that exchange occurs from random-coil conformations only.

Comparison to Experimental Results on Copolymers. The only experimental study on exchange with copolymers is that of Ikegami and Kono (1967) using alanine–glutamic acid copolymers. Copolymers containing 20, 40, and 70 mole % alanine were investigated as a function of pH. We summarize their results, first for the 20 and 40% alanine copolymers, and then for the 70% alanine copolymers.

In the 20 and 40% copolymer, in the high pH range where most of the carboxyls are ionized optical rotation data show little evidence of helical structure, and in general the exchange curves are sensibly simple first order, Figures 11–13. In the low pH region there is observable curvature in the 40% copolymer carried to high extent of exchange, Figure 13. The conformational contribution to the initial exchange rate constant is approximately proportional to the average random-coil content.

In the 70% polymer the exchange curves noticably deviate

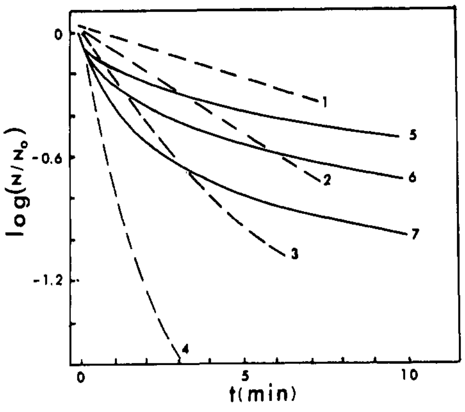

FIGURE 12: Exchange curves for a 40% alanine–60% glutamic acid copolymer at pH 5.84(1), 6.06(2), 6.08(3), and 6.52(4), and for a 70% alanine–30% glutamic acid copolymer at pH 6.26(5), 6.42(6), and 6.54(7) (Ikegami and Kono, 1967).

from simple first order at any pH, Figures 12 and 13. At high pH the polymer is still predominantly helical. At low pH the exchange curves were separable into contributions from "rigid" and from "flexible" helical regions. The exchange kinetics from the 70% alanine copolymer thus appear to be qualitatively different from the other copolymers.

Calculations relevant to these copolymers may be made at several degrees of approximation. Assuming k_0(Ala) = k_0(Glu), s(Ala) = s(Glu), and σ(Ala) = σ(Glu), Figures 5–8 are appropriate for comparison. For the 20% polymer exchange curves at low and at high pH are similar to the homopolymer curves, *i.e.*, sensibly linear and consequently in agreement with the experimental data. In the helix–coil transition region, *e.g.*, Figure 6, a slightly greater deviation from linearity is found in the calculated curves than in the experimental data, Figure 11. As previously discussed better accounting of the electrostatic interactions will tend to straighten the computed curves. In as much as alanine–glutamate copolymers of varying composition have comparable exchange rate constants when in random-coil conformations (Ikagami and Kono, 1967) suggests that k_0(Ala) = k_0(Glu). For the conformational parameters experimental evidence indicates that s(Ala) $\leq$ s(Glu) and that σ(Ala) < σ(Glu) (Nitta and Sugai, 1967; Ingwall *et al.*, 1968). Lowering both s(Ala) and σ(Ala) has little effect on the $f_{c,i}$ distribution as the effects are compensating.

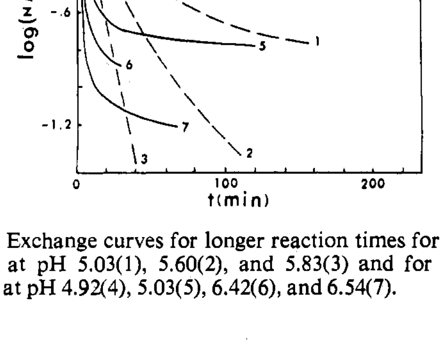

FIGURE 13: Exchange curves for longer reaction times for the 40:60 copolymer at pH 5.03(1), 5.60(2), and 5.83(3) and for the 70:30 copolymer at pH 4.92(4), 5.03(5), 6.42(6), and 6.54(7).

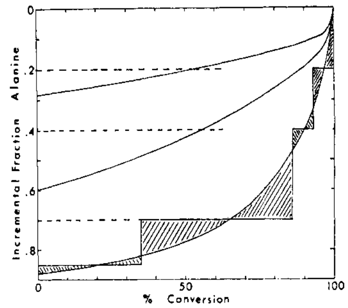

FIGURE 14: Incremental copolymer composition as a function of per cent monomer conversion in the polymerization of alanine and benzylglutamate N-carboxyanhydrides. Average composition at complete conversion given by dashed line. Hatched areas bordering the 70% alanine copolymer show the simulation of this copolymer by four fixed composition copolymers.

Calculated rate curves for the 40% copolymer are similar to the 20% except that nonlinearity is more pronounced and it also extends further into the low pH region. In order to save computing time extensive calculations were not made for this composition. However, the trends are in line with the experimental data of Ikegami and Kono (1967).

The calculated $f_{c,i}$ distributions for the 70% alanine copolymer at high pH are qualitatively different from the copolymer of lower alanine content. At complete ionization regions of a chain rich in alanine are still largely helical.

Significant deviation from simple first-order exchange thus extends over a far wider pH range. Changing electrostatic, σ, or s parameters to correspond more appropriately to alanine–glutamate copolymers at low ionic strength (Nagasawa and Holtzer, 1964; Nitta and Sugai, 1967; Olander and Holtzer, 1968; Ingwall *et al.*, 1968), still gives exchange curves qualitatively similar to those in Figure 8, and not in very good agreement with the experimental data, Figures 12 and 13. The striking features of the experimental curves are the initially rapid exchange followed by a slow exchange. We then decided to consider the monomer distribution in the copolymers. Ikegami and Kono (1967) determined the reactivity ratios for the copolymerization, thereby providing the necessary information to calculate the incremental copolymer composition as a function of per cent monomer conversion. Using standard methods (Flory, 1953) and their reactivity ratios the incremental copolymer composition was calculated for 20, 40, and 70 mole % copolymers. The calculated curves are shown in Figure 14. It is furthermore known that the polymerization mechanism for the initiator and solvent systems utilized by Ikegami and Kono leads to polymers whose chain length is sensibly independent of the per cent conversion of monomer into polymer (Blout and Karlson, 1956). Thus, the incremental composition is a measure of the average monomer composition of the polymer molecules formed at each stage in the reaction. The monomer sequence within a molecule may be considered to be random with a composition governed by the incremented composition at that point in the reaction.

Since the molecular discription of the copolymer is a set of essentially random copolymers whose compositions parallel the incremented composition, a better view of the exchange is afforded by considering the exchange from such a collection of molecules. As it is impractical to calculate the exchange from a continuous set, a discrete set was chosen. Simulating the 20 and 40% alanine polymers in this manner gives composite exchange curves differing insignificantly from those based on a single composition, whereas the 70% alanine results were considerably altered. The reason for this can be seen by comparing the random-coil probability curves in Figures 1, 5, and 7. If the alanine content is not too high, charging the glutamate side chains will disrupt the helix in a relatively even manner allowing both glutamate and alanine amide protons to exchange. In the 20% alanine and in most of the molecules in the 40% alanine polymer the random-coil probability chain position curves are relatively smooth. However, in the 70% alanine copolymer, 65% of the molecules have less than 30% glutamic acid, and 20% less than 15% glutamic acid. Charging up the side chains of the low glutamate molecules increases the exchange rate very little, whereas those molecules rich in glutamic acid will show considerable dependence of the exchange on pH. We can thus see why the 70% alanine copolymer might show qualitatively different exchange kinetics from the 20 and 40% polymers.

Simulating the 70% alanine copolymer with random copolymers containing 80% glutamate (7%), 60% glutamate (7%), 30% glutamate (51%), and 15% glutamate (35%), calculated exchange curves are shown in Figure 15 using a set of parameters thought to be realistic for this copolymer. The parameters describing glutamic acid were those appropriate at low ionic strength. For alanine we took $\sigma(\text{Ala}) = 10^{-4}$, $s(\text{Ala}) = s(\text{Glu})$, and $k_0(\text{Ala}) = k_0(\text{Glu})$. The time scale for each pH is adjusted to reflect the pH dependence of the apparent rate constant observed by Ikagami and Kono (1967) for the presumably random-coil L-alanine, D-glutamic acid copolymer. Taking into account the nonrandom character of the copolymerization results in exchange curves which begin to have the general features of the experimental ones. The chief difference is that the fraction of amides with a high rate constant is not large enough. It is the alanine protons in the high alanine content molecules which are the last to exchange. Letting $k(\text{Ala}) < k(\text{Glu})$ will tend to flatten out the exchange curves when τ is large. A more proper accounting of the electrostatic interactions would also be expected to alter the exchange curves. Although the calculations are based on some rather crude estimates of parameters, it seems reasonable to conclude that the general features of the exchange kinetics can be understood assuming equilibrium conformation in the random-coil population. Contrary to the conclusions of Ikegami and Kono we believe it is unnecessary at this time to invoke new structural properties to the 70% alanine copolymer.

Exchange in Proteins. Information about structure, and structural changes resulting from environmental changes has been the goal of those studying amide exchange in proteins. The experimental data are never resolved meaningfully to yield more than three or four rate constants (Hvidt and Nielsen, 1966; Segal and Harrington, 1967), irrespective of the actual rate constant distribution. Even proteins which appear to be random coil, devoid of native structure, show complex exchange kinetics (Woodward and Rosenberg, 1970) indicating the difficulty of separating macromolecular structural effects from intrinsic differences among the various amino acid residues.

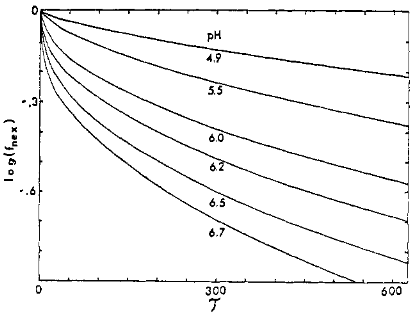

FIGURE 15: Exchange curves for a 30% glutamic acid–70% alanine copolymer taking into account the nonrandom character of the synthesis.

The calculations presented herein further emphasize the difficulty in separating structural from nonstructural effects. Without a very detailed model from which one can predict the exchange kinetics under a variety of conditions, it is not possible to recognize conformational thermodynamic effects. If conformational kinetics must be included, as appears to be the case with some proteins, an even more detailed model must be at hand to aid data interpretation.

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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, $k_{2,\mathrm{app}}$, 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 $k_{3,\mathrm{app}}$ 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

$$\mathrm{HRP} + H_2O_2 \xrightarrow{k_{1,\mathrm{app}}} \text{HRP-I} \tag{1}$$

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

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

(George, 1952, 1953a; Chance, 1952a). No particular states of ionization of the reactants are implied. The rate constants are labeled apparent rate constants as $k_{2,\mathrm{app}}$ and $k_{3,\mathrm{app}}$ 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 $k_{2,\mathrm{app}}$ and $k_{3,\mathrm{app}}$ 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

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[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 $\mathrm{HFe(CN)_6}^{3-}$; K_1, K_2, etc., single acid dissociation constants either on HRP-I or HRP-II as indicated; $k_{2,\mathrm{obsd}}$ and $k_{3,\mathrm{obsd}}$: pseudo-first-order rate constants for the reactions of HRP-I and HRP-II with ferrocyanide; $k_{2,\mathrm{app}}$, $k_{3,\mathrm{app}}$: 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.