to the side chain alone since fumarate ion which is very close in structure to the fumaranilate ion does not combine with anti-$F_p$ antibody. The antibodies against the fumaranilate group must not be as closely fitting as those against maleanilate since they can accommodate the larger methylene groups of the succinanilate ion. Changes other than just saturation of the double bond to form succinanilate ion decrease the combining power still further. Other substances such as phenylhydantoate and benzoylpropionate which have been shown to exist in the coiled configuration[4] are also able to exist in an extended configuration since they combine with the anti-fumaranilate antibodies. The replacement of a $CH_2$ group of succinanilate by NH (in phenylhydantoate) decreases combination by increasing $\Delta F_{rel}$ by 800 cal. Placing a methyl group on the anilino NH (N-methylsuccinanilate) increases $\Delta F_{rel}$ by another 800 cal. Replacing the anilino group to give fumarate ion decreases the strength of combination by at least 3200 cal. The importance of the $\gamma$-carbonyl group is also shown by the fact that $\gamma$-phenylbutyrate ion ($\Delta F_{rel}$ 3200 cal.) combines to a lower extent than does the $\beta$-benzoylpropionate ion ($\Delta F_{rel}$ 2300 cal.).

The importance of the correct benzene-carboxyl distance is reflected by the fact that phenylvalerate combines more strongly (by 500 cal.) than does phenylbutyrate, which is one carbon short of the distance between the groups in the homologous hapten. The effect of substitution in the benzene ring in the *para* position (the position homologous to that for attachment of the immunizing hapten) is to increase the combining power. The presence of a nitro group in the *p*-position increases the extent of combination by decreasing the relative free energy by 1600 cal. in the case of *p*-nitrofumaranilate ion, and 1200 cal. in the case of *p*-nitrosuccinanilate. The acetamino group has a similar effect (1000 cal.) in the fumaranilate system.

The results of these experiments show that succinanilate, N-methylsuccinanilate, benzoylpropionate, and phenylhydantoate ion can exist in aqueous solution in the *trans* configuration (or readily assume that configuration) as well as in the *cis* configuration which is responsible for their combination with antibodies to succinanilate ion.

NEW YORK, N. Y.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

# Effect of Temperature on the Reversible *p*H-dependent Denaturation of Horse Ferrihemoglobin[1]

BY ETHEL M. ZAISER AND JACINTO STEINHARDT

RECEIVED JANUARY 26, 1954

Velocity and equilibrium constants for the acid denaturation of horse ferrihemoglobin have been reported previously for *p*H 3.1 to 4.6 at 25°. It was shown that denaturation as characterized by loss of solubility at the isoelectric point can also be followed quantitatively by measuring the absorption of light of 4060 Å. wave length, and that denaturation is accompanied by the all-or-nothing appearance in each molecule of 36 acid-binding groups. In new experiments the effect of temperature on the kinetics and equilibria is examined in formate buffers over the range 15 to 35°. A formulation of the equilibrium constant based on a proposed cyclic model of the reaction leads to an explanation of the observed zero heat of reaction as an over-all heat of a reaction comprising dissociation as well as denaturation and regeneration steps. Thus, the observed $\Delta H$ (zero between 15 and 25°) gives no direct information as to the heat of transformation of native protein into denatured protein in the same ionic state. The observed rates of approach to equilibrium are analyzed for the contributions of the opposing denaturation and regeneration reactions. According to the model, different rate-determining acid dissociations contribute to the effect of temperature on denaturation and regeneration rate constants. In the case of denaturation, the earlier identification of the "trigger" groups as carboxyls (which have negligible heats of dissociation) makes it possible to attribute all of the small energy of activation, 16.2 kcal./mole at 15 to 25°, to the transformation of native into denatured protein in the same state of ionization. The apparent energy of activation for regeneration, however, contains heats of dissociation which are not yet evaluated. Some differences in the effect of *p*H at 35° as compared with 15 or 25° are examined in the light of the reaction model.

## Introduction

Consideration of the kinetics and equilibria observed in the reversible denaturation of horse ferrihemoglobin with acid[2] has led to the view that the combination of hydrogen ion with a small number (2 to 3) of trigger groups suffices to initiate the appearance (as a unit) in each molecule of 36 acid-binding groups which were formerly unreactive. This change occurs simultaneously with a loss of solubility at the isoelectric *p*H and a decrease in spectroscopic absorption at the 4060 Å. band. The 36 extra acid-binding groups combine quantitatively with hydrogen ion at the *p*H at which they are unmasked[3]; thus it appears likely that they are the $\epsilon$-amino groups of the 36 lysine residues, or possibly, in part, guanidino groups of arginine. In unbuffered solutions these 36 basic groups combine with hydrogen ion at the expense of carboxylate and imidazole groups which have already been titrated, and the net effect actually appears to be an increase in carboxylate and imidazole groups. All information available as to the identity of the 2 to 3 trigger groups whose titration determines the rate of denaturation is consistent with the possibility that they are carboxylate groups.

This paper is concerned with the effect of temperature on the kinetics and equilibria of the denaturation reaction and provides further information about the reaction. For convenience, the spectro-

(1) A brief account of this work was presented at the meeting of the American Chemical Society at Chicago, Ill., in September, 1953.

(2) J. Steinhardt and E. M. Zaiser, THIS JOURNAL, **75**, 1599 (1953).

(3) E. M. Zaiser and J. Steinhardt, *ibid.*, **76**, 1788 (1954).

scopic criterion of denaturation was employed, since it has already been shown[2,3] to give results that are, for this protein at least, identical with both titrimetric and precipitation methods.

## Experimental

The methods used in preparing horse ferrihemoglobin and in determining rates and equilibrium points by spectrophotometric measurements at 4060 Å. already have been described.[2] In this paper the results reported earlier for the acid denaturation of 0.06% ferrihemoglobin in formate buffers of ionic strength 0.02 at 25°[2,3] have been extended to include 15.5 and 34.9°. The temperature in the cell compartment of the Beckman spectrophotometer was controlled during runs to within ±0.1°, a sufficient degree of precision in view of the small temperature coefficient of the reaction. The measurement of hydrogen ion activity was carried out with a MacInnes–Belcher type glass electrode at 30 to 32° for the 34.9° runs, and at 24 to 25° for the 15.5° runs. The small variation with temperature of $pK_A$ for formic acid[4] between 15 and 35° results in the introduction of an error of less than 0.005 $p$H unit in determining the actual $p$H during a run at these other temperatures.

Since the reaction is first order, half periods and values of $D_\infty$ (optical density at equilibrium) could be obtained by Guggenheim's method.[2,5] The effects of a small temperature-dependent irreversible loss of protein during denaturation were largely eliminated by the use of small values of the Guggenheim $\tau$ (time interval between pairs of readings) and by the fact that this method permits the calculation of $D_\infty$ from the early course of the denaturation. By plotting the logarithm of the difference between the observed optical density $D$ and the equilibrium value $D_\infty$ found by Guggenheim's method, the existence of an equilibrium which makes the end-point as well as the velocity dependent on $p$H is taken into account. Figure 1 demonstrates the linearity, persisting through at least three half-periods, of first-order plots of log $(D - D_\infty)$ at 15.5 and 34.9°, similar to those found at 25.0°. From the slopes of such curves are calculated half-periods and values of the first-order rate constant $(k_1 + k_2)$, where $k_1$ refers to denaturation and $k_2$ to regeneration of native protein. As in earlier work,[2,3] $D_\infty$ was used to calculate the fraction of protein denatured at equilibrium.

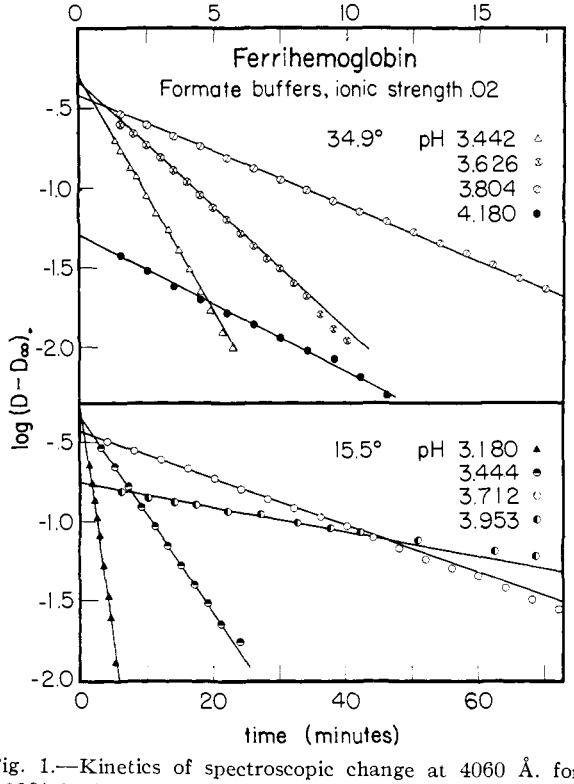


Fig. 1.—Kinetics of spectroscopic change at 4060 Å. for 0.06% ferrihemoglobin in formate buffers of ionic strength 0.02 at 15.5 and 34.9°.

(4) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, N. Y., 1943, p. 510.

(5) E. A. Guggenheim, *Phil. Mag.*, Ser. 2, **7**, 538 (1926).

## Results and Discussion

**Kinetics of Denaturation.**—Figure 2 shows the dependence of log half-period and log $(k_1 + k_2)$ on $p$H at 15.5, 25.0, and 34.9°. The results are much the same at all three temperatures, except for the expected differences in velocity, which are much smaller than those usually found for protein denaturation. At the two lower temperatures, in fact, the resemblance is quite complete. Thus, the limiting slope at low $p$H is 2.5 at 15.5 and 25.0° (although only 2.1 at 34.9°). It is apparent that temperature has little effect on the $p$H for minimum log $(k_1 + k_2)$. Ordinarily, regions of curvature in logarithmic plots of denaturation rate constants as a function of hydrogen ion activity permit significant comparison of such curves at different temperatures by making the curves superimposable by translation on the coördinate scales in a single unique fashion. In the case of pepsin, for example,[6] where denaturation at $p$H 5–7 is practically irreversible, curvature set in as the proportion of molecules present in the reactive ionic form approached unity, and it was found that most of the temperature effect was manifested as a horizontal shift whose magnitude, in $p$H units, was related to the heat of dissociation of the groups which characterize the unstable ionic form.

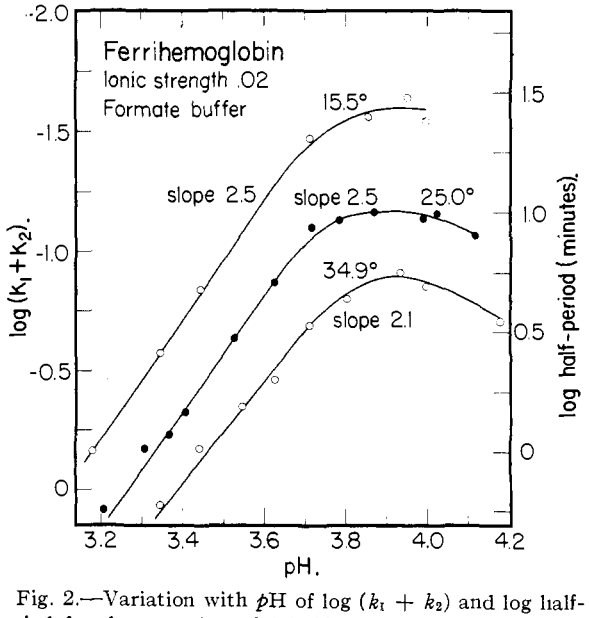


Fig. 2.—Variation with $p$H of log $(k_1 + k_2)$ and log half-period for denaturation of 0.06% ferrihemoglobin at 15.5 25.0 and 34.9°.

In the present reversible case, however, where the rates are determined by $(k_1 + k_2)$, the curvature and the position of the minimum on the $p$H scale in Fig. 2 are determined solely by the relative rates

(6) J. Steinhardt, *Kgl. Danske Videnskab. Selskab., Mat.-fys. Medd.*, **14**, No. 11 (1937).

of the forward and reverse reactions and their dependence on $p$H. Since $k_1$ depends directly and $k_2$ inversely on $p$H, the curvature in the log $(k_1 + k_2)$ arises from the mutual opposition of $k_1$ and $k_2$ in a region of $p$H where their magnitudes are sensibly the same. In general, if denaturation is proportional to the $x$th power and regeneration to the inverse $y$th power of the hydrogen ion activity, $(k_1 + k_2)$ can be expressed as $\{k'_1[H^+]^x + k'_2[H^+]^{-y}\}$. The $p$H of the minimum value of $(k_1 + k_2)$ is thus $[1/(x + y)] \log [xk'_1/yk'_2]$. We may, however, conclude from the fact that the position of this minimum is little altered by temperature that the effect of temperature on $k_1$ and $k_2$ cannot be widely different. Thus, we would expect very little if any effect of temperature on the equilibrium between native and denatured protein at each $p$H. No definite conclusion as to the heat of dissociation of the trigger groups can be drawn.

**Equilibrium between Native and Denatured Protein.**—The effect of $p$H on the fraction $(X/A)$ of protein denatured at equilibrium in the acid denaturation of ferrihemoglobin at three temperatures is shown in Fig. 3. The points represent true equilibrium states, confirmed by approaching them by way of the regeneration of initially denatured protein. The equilibrium is the same at 15.5 and 25.0°, as predicted above, while at 34.9° the curve is displaced slightly in the direction of increased denaturation at a given $p$H. This shift cannot be an anomaly caused by a temperature-dependent irreversible loss of protein, since a reaction mixture equilibrated at 34.9° can be partially regenerated by cooling to 15 to 25°, with an extent of reversal which is compatible with the difference between the two equilibrium curves in Fig. 3. Figure 4 shows the dependence on $p$H of the equilibrium constant, defined as $K_{eq}$ = denatured/native = $X_\infty/(A - X_\infty)$, and calculated from the equilibrium points in Fig. 3. It is evident that $K_{eq}$ is proportional to the fifth power of the hydrogen ion activity and is the same at 15.5 and 25.0°. At 34.9°, however, $K_{eq}$ is higher than at the lower temperatures at all $p$H values, and its proportionality to the hydrogen ion activity at 34.9° decreases to a power of 4.6.

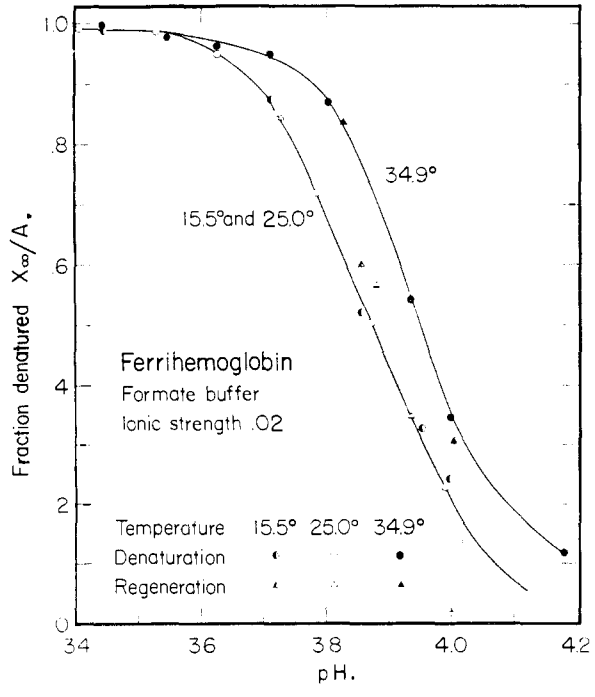


Fig. 3.—Dependence on $p$H of fraction of protein denatured at equilibrium for 0.06% ferrihemoglobin in formate buffers at 15.5, 25.0 and 34.9°.

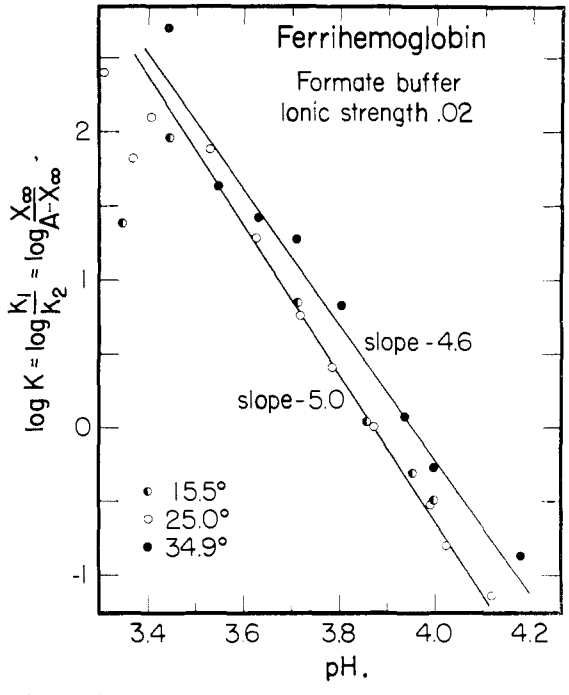


Fig. 4.—Log equilibrium constant as a function of $p$H for 0.06% ferrihemoglobin in formate buffers at 15.5, 25.0 and 34.9°.

**Model of the Reaction.**—The molecular kinetic interpretation of the thermodynamic functions which may be derived from the effects of temperature on the equilibria depends on postulates as to the reactions to which the measured equilibria refer. The following model is an extension of the scheme proposed earlier[2] to account for the effects of $p$H on both equilibria and velocities. In it we distinguish between native and denatured protein, N and D, and the ions, $NH_x$ and $DH_x$, formed by the combination of hydrogen ion with the trigger groups, $x$ in number

$$\begin{array}{ccc} N + xH^+ & \overset{K_{NH_x}}{\rightleftharpoons} & NH_x \\ k_2^0 \uparrow\downarrow k_{-2}^0 & & k_1^0 \uparrow\downarrow k_{-1}^0 \\ D + xH^+ & \underset{K_{DH_x}}{\rightleftharpoons} & DH_x \end{array}$$

Besides actual denaturation and regeneration steps (vertical arrows), dissociation equilibria (horizontal arrows) characterized by constants $K_{NH_x}$ and $K_{DH_x}$ are postulated, where $K_{NH_x}$ and $K_{DH_x}$ are each compound constants for $x$ steps, *i.e.*, $K_1K_2 \ldots K_x$. The equilibrium which is measured spectroscopically is between total native protein (N +

$NH_x$) and total denatured protein ($D + DH_x$), so that the observed $K_{eq} = (D + DH_x)/(N + NH_x)$. At equilibrium

$$d(D + DH_x)/dt = k_1^0(NH_x) - k_{-1}^0(DH_x) + k_2^0(N) - k_{-2}^0(D) = 0$$

In order to account for the effect of $p$H on the observed equilibrium it is necessary to assume that denaturation of a molecule occurs only when $x$ groups (2.5 on the average) involved in the trigger reaction have combined with $H^+$, and that regeneration can occur only when these groups dissociate again, *i.e.*, that $K_{NH_x} \neq K_{DH_x}$ and that $k_1^0 >> k_{-1}^0$ and $k_2^0 >> k_{-2}^0$. The formulation of the denaturation equilibrium will then contain not only the ratio of forward and reverse velocity constants but also a factor arising from the controlling influence of $p$H on the dissociation of the trigger groups

$$K_{eq} = \frac{D + DH_x}{N + NH_x} \sim \frac{k_1^0}{k_2^0} \times \frac{1}{K_{NH_x}K_{DH_x}} \times [H^+]^{2x} = \frac{k_1}{k_2} \quad (1)$$

where $k_1$ and $k_2$ are velocity constants equal to $k_1^0 \cdot [H^+]^x/K_{NH_x}$ and $k_2^0 K_{DH_x}/[H^+]^x$, respectively.

**Thermodynamic Functions.**—The heat of reaction which is observed to be zero, at least between the two lower temperatures, is, according to the above model, an over-all $\Delta H$ for the transformation of $(N + NH_x)$ into $(D + DH_x)$, and is made up of the contributions of the heats of dissociation of $NH_x$ and $DH_x$ as well as the heats of reaction for the transformation of $NH_x$ into $DH_x$ and D into N. Accordingly, to state that $\Delta H = 0$ gives little information about the heat of transformation of N into D unless the dissociation constants $K_{NH_x}$ and $K_{DH_x}$ are identical. Since the equilibrium is affected by $p$H, $K_{NH_x}$ and $K_{DH_x}$ cannot be the same, in terms of the model above; the most that can be said is that $\Delta H$ for the conversion of N to D is probably small.

The standard free energy change $\Delta F^0$, defined formally as the change in available energy in going from a particular value of $K_{eq}$ *without changing pH* to an arbitrarily chosen standard state where $(D + DH_x)/(N + NH_x) = 1$ is equal to $-RT \ln K_{eq}$. In the $p$H range studied, $\Delta F^0$ for 15.5 and 25.0° is small, ranging from −3.2 kcal. at $p$H 3.4 to +1.5 kcal. at $p$H 4.1. This is to be expected since observations were confined to the region of $p$H where a measurable equilibrium point is found, and where $K_{eq}$ therefore does not depart grossly from unity. These small values of $\Delta F^0$ do not, however, represent the free energy change in going from native to denatured protein, and the $\Delta F$'s for the processes $D \rightarrow N$ and $NH_x \rightarrow DH_x$ and for the ionization steps may well be large. A similar difficulty is encountered in the interpretation of the entropy of reaction. Since $\Delta H = 0$ for the interval 15.5 to 25.0°, $\Delta S$ varies with $p$H as $\Delta F^0$ does, and ranges from +10.9 e.u. at $p$H 3.4 to −5.1 e.u. at $p$H 4.1. Again this tells nothing about $\Delta S$ for the steps of the reaction that are of particular interest in studying denaturation, *i.e.*, $D \rightarrow N$ and $NH_x \rightarrow DH_x$. It appears likely that these limitations in significance are applicable to numerous other thermodynamic data on denaturation in the literature.

Thermodynamic functions have not been calculated for the temperature interval 25.0 to 34.9° because the power of the dependence of $K_{eq}$ on hydrogen ion activity is different at these two temperatures (Fig. 4) and the thermal functions thus depend on $p$H. The observed difference is not inconsistent with the model indicated above. Thus, beyond some particular temperature the assumption that the reactions $NH_x \rightarrow DH_x$ and $D \rightarrow N$ go to completion, subject only to the control of the dissociation constants $K_{NH_x}$ and $K_{DH_x}$, may no longer be valid. The maximum possible extent of denaturation or regeneration at any $p$H would then be less than 100%, and the order of dependence of $K_{eq}$ on the hydrogen ion activity would be lessened. If this is the case at 34.9°, the calculated $\Delta H$ for the reaction between 25.0 and 34.9° at each $p$H cannot have the same meaning as in the 15–25° region discussed above. An alternative possibility is that the identity of the ionic species which most readily undergoes denaturation at 15.5 and 25.0° is not the same at the highest temperature (*i.e.*, that the average number of trigger groups changes). The second possibility is supported by the observed decrease in the dependence of log $(k_1 + k_2)$ on $p$H at 34.9° (Fig. 2).

**Velocity Constants and Energy of Activation.**—In order to obtain the energy of activation for denaturation it is necessary to separate $k_1$ for denaturation from the measured velocity constant $(k_1 + k_2)$ which contains the velocity constant of regeneration. Figure 5 shows the dependence on $p$H of log $k_1$ and log $k_2$ at three temperatures, as calculated from the measured rates and equilibria.[2] At low $p$H, $k_2 << k_1$, and the calculated value of $k_1$ is practically the same as the observed rate constant, and exhibits the same order of dependence on the hydrogen ion activity (2.5 at 15.5 and 25.0°, and 2.1 at 34.9°). Since the relation of $k_1$ to $p$H remains linear throughout the range of $p$H studied it is not possible to calculate the $pK$'s of the trigger groups from the position of this function on the $p$H axis. In view of the tentative identification of these groups as carboxyl,[2] however, it is likely that their heats of dissociation are negligible, and that the displacement of the curve for log $k_1$ when the temperature is changed has no appreciable horizontal ($\Delta pK$) component. Thus, although the model for the reaction indicates that $k_1 = k_1^0[H^+]^x/K_{NH_x}$, differences in log $k_1$ at two temperatures can be ascribed directly to the energy of activation, without the correction for a shift in $p$H which is sometimes required.[6] Figure 6 shows values of the Arrhenius energy of activation for denaturation calculated from the effect of temperature on $k_1$

$$E_1 = 2.303R[(T'T)/(T' - T)] \times \Delta \log k_1$$

In the lower temperature interval (15.5 to 25.0°), $E_1 = 16.2 \pm 0.2$ kcal./mole over the entire range of $p$H. The difference in the $p$H dependence of the velocities at 25.0 and 34.9° causes the energy of activation calculated in this interval to lose its usual meaning, and in fact to appear to depend on $p$H. No attempt will be made here to interpret the rather ambiguous result of such a calculation.

The value of the energy of activation, 16.2 kcal.,

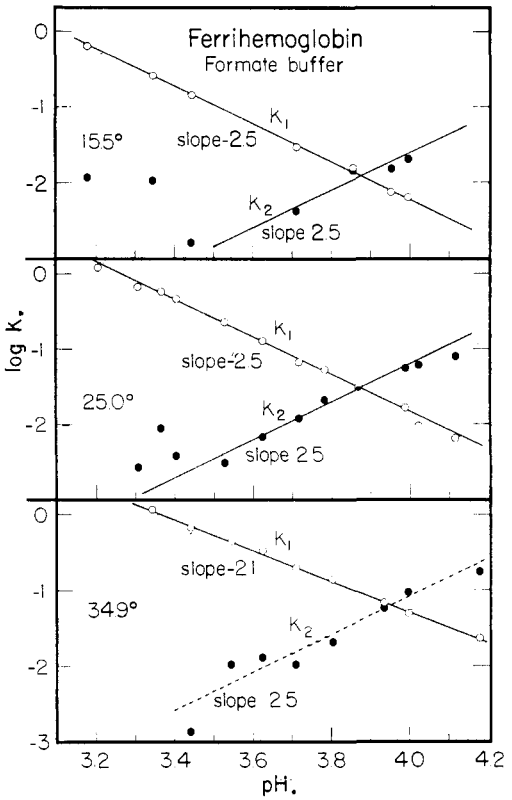


Fig. 5.—Dependence on $p$H of log $k_1$ (denaturation) and log $k_2$ (regeneration), calculated from kinetics and equilibria for 0.06% ferrihemoglobin in formate buffers at 15.5, 25.0 and 34.9°.

falls well within the range commonly encountered in such chemical reactions as hydrolyses of ester or amide linkages. Reactions that depend solely on the rupture of a single hydrogen bond require much smaller energies. When the energy of activation is used in conjunction with the rates observed at 25.0° to calculate the apparent collision frequency factor $Z = k_1 e^{E_1/RT}$, the values of $Z$ of course vary with $p$H, reflecting the dependence on $p$H of the concentration of the ionic species which can undergo activation. The order of magnitude of $Z$ is $10^{10}$ to $10^{12}$ sec.$^{-1}$, reasonably close to the value of $10^{13}$ sec.$^{-1}$ which collision theory would predict for the frequency of collision of a molecule of the size and mass of the half-molecule[7] of ferrihemoglobin with water molecules in dilute solution. It is important to note that the anomalously high values of $E$ and $Z$, which are sometimes found[6,8] for data on protein denaturation when uncorrected for the effects of ionic equilibria characterized by large $\Delta H$ values, are absent here (therefore the Eyring–La Mer entropy of activation is negligible). This further confirms the view that carboxyl groups are the ionizing groups responsible for the $p$H dependence of the reaction rates, since their heats of dissociation are small or zero at 15–25°, and would contribute very little to $E$ or $Z$.

(7) The dissociation of hemoglobin into half-molecules on even mild treatment with acid was demonstrated by N. Gralen, *Biochem. J.*, **33**, 1907 (1939).

(8) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

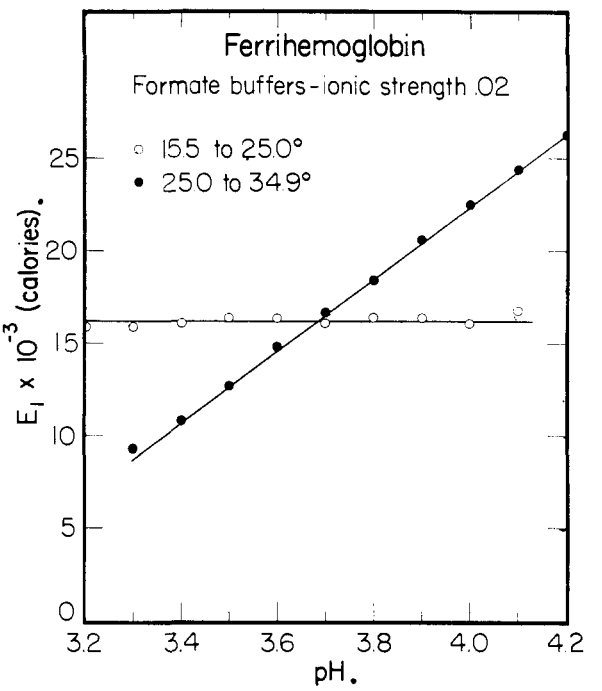


Fig. 6.—Energy of activation as a function of $p$H, calculated from denaturation $k_1$.

The application of absolute rate theory[9] to values of $k_1$ at 15.5 and 25.0° yields thermodynamic analogs as follows: $\Delta H^* = 15.7$ kcal./mole, $\Delta F^*$ varies from 17.2 kcal./mole at $p$H 3.2 to 20.2 kcal./mole at $p$H 4.1, and $\Delta S^*$ varies from $-5.1$ e.u. at $p$H 3.2 to $-15.3$ e.u. at $p$H 4.1. The precise meaning of such analogs is not clear, however, since the frequency factor $kT/h$, which is assumed in the theory of absolute rates and on which the values just given depend critically, may not be appropriate for the collision of very large molecules (such as proteins) with solvent.

It would be of interest to determine the energy of activation for the reverse reaction, $E_2$. According to the cyclic model proposed above, the activated complexes for denaturation and regeneration are not identical; thus $E_2$ cannot be calculated from the known values of $E_1$ and the over-all heat of the reaction, *i.e.*, $\Delta H \neq E_1 - E_2$. The alternative possibility of calculating $E_2$ from the variation in log $k_2$ with temperature (Fig. 5) is not attractive because the values of $k_2$ given are very small at low $p$H, while they are subject to considerable error at $p$H > 4 where the extent of denaturation is small and the precision necessarily limited. Furthermore, eq. 1 implies that the effect of temperature on $k_2$ will include an effect on $K_{DH_2}$ of unknown magnitude. No attempt will be made in the present paper, accordingly, to calculate the energy of activation for the regeneration of native ferrihemoglobin. Preliminary examination of data on regeneration at 15 and 25°[10] suggests that the values of log $k_2$ shown in Fig. 5 are reasonable, and that the energy of activation for regeneration $E_2$ may be

(9) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., New York, N. Y., 1941 Chapter VIII.

(10) J. Steinhardt and E. M. Zaiser, in preparation.

between 5 and 10 kcal. higher than $E_1$ for denaturation.

**Acknowledgment.**—Thanks are due to Professor G. Scatchard of the Department of Chemistry, Massachusetts Institute of Technology, for extension of the Department's facilities, and for helpful discussions.

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[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY AND BIOLOGICAL CHEMISTRY OF WASHINGTON UNIVERSITY]

# The Mechanism of the Alkaline Hydrolysis of Ribonucleic Acids

BY DAVID LIPKIN, PRESTON T. TALBERT AND MILDRED COHN[1]

RECEIVED JANUARY 6, 1954

Yeast ribonucleic acid was subjected to hydroxide ion catalyzed hydrolysis in $H_2O^{18}$. The phosphate groups of the mononucleotides formed in the hydrolysis were found to contain one atom of $O^{18}$ per atom of phosphorus. A mechanism for the hydrolysis of ribonucleic acid which is in accord with these data is discussed.

The discovery of two isomeric forms of each mononucleotide,[2] as well as cyclic nucleoside-2′,3′-phosphoric acids,[3] in alkaline hydrolysates of yeast ribonucleic acid stimulated interest in the mechanism of alkaline hydrolysis of this acid. To explain the formation of isomers and cyclic phosphates, Brown and Todd[4] invoked Fonó's interpretation of the alkali lability of ribonucleic acids[5] and elaborated a hydrolysis mechanism which involved the intermediate formation of a cyclic *triester*. In an effort to obtain more detailed information concerning the reaction mechanism, the alkaline hydrolysis of ribonucleic acid has been carried out in $H_2O^{18}$. The results of this work, taken in conjunction with the known properties of phosphate esters, are in agreement with a modification of the mechanism originally suggested by Brown and Todd.[4]

## Experimental

**Materials.**—A sample of yeast ribonucleic acid (Schwarz Laboratories, Inc., Mount Vernon, N. Y.) was purified by precipitating twice with sodium chloride–ethanol.[6] This purified product (RNA), which was shown by dialysis to contain no free mononucleotides, was used in the hydrolysis experiments.

Dry Dowex A-1 chloride (Dow Chemical Company, Midland, Mich.), 200–400 mesh, was extracted with 95% ethanol in a Soxhlet extractor. The extraction was continued until the optical density at 260 mμ of two successive samples of ethanol from the extraction thimble was the same.[7] After drying the extracted resin to constant weight, it was converted to the formate form by means of a solution 1 *N* in both formic acid and sodium formate.

The charcoal used for adsorbing the nucleotides from the ion exchange column eluates was prepared as follows: About 50 g. of Darco G-60 (Darco Corporation, New York, N. Y.) was added to 1 l. of 6 *N* hydrochloric acid. After boiling this mixture for 45 minutes, the supernatant liquid was removed by decantation. This operation was repeated twice. The charcoal next was boiled for 45 minute periods with 1-l. portions of distilled water until the supernatant gave a negative chloride test. Finally, the charcoal was removed by filtration through a sintered glass funnel and air-dried at 120° for 24 hours. Samples of Darco G-60 treated in this way were found to contain 0.0% phosphorus.

The intestinal phosphatase employed in these experiments was a commercial preparation (Armour Laboratories, Chicago, Ill.) containing 15 units/mg.[8]

The $O^{18}$-enriched water used in this work contained 1.97 atom per cent. excess $O^{18}$.

**Hydrolysis of RNA.**—A 350-mg. sample of the sodium salt of RNA was dissolved in 20 ml. of 0.5 *M* sodium hydroxide in $H_2O^{18}$. This solution was kept at 37° for 17 hours. The resulting hydrolysate was concentrated to a small volume *in vacuo* at room temperature.

**Chromatographic Separation and Isolation of the Nucleotides.**—The concentrated hydrolysate was taken up in water and chromatographed on a 3.8 sq. cm. × 15 cm. long Dowex A-1 formate column.[2c] For the elution, the following solutions were used successively to displace the stated nucleotides from the column: cytidylic acids, 0.01 *N* formic acid; adenylic acids, 0.070 *N* formic acid plus 0.0070 *N* sodium formate; uridylic acids, 0.0017 *N* formic acid plus 0.030 *N* sodium formate; guanylic acid-*a*, 0.0042 *N* formic acid plus 0.103 *N* sodium formate; and, finally, guanylic acid-*b*, 0.0042 *N* formic acid plus 0.171 *N* sodium formate. All of the nucleotides except the uridylic acids-*a* and -*b* were eluted as distinct peaks. The uridylic acids were eluted as a single peak with two distinct maxima. The fractions containing this peak were divided at the midpoint between the maxima to yield two fractions, one containing predominantly *a*-isomer the other predominantly *b*-isomer.

The individual nucleotides in the various fractions of the ion exchange column eluate were concentrated as follows: A portion of acid-treated Darco G-60 was added to a given fraction[9] and the mixture stirred for a few minutes. The charcoal[5] containing the adsorbed nucleotides then was removed by filtration, was washed free of formic acid and formate with two portions of distilled water and finally was dried in a vacuum desiccator containing activated alumina.[7]

The nucleotides were recovered from the charcoal samples by exhaustive elution with a 5% aqueous solution of pyridine. The combined pyridine eluates from a given charcoal sample were extracted three times with equal volumes of chloroform to remove the pyridine and then were distilled almost to dryness under reduced pressure. These concentrates, containing individual nucleotides, subsequently were hydrolyzed in order to convert organically-bound phosphate to inorganic phosphate.

**Hydrolysis of the Mononucleotides.**—A concentrate containing a single nucleotide was dissolved in 10 ml. of a solution containing 0.1 *M* veronal buffer, *p*H 8.9, and 0.008 *M* $MgCl_2$. The resulting mixture was incubated for 2 hours at 37° with 5 mg. of intestinal phosphatase. At the end of the incubation, the protein was precipitated with trichloroacetic acid and the inorganic phosphate was isolated and analyzed for $O^{18}$ as described previously.[10]

(1) Part of this work was done during the tenure by one of us (M.C.) of an Established Investigatorship of the American Heart Association.

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