that the degree of oxidation of methanol to formaldehyde is increased by sodium carbonate, reaching one hundred per cent. oxidation at a carbonate concentration of one-half molar.

The influence of the anodic material on the course of the electrolysis is summarized in Table III. Platinum and graphite give the best results for both methanol and ethane, while nickel, iron, lead peroxide and platinized platinum are not suitable for the production of methanol. Graphite appears to be superior to platinum if methanol is the desired product, since there is less loss of methanol by oxidation. Platinized platinum is inferior to platinum; the addition of sodium fluoride did not improve the efficiency as was observed by Glasstone.[5]

(5) S. Glasstone and A. Hickling, *J. Chem. Soc.*, 1878 (1934).

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

Kinetic Study of the Reversible Acid Denaturation of Ferrihemoglobin[1]

BY JACINTO STEINHARDT AND ETHEL M. ZAISER

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The denaturation of horse ferrihemoglobin, like that of carbonylhemoglobin, involves the liberation of 36 acid-binding groups, half of the total available for combining with acid, in the same *p*H range in the denatured protein. Detailed equilibrium and rate measurements show that, unlike the case of carbonylhemoglobin, the denaturation of ferrihemoglobin is fully reversible, whether measured by changes in spectroscopic absorption, loss of solubility or acid-binding capacity. From the rates of denaturation and the equilibrium points the magnitudes of the rate constants for both denaturation and regeneration processes have been obtained as a function of *p*H. The rate of liberation of basic groups is faster than the rate of denaturation measured by the other, more conventional, methods. It is shown that hydrogen-ion does not act as a perfect catalyst but must combine with protein in the process of denaturation. A partial physical model is proposed, which accounts for the effects observed.

Equilibrium[2] and kinetic[3] measurements have shown that exposure of horse carbonylhemoglobin to dilute acid solutions (*p*H < 4) initiates a time-dependent reaction which liberates 36 acid-binding groups. Half of these groups appear to have *pK* near 4.4 and half *pK* near 6.1. Indications that the rates of liberation of these groups at any *p*H were identical with rates of loss of characteristic absorption at 5380 Å., and loss of solubility at the isoelectric point, made it appear probable that the liberation of these groups was concurrent with denaturation, as usually defined. The liberation appears to occur essentially as an all-or-nothing phenomenon, *i.e.*, no liberated groups are found in undenatured molecules; and denatured molecules always have the full complement of 36.[3]

The apparent first-order kinetics of the denaturation process (measured in any of the three ways) evidently concealed a more complex process. The rate of denaturation proved very sensitive to traces of oxygen—so sensitive, that reproducible data were obtained only if certain minimal amounts of air were deliberately added. Since ferrihemoglobin is formed rapidly when oxygen is present, and is denatured more rapidly than COHb itself, the transformation to denatured protein occurred at least in part through the intermediate formation of ferrihemoglobin. It seemed more promising, therefore, to investigate the connection between the appearance of 36 acid-binding groups and denaturation on the oxidized form of hemoglobin, in order to minimize the complication of successive or parallel reactions. The choice of ferrihemoglobin soon presented an additional attraction in that its denaturation by acid, unlike that of COHb, was found to be completely rather than partially reversible.[4] With COHb only the rates measured by the three methods could be compared. With ferrihemoglobin equilibria could be compared also. A direct comparison of equilibrium and kinetic constants may thus be made.

This paper therefore presents measurements on ferrihemoglobin similar to those already reported for COHb.[2,3] Direct measurement of the kinetics of the regeneration process, and examination of the identity of the regenerated material with relation to the native protein are given in a paper to follow.

Experimental

Except for the work with titration curves, which was straightforward,[1] working with ferrihemoglobin rather than COHemoglobin required overcoming three main difficulties: (a) close similarity of the spectra of both native and denatured forms over the greater part of the visible spectrum; (b) a marked dependence of absorption on *p*H in both native and denatured forms; and (c) practically complete and rapid reversal of denaturation, on returning solutions to a higher *p*H, in order to precipitate denatured protein.

The first of these difficulties was circumvented by measuring the change in absorption at either 4060 Å. (Soret band) or at 6300 Å., where there are major differences in absorption between native and denatured protein. The larger changes occur at the former wave length, and all the measurements reported here were made at 4060 Å. Calculations based on results obtained at 6300 Å., however, lead to the same conclusions.

The second difficulty was dealt with by taking into account the effect of *p*H on absorption, in calculating concentrations. This effect, over most of the visible region of the native ferrihemoglobin spectrum, is shown in Fig. 1.[5]

(1) A preliminary account of this work was presented at the Meeting of the American Chemical Society at New York, N. Y., in September, 1951.

(2) J. Steinhardt and E. M. Zaiser, *J. Biol. Chem.*, **190**, 197 (1951).

(3) E. M. Zaiser and J. Steinhardt, THIS JOURNAL, **73**, 5568 (1951).

(4) *Cf.* H. F. Holden, *Australian J. Exptl. Biol. Med. Sci.*, **14**, 291 (1936).

(5) All extinction coefficients (ε) are expressed as log I_0/I for a 1-cm. thickness of solution containing 1 mmole of heme iron per liter. It will be observed that there is no measurable change with *p*H in this part of the spectrum of native ferrihemoglobin over most of the *p*H range of the kinetic experiments (3.5–4.2); or of denatured ferrihemoglobin at *p*H more acid than 3.5. The data differ in several important respects from those given for hog hemoglobin by W. M. Urbain and D. A. Greenwood (*Food Research*, **5**, 617 (1940)).

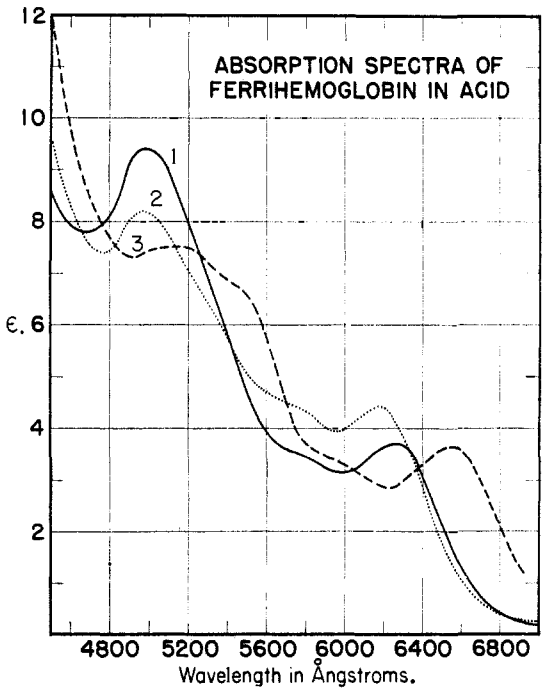

Fig. 1.—Effect of *p*H on absorption by ferrihemoglobin, as measured with Hardy recording spectrophotometer in formate and acetate buffers (ϵ units = cm.$^{-1}$ mmole Fe^{-1} liter): 1, *p*H 4.7 to 6.6 (no change with time); 2, *p*H 3.5 to 4.2 (initial spectrum); 3, *p*H 2.6 to 3.5 (final spectrum).

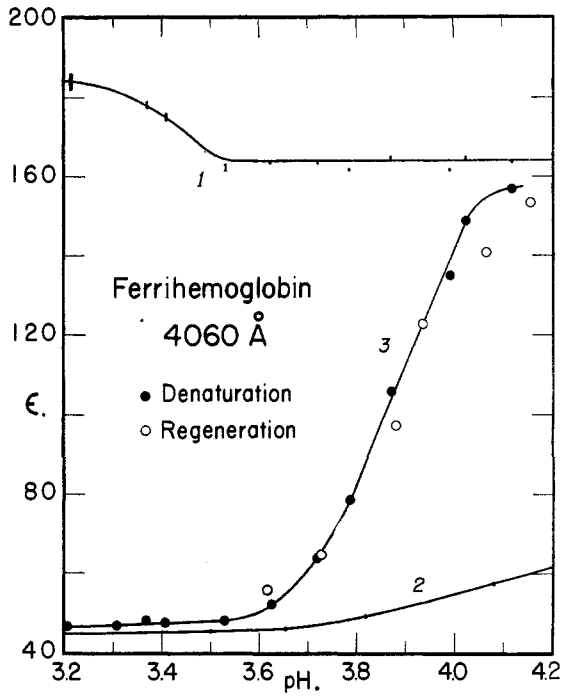

Fig. 2.—Effect of *p*H on absorption by ferrihemoglobin at 4060 Å.: 1, native; 2, denatured; 3, equilibrium mixture of native and denatured, prepared both by denaturation and regeneration. Accurate equilibrium values are obtained by the methods described in the text.

In Fig. 2 the effect at 4060Å. for both native and denatured ferrihemoglobin, is shown in curves 1 and 2. These data were obtained by extrapolating to zero time the first-order kinetic data obtained spectrophotometrically in experiments in which initially native ferrihemoglobin was denatured, or initially fully denatured ferrihemoglobin was transformed to the native form as described elsewhere in this paper. The fraction of protein denatured, x/a, at any time t, at a given *p*H, is equal to $[(D_n - D)/(D_n - D_d)]$ where D is the optical density at t, and D_n and D_d are the optical densities of native and denatured protein, respectively, at the same *p*H.

The third difficulty yielded to essentially the same procedure devised for COHb, *i.e.*, rapid addition of sodium sulfate to 12.5% before raising the *p*H,[3] even though the tendency to regenerate native protein was much more marked than in the case of COHb. Both salt and base were added very rapidly within periods of a second or less, using syringes, to avoid either regeneration by base, or salt-accelerated denaturation during the mixing process.

Except as described above, and elsewhere in the text, experimental conditions, procedures, and methods of calculation, were as previously described.[2,3] All buffers (formate) were at 0.02 ionic strength.

Preparation of Ferrihemoglobin.—Since Holden has shown,[4] that the use of ferricyanide as an oxidant yields a product from which dialysis fails to remove all of that ion, ferrihemoglobin was prepared from solutions of twice recrystallized horse COHb by displacing the CO with air or O_2 after adjusting the *p*H to 4.9–5.0 with HCl. At this *p*H, denaturation is exceedingly slow. After 4 to 5 days at 24–28°, conversion to ferrihemoglobin was > 99% complete. A small amount of insoluble material which appeared when the *p*H was returned to 6.6 was discarded. No differences have been found between preparations made up in this way, and by action of ferricyanide, except for the presence of ferricyanide in the latter, which leads to the formation of a slight precipitate when acid is added.

Reversibly denatured ferrihemoglobin was prepared by exposing the native protein in solution to *p*H values between 2.96 and 3.52 for periods from a few minutes (at 2.96) to several hours. Over this wide range of conditions practically complete conversion to denatured protein occurs, but the time and *p*H of denaturation have important effects on regeneration, which are to be described elsewhere. The mildest treatment which produced 95% denaturation (20 minutes at *p*H 3.5) was used unless otherwise noted.

Concentrations Used.—A gradual irreversible loss of protein complicates the results obtained with dilute solutions. The rate of this irreversible reaction is nearly independent of protein concentration. Thus, since it is slow, its effect can be made negligible by working at sufficiently high concentration. At 0.06%, the concentration used in most of the experiments, considerably less than 5% of the total protein is lost irreversibly in 20 min. at *p*H 3.5 at 25°, during which time over 90% of the protein reacts reversibly. In 10 times more dilute solutions about 20% is lost under the same conditions. This irreversible loss affects both denaturation and regeneration experiments. However, at 0.06% the kinetic errors and uncertainties in equilibrium points due to this cause are small enough to be disregarded. By using a 1-mm. absorption cell, 0.06% protein can be measured at 4060 Å. without loss of precision.

Results and Discussion

Increase in Acid-binding Groups.—Titration curves with HCl of 0.4% solutions of ferrihemoglobin at three different time intervals after mixing are shown in Fig. 3. A back-titration curve with KOH of protein initially exposed to HCl at *p*H 2.6 for 1 hour is also shown. The direct titration curves were made at 0.02 *M* total chloride (KCl added) to permit direct comparison with the back-titration curve. It will be noted that the *back-titration* curve as well as a titration curve is given at 3 seconds after mixing. This was done because, unlike the case of COHb, *p*H drifts are encountered on back-titration which are about as rapid as the drifts which made the 3-second technique necessary with both COHb and ferrihemoglobin for the direct titration. After less than two hours, the position of the back-titration curve approaches closely to

the position of the 2.5 and 22 hour (equilibrium) titrations (dotted curve). The approach is closer the milder the conditions of the initial denaturation. The dotted curve was obtained after initial exposure to *p*H 3.4 for ten minutes, resulting in about 95% denaturation, before back-titration. The close approach of the dotted curve to the 2–22 hour curves strongly suggests that the latter represent a *p*H-dependent equilibrium between native and denatured ferrihemoglobin.

The results shown in Fig. 3 are in most other respects similar to those already reported for COHb.[2] This similarity extends to practical identity of the "instantaneous" (3 second) curves for native and denatured protein with the corresponding curves obtained with COHb, over the *p*H range in which the 3 second technique is valid. The acid denaturation of ferrihemoglobin, however, is much more rapid than that of COHb, and the 3 second curve for native protein cannot be regarded as the instantaneous curve, representing unreacted protein, below about *p*H 4.0 (0.41 mmole acid bound).[6] With COHb the 3 second technique gave the true titration curve with good approximation to *p*H 3.2 (0.72 mmole bound). The true titration curve to somewhat lower *p*H values for both native proteins, may be obtained by extrapolation as explained below.

A second difference is in the position of the "equilibrium" (22 hr.) data. For example, ferrihemoglobin binds 0.50 mmole at *p*H 4.18. With COHb, the amount bound at this *p*H value is less than 0.42 mmole. If the 22 hour curve for ferrihemoglobin, like that of COHb, represents equilibrium mixtures of molecules in which 36 groups have been liberated with molecules which are unchanged, it is evident that a larger proportion of the ferrihemoglobin molecules are transformed at any *p*H value than is the case with COHb. This inference is consistent with the more rapid *rate* of denaturation characteristic of ferrihemoglobin, to be described below.

The inserted curves at the bottom of Fig. 3 show the *difference* between the acid bound in 3 seconds, on titration with HCl, and on back titration with KOH, for both COHb and ferrihemoglobin. The upper continuous curve represents the results previously published for COHb. The curve traced by the open triangles at equal *p*H intervals represents the differences with ferrihemoglobin. The two sets of data agree down to *p*H values at which the 3 second technique breaks down, as explained above. Thus, in this range, the groups liberated in both proteins are probably alike (same dissociation constant) and equal in number.

Also shown on the upper curve of the insert are small circles representing the 3 second titration data from the main figure. It will be observed that the *difference* data and the 3 second data are practically superimposed to *p*H values below 4 (the range of validity, with ferrihemoglobin, of the 3 second technique). Thus, the number of groups liberated by acid is equal to the number of initially titratable groups—*i.e.*, *just one-half* of the groups in native ferrihemoglobin (and COHb) which combine with acid in this *p*H range are initially unavailable for combination with acid.

(6) Solutions at *p*H values below about 4 are exposed briefly to much more acid reactions, at which appreciable denaturation occurs in three seconds.

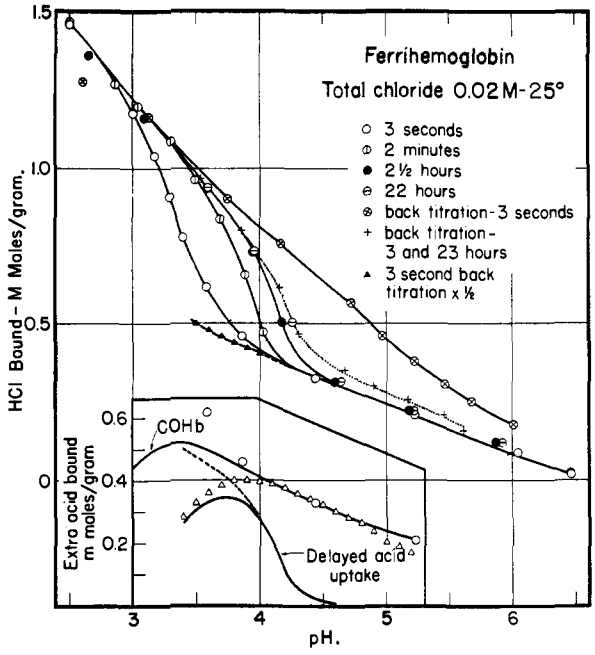

Fig. 3.—Amount of HCl combined with 0.4% ferrihemoglobin. *Inset lower curves* are obtained by subtracting titration curve at 3 seconds from "equilibrium" curve (2 to 22.5 hours) without correction (solid line) and with correction (see text) of 3 second data (dotted line). *Inset upper curves* are obtained by subtracting 3 second titration from 3 second back-titration curves of COhemoglobin (solid line) and ferrihemoglobin △, with 3 second data for ferrihemoglobin ⊙ included for comparison.

Advantage has been taken of this simple relation (delayed acid uptake equal to initial acid uptake) to extrapolate the 3 second titration data (and therefore the *difference* curve as well) to *p*H 3.5 (broken line through solid triangles). The extrapolation of the difference curve lies so close to the COHb difference curve that it has not been drawn; this similarity serves further to demonstrate that the maximum number of groups liberated is about the same (36) in both proteins, and corresponds to approximately 0.52 mmole of acid bound per gram of protein.

The lower solid curve in the insert shows the difference between the instantaneous data (3 seconds) and the equilibrium (22 hr.) data. Thus it should represent a product of two functions of *p*H: (a) the fraction of the total protein in which 36 groups have been liberated at equilibrium, and (b) the ionization equilibrium of the liberated groups. If the first function could be measured in other ways (as, *e.g.*, spectrophotometrically) the second function could be calculated.

As in the case of the upper solid curve, the lower solid curve falls at low *p*H, because the 3 second technique does not give truly instantaneous values at *p*H much below 4. By making use of the same extrapolation device previously referred to, the broken curve is obtained, with no indication

that a maximum of 0.52 mmole per gram is not obtained here also.

Because of the high exponential dependence of rate of liberation on $[H^+]$, a more than hundred-fold reduction in the 3 second time interval would be required to dispense with the extrapolation described above. Simple analysis of the *kinetics* of the liberation of acid groups therefore did not appear feasible; the fact that under the conditions of any *titration* technique, the *p*H changes as groups are liberated also adds to the difficulties.

Spectroscopic Changes.—Such an analysis is easily made, however, by following spectroscopic changes, or solubility changes, in buffered solutions, as previously reported for COHb.[3] The results of these spectrophotometric experiments strongly suggest the existence of an equilibrium involving hydrogen-ion since the reactions stop short of completion, at end values which depend on *p*H. The time course of the reaction is given by the reaction isotherm of a reversible first-order process

$$k_1 + k_2 = \frac{1}{t} \ln \frac{x_\infty}{x_\infty - x} = \frac{1}{t} \ln \frac{D_n - D_\infty}{D - D_\infty} \qquad (1)$$

where k_1 is the first-order constant for the denaturation reaction, k_2 is the first-order constant for the reverse process, x_∞ and D_∞ are the final values for denatured protein, and x and D are values for the denatured protein at time t. Representative kinetics experiments are shown in Fig. 4. Although all the experiments shown were performed with the same concentration, the straight lines cross one another because the quantity plotted is log $(D - D_\infty)$ and D_∞ is different at each *p*H. D_∞ has been determined either by waiting for at least six half-periods, or by curve-fitting (determining the value that gives the best straight line when log $(D - D_\infty)$ is plotted against time. When the first of these two methods is used, a small correction must be applied to take into account the existence of a very slow irreversible zero-order loss of native protein, minimized by using as concentrated solutions as possible (see Experimental). The change in equilibrium extinction coefficient with *p*H is shown as curve 3 in Fig. 2. As would be expected with first-order processes the rates obtained and the values of x_∞ are independent of protein concentration over a very wide range (0.002 to 0.1%).

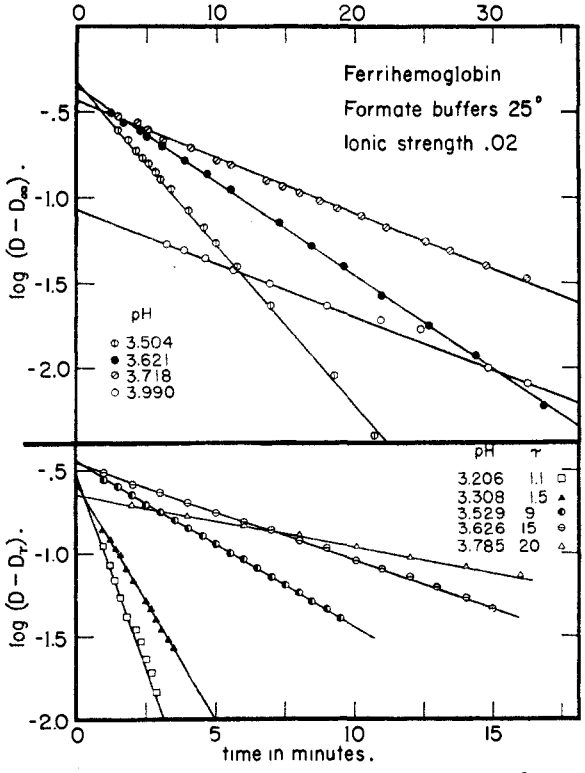

Fig. 4.—Kinetics of spectroscopic change at 4060 Å. with 0.06% ferrihemoglobin: upper curves, D_∞ chosen by curve-fitting for best linear relation of log $(D - D_\infty)$ to time; lower curves, Guggenheim's method, where τ = fixed time interval between density readings.

In order further to improve the precision of the rate constant, and to make it independent of assumptions as to final equilibrium values, recourse was had to Guggenheim's method,[7] in which differences in optical density between readings taken at a fixed time interval τ, are plotted (as in the lower half of Fig. 4) instead of absolute densities. A value of x_∞ can then be chosen which will give the same rate constant (slope) when log $(x_\infty - x)$ is plotted against time. Alternatively, if D_n is known (as by extrapolation to zero time), D_∞ may be calculated directly since

$$(D_n - D_\tau) = (D_n - D_\infty)[1 - e^{-(k_1 + k_2)\tau}]$$

Values obtained for x_∞ and $(k_1 + k_2)$ by both methods have agreed very closely.

Dependence of $(k_1 + k_2)$ and Equilibrium Point on *p*H.—The effect of *p*H on log $(k_1 + k_2)$ determined spectrophotometrically is shown by the open circles in Fig. 5. The approximation that the rate is proportional to the 2.5 power of the hydrogen-ion activity, while true over a fifteen-fold variation in half-period, fails when the *p*H exceeds about 3.75. The results previously obtained with COHb

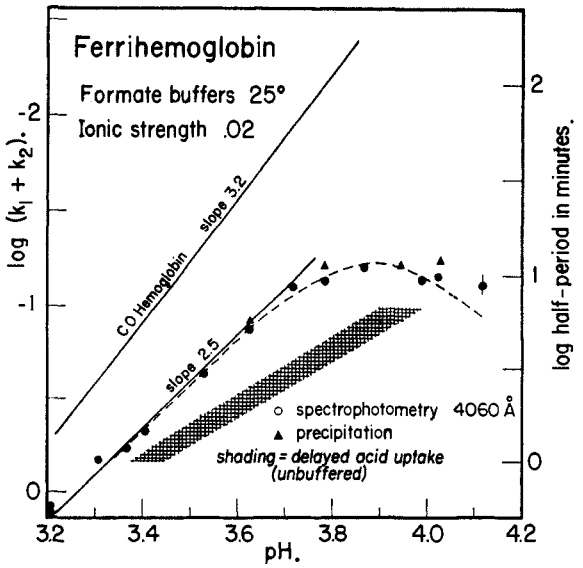

Fig. 5.—Variation with *p*H of log $(k_1 + k_2)$ and log half-period as measured by rate of denaturation of ferrihemoglobin.

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

are shown also. With COHb the dependence on pH is greater (slope 3.2 instead of 2.5) and the reaction is slower throughout the pH interval. Because of the different dependence, the difference is smallest at the most acid pH, and may reverse in sign at still lower pH.

The levelling-off of the rate constant at high pH is not unexpected: The log of the sum of *two* rate constants is unlikely to be linear over wider intervals of pH. It is shown later that $k_2 \sim [H^+]^{-2.5}$. Thus

$$k_1 + k_2 = k_1'[H^+]^{2.5} + k_2'/[H^+]^{2.5}$$

and log $(k_1 + k_2)$ cannot be linear in pH over a wide range. The broken curve in Fig. 5 is calculated for $k_1' = 1.51 \times 10^8$ min.$^{-1}$ and $k_2' = 5.62 \times 10^{-12}$ min.$^{-1}$.

The practical upper limit of pH at which the rate constant $(k_1 + k_2)$ can be measured is set by the small extent of reaction at pH values much above 4. The total change in density is only slightly outside the experimental error, and calculated values of $(k_1 + k_2)$ become very sensitive to errors in determining D_n and D_∞.

The combined use of curves 1, 2 and 3 of Fig. 2 permits the calculation of x_∞ as a function of pH. The result of this calculation is shown in Fig. 6.

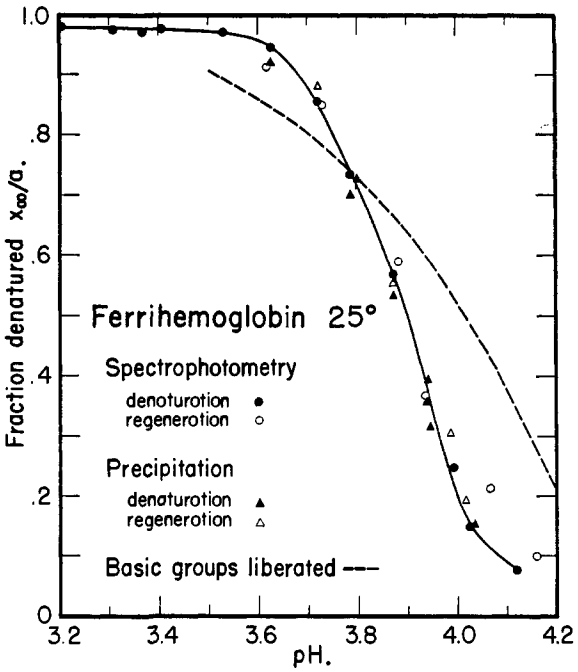

Fig. 6.—Dependence on pH of the fraction of ferrihemoglobin denatured at equilibrium in formate buffers, ionic strength 0.02.

Since the equilibrium constant K should equal k_1/k_2, while the "rate constant" is $(k_1 + k_2)$, it is of interest to determine the former quantity, in order to determine separately the dependence of k_1 and k_2 on pH. In Fig. 7 the data of Fig. 6 are replotted as log K against pH. Over a wide range K varies approximately inversely with the 5th power of $[H^+]$. The fact that this relation breaks down and K appears to become constant in the low pH range can be accounted for if about 2% of the total protein is resistant to denaturation, or if only 2% of the density attributed to the native protein is contributed by a stable component.

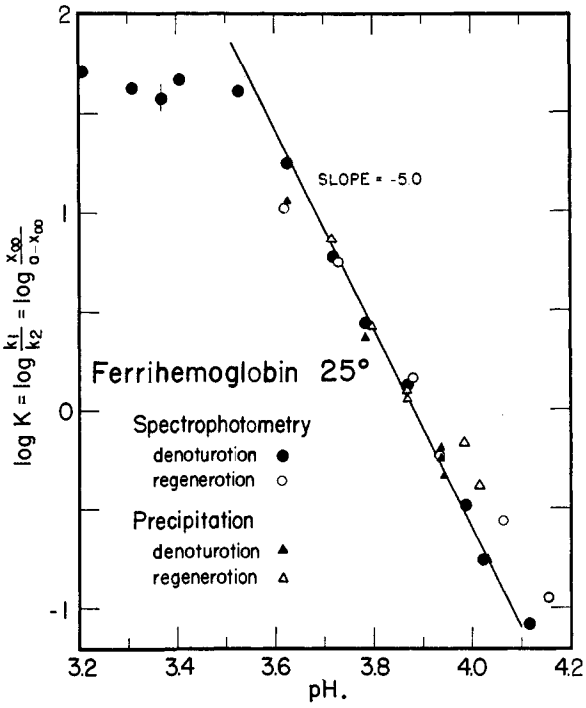

Fig. 7.—Effect of pH on the equilibrium constant for denaturation of ferrihemoglobin in formate buffers, ionic strength 0.02.

It should be noted that in Figs. 6 and 7 data are given for the approach to equilibrium from the denatured side as well as from the native side. The kinetics of the regeneration process and proof that the regenerated material is identical with native ferrihemoglobin will be given elsewhere.

Precipitation of Insoluble Protein.—Denaturation is characteristically defined in terms of virtually complete loss of solubility in salt solutions at the isoelectric point. Sufficient rate experiments and measurements of final values were therefore made by precipitating denatured protein and determining the soluble residue (spectrophotometrically) to establish that the spectroscopic measurements described above were measures of protein denaturation. The precautions, previously described,[3] that must be taken are amplified in the Experimental section of the present paper. The results of typical rate measurements are given in Fig. 8, together with spectrophotometric measurements made under identical conditions. The data are essentially similar.

In Fig. 5 points are included which represent rate constants determined by the solubility method. It is clear that rates so determined are the same as those found by following the changes in spectroscopic properties of the reaction mixture—throughout the pH range investigated. Figures 6 and 7 also include data which show that values determined by precipitation of denatured protein are also in good agreement with those determined by spectrophotometric determinations on the unfractionated reaction mixture. It is evident that both spectrophotometric and solubility methods meas-

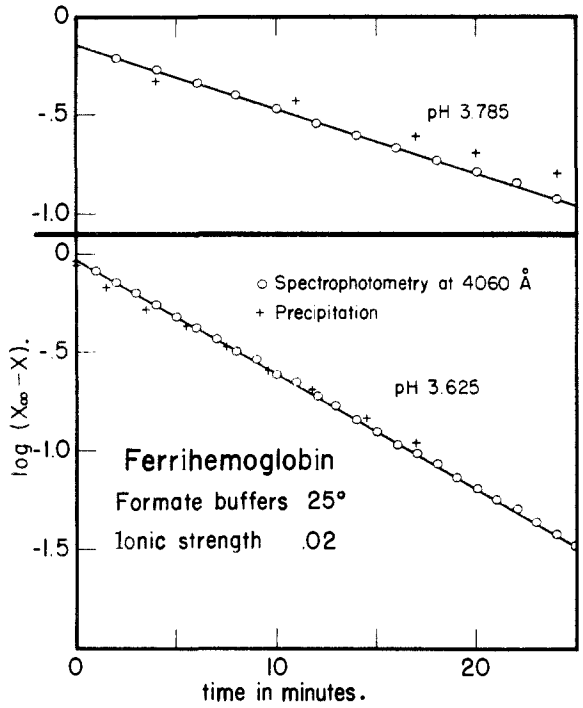

Fig. 8.—Comparison of the rates of denaturation of ferrihemoglobin as measured by decrease in absorption at 4060 Å. and by loss of solubility at the isoelectric *p*H.

ure the rate at which molecules are transformed to denatured protein.

Rate of Increase in Acid-binding Groups.—The difficulty previously noted in making a complete kinetic analysis of the liberation of acid-binding groups (the fact that *p*H changes necessarily occur in the unbuffered solutions used for titration) can be partially circumvented by examining the *p*H drifts observed over relatively short intervals of *p*H (0.1 unit) and correspondingly short intervals of time (12–60 seconds). In this way approximate rate constants have been obtained from the initial rates of liberation of new groups. One set of values so determined is shown in Fig. 5 for comparison with those determined by spectrophotometric and solubility methods. While only approximate, they appear to indicate that the reaction which unmasks acid-binding groups is consistently faster than the other changes studied, *i.e.*, delayed acid uptake precedes actual denaturation.[8] Determinations by this method are subject to great error at either higher or lower *p*H values than those shown. About the exact relation of rate of the liberation of basic groups to *p*H, however, no more can be said therefore than that it increases with the acidity as do the other reactions.

There is thus some indication that the reactions liberating basic groups and causing denaturation may not be identical, although closely interdependent. Further support of this possibility is to be seen in Fig. 6 where the broken line indicates the "equilibrium" values for the liberation of acid-binding groups, taken from Fig. 3 (insert) normalized by setting 0.52 mole equal to 100% denaturation. The probability that the reactions are distinct (although interconnected) eliminates the possibility, previously referred to, of calculating the ionization equilibrium of the liberated groups.

(8) The result of an earlier attempt to demonstrate that these rates were the same[3] for COHb is not considered unequivocal because of the sensitivity of the rates to traces of oxygen present during the titration of that protein; the changes of *p*H referred to above were also inadequately allowed for.

NOTE ADDED IN PROOF.—More recent experiments with several buffers other than formate and with HCl have removed the discrepancy between the rates of the two reactions, and restored the possibility of calculating the ionization constants of the acid-binding groups involved. The discrepancy noted in the text is due to a peculiarity of formate which was used in the spectrophotometric and solubility work, but not in the titrimetric experiments.

Dependence of k_1 and k_2 on *p*H.—Since K and $(k_1 + k_2)$ are both known as functions of *p*H, it is possible to determine for the *p*H of each experiment, separate values of k_1 and k_2. These values are shown in Fig. 9. The results show that the rate of the denaturation reaction (k_1) is proportional to the 2.5 power of $[H^+]$ over the entire *p*H range investigated. They also indicate that the rate of the regeneration process (k_2) is *inversely* proportional to the 2.5 power of $[H^+]$.

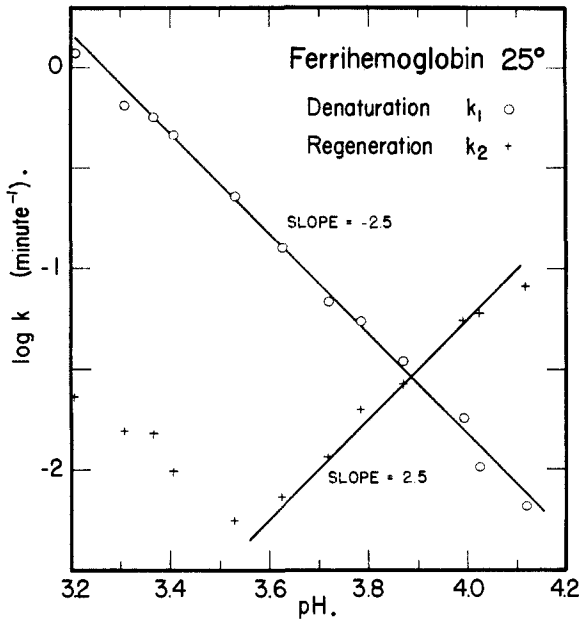

Fig. 9.—Variation with *p*H of calculated first-order rate constants for denaturation (k_1) and regeneration (k_2) of ferrihemoglobin in formate buffers, ionic strength 0.02.

The departure at low *p*H of the points for k_2 from the straight line of slope 2.5 is a consequence of the leveling off of K in this *p*H range (Fig. 7). If the explanation suggested for the latter (2% of protein resistant to acid-denaturation) is valid, the scatter of these points below *p*H 3.6 has no significance for k_2. This conclusion has been confirmed by determining $(k_1 + k_2)$ by measuring the rate of the regeneration reaction as will be reported elsewhere.

Model of the Reaction.—The fact that k_1 and k_2 both depend on *p*H, but in different (inverse) ways suggests that the role of $[H^+]$ is not a simple catalytic one: *i.e.*, $[H^+]$ must combine with protein in the process of denaturation, and be dissociated from protein in the process of regeneration. This qualitative conclusion, of course, has already been

reached quantitatively on the basis of the titration data (Fig. 3). In its simplest form the equilibria involved must be of the form

$$\text{denaturation}\quad N + xH^+ \xrightleftharpoons{\text{instantaneous}} NH_x \xrightarrow{k_1} DH_x$$

$$\text{regeneration}\quad DH_x - xH \rightleftharpoons D \xrightarrow[k_2]{} N$$

where N and D are native and denatured protein, respectively; and x is the number of equivalents of $[H^+]$ involved in the trigger reaction. It is important to note that x is not necessarily 36; the kinetic data suggest a much smaller number (2.5) of groups which affect the stability of N. Furthermore it is not necessary to assume that N and NH_x, or D and DH_x are characterized by the same ϵ. It is only necessary that the ratios $[N]/[NH_2]$ and $[D]/[DH_2]$ be the same in equilibrium mixtures of all four components, as they are in pure native or pure denatured protein at the same pH, so that the ratios measured by the absorption measurements are $([N] + [NH_2])/([D] + [DH_2])$ rather than $[N]/[D]$.

It is attractive to inquire how the combination of 2.5 equivalents (average) of H^+ results in a rapid doubling of the groups available to combine with acid, and in the spectroscopic and solubility changes which follow. Without contradicting conclusions drawn from X-ray data and molecular constants, we may consider the ferrihemoglobin molecule to consist in a singly folded flat molecular fabric, with the prosthetic groups on the inner surface of the fold, and acid-binding groups distributed uniformly over both surfaces of each disc.[9] Combination with H^+ of an average of 2.5 COO^- groups per molecular unit of folded fabric may then cause opening of the fold, and separation of the inner surfaces; thus the COO^- groups on the inner surfaces become available to combine with H^+. At the same time, but either more slowly or only after combination with H^+, the molecular rearrangements which affect the absorption, take place. Aggregation of the fabric units into the large particles of insoluble denatured protein takes place only after COOH groups are ionized again by removal of H^+. If the COOH groups are not completely ionized (pH range near 4) all the changes are reversed and orderly refolding of single units gradually occurs, and no precipitation results. If all the COOH groups dissociate, including those that participate in bonding the discs, orderly folding is less likely to occur than the building up into large aggregates that is always possible when polyvalent units are involved.

In view of the demonstration by Gralén[10] that hemoglobin is dissociated into units of half the normal molecular weight (two hemes per unit) at pH values above those studied in this paper, the folded units postulated above must be thought of as containing only two hemes each.

(9) A folded fabric is indicated rather than paired flat discs, since the regeneration reaction, as well as the denaturation reaction, is first order.

(10) N. Gralén, *Biochem. J.*, **33**, 1907 (1939).

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The Chemistry of the Perfluoro Acids and their Derivatives. V. Perfluoropropionic Acid

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A number of derivatives of perfluoropropionic acid have been prepared and their properties determined. Data are reported for the first time on the anhydride, acid chloride, methyl, ethyl and isopropyl esters, dihydro amine and dihydro alcohol. Many compounds containing the perfluoroethyl group, C_2F_5-, differ sharply in chemical or physical properties from the adjoining compound containing one CF_2 group more, or one CF_2 group less.

We started our investigation of the properties of the perfluorinated acids and their derivatives with perfluorobutyric acid. Numerous derivatives were made from that acid, and were reported earlier.[1] As the other perfluoro acids became available it was desirable to make the corresponding derivatives from these acids in order to study changes in properties as affected by changes in fluorocarbon chain length. In making these comparisons a number of derivatives of perfluoropropionic acid were prepared. The syntheses and properties of some of these derivatives are reported in this paper.

Our original supply of acid for this investigation was obtained by distillation of the forerun from the electrolytic preparation of perfluorobutyric acid. For later work, the acid was obtained by the electrolytic fluorination of propionic acid.[2] This acid forms a hydrate, containing about 10% water, boiling at 108.5°. Pure perfluoropropionic acid is easily obtained from the hydrate upon distillation from a little phosphorus pentoxide. The small amounts of anhydride formed are removed as the low-boiling fraction.

The preparation of perfluoropropionic anhydride itself is easily accomplished by adding an excess of phosphoric anhydride to the acid, refluxing the mixture for several hours and then recovering the anhydride by distillation. The acid chloride is conveniently prepared by the reaction of the anhydrous acid with benzoyl chloride. The amide is prepared by addition of anhydrous ammonia to an ether solution of the ethyl or methyl ester, or by the reaction of the acid chloride with ammonia. The

(1) D. R. Husted and A. H. Ahlbrecht, 116th American Chemical Society Meeting, Atlantic City, N. J., September, 1949, Fluorine Symposium Paper No. 17, page 10K; paper for publication in preparation.

(2) E. Kauck, A. Diesslin, J. H. Simons, 116th American Chemical Society Meeting, Atlantic City, N. J., September, 1949, Paper No. 15, page 9K; *Ind. Eng. Chem.*, **43**, 2332 (1951).