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

A Specific Effect of Formate Ion on the Reversible Acid Denaturation of Horse Ferrihemoglobin[1]

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Exposure of native horse carbonylhemoglobin or ferrihemoglobin to acid results in the liberation of 36 acid-binding groups which are unavailable for titration in the native protein. With ferrihemoglobin, a study of reaction rates and equilibria showed that the liberation of basic groups in unbuffered HCl solutions occurred somewhat more rapidly at a given *p*H than denaturation in formate buffers, as defined by spectral change or loss of solubility. New experiments show that this difference in rates disappears when HCl or buffers such as acetate, lactate or monochloroacetate are substituted for formate. The specific effect of formate ion does not alter the functional dependence of the equilibria or rates on *p*H, but merely translates them to a lower range of *p*H. Removal of the discrepancy in rates has restored the earlier conclusion as to the all-or-nothing character of the unmasking of 36 basic groups, and made possible the estimate that their pK is so high that the newly liberated groups combine quantitatively with acid at the *p*H of liberation. Consequently, they have been tentatively identified as the ϵ-amino groups of lysine (or the guanidino groups of arginine).

Introduction

Studies of the rates and equilibria involved in the reversible acid denaturation of horse carbonylhemoglobin[2,3] and ferrihemoglobin[4] have shown that half of the titratable acid-binding groups are unable to combine with acid until after a very brief preliminary exposure of the native protein to acid. The appearance of 36 extra basic groups is accompanied by spectral changes (the most prominent of which is a large decrease in extinction at the 4060 Å. band) and by loss of solubility at the isoelectric *p*H. Rates and equilibrium points determined in formate buffers by following changes in both spectrum and solubility proved to be identical.[4] However, a comparison with rates and equilibria determined from the increase in acid-binding groups on exposure to unbuffered HCl solutions of the same ionic strength appeared to indicate that the appearance of titratable basic groups occurred more rapidly than denaturation as measured in formate buffers by the other criteria. To determine whether this difference reflected a real stepwise nature of the denaturation process or whether it was a consequence of the difference in conditions (formate buffers instead of HCl) it appeared desirable to make similar absorption and solubility measurements in other buffer systems and in HCl. This paper therefore reports new spectrophotometric observations of the *p*H-dependent equilibrium between native and denatured ferrihemoglobin in unbuffered HCl–KCl solutions, and of the rates and equilibria in buffers other than formate. Since earlier work has shown that the loss of solubility at the isoelectric *p*H occurs simultaneously with the spectroscopic change, no new data by the precipitation method are presented here.

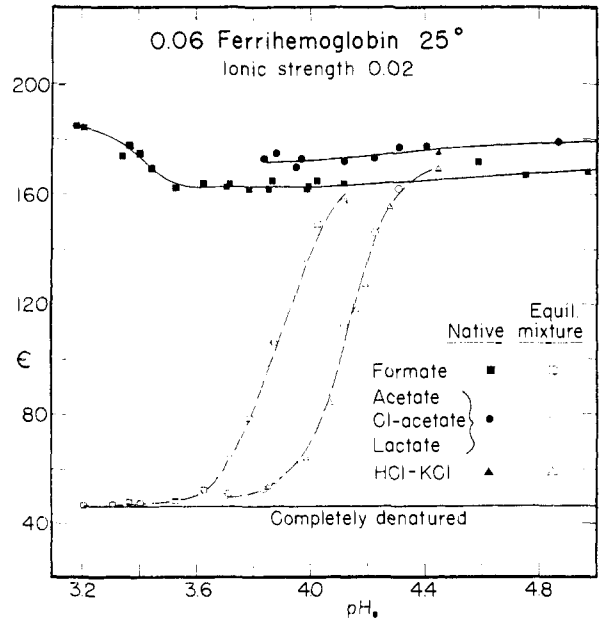

Fig. 1.—Effect of *p*H on extinction coefficient (cm.$^{-1}$ mmole Fe^{-1} liter of native, denatured, and equilibrium mixtures of ferrihemoglobin in various buffers and HCl–KCl.

Experimental

The preparation of horse ferrihemoglobin stock solutions and the measurement of rates and equilibrium points by titrimetric and absorption methods (4060 Å.) all have been described earlier.[4] All spectrophotometric data in the present paper were obtained at 25° on 0.06% solutions of ferrihemoglobin of ionic strength 0.02. In the present work measurements previously reported for formate buffers (0.02 ionic strength) are extended to acetate, monochloroacetate and lactate buffers.[5] The equilibrium values of the extinction coefficient ϵ (cm.$^{-1}$ mmole Fe^{-1} liter) at 4060 Å. as a function of *p*H in these buffers and in HCl–KCl solutions are given in Fig. 1. The same figure shows the *p*H dependence of ϵ for both native and acid-denatured ferrihemoglobin in formate and in acetate and other buffers. The values given for formate differ slightly from those published earlier, for reasons discussed below. The curve for native ferrihemoglobin in formate buffer has been determined beyond the range of *p*H represented in Fig. 1; it is noteworthy that the slight difference between it and the corresponding values in other buffers persists at least up to *p*H 6.

Results for denatured protein, however, are the same in all buffers. The value of ϵ for the denatured protein, which differs slightly from those previously published,[4] is obtained by extrapolation to zero time of measurements of ϵ during the reversal of denaturation of initially completely denatured protein. An improved extrapolation over very short intervals of optical density and time, under conditions where reversal of denaturation is slow, has eliminated the appearance of the slight *p*H dependence in ϵ for the dena-

(1) A preliminary account of this work was presented at Chicago, Ill., on April 10, 1953, before the American Society of Biological Chemists.

(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) J. Steinhardt and E. M. Zaiser, *ibid.*, **75**, 1599 (1953).

(5) Buffers of benzoic acid and its derivatives could not be used for this work because of their highly specific reactions with heme compounds.

tured protein which was previously described, and has established the value $\epsilon = 46.4$ over the pH range 3.2–5.2.

Values for the fraction of protein denatured at equilibrium, X_∞/A (where X_∞ is the concentration of denatured protein at equilibrium, and A is the total protein concentration) are calculated with the use of the curves in Fig. 1. The differences between such recalculated values of X_∞/A and the values previously reported for formate buffers[4] are very small. However, these differences suffice to eliminate the earlier conclusion that about 2% of the protein was resistant to acid denaturation.

Results and Discussion

Dependence of $(k_1 + k_2)$ on pH in Buffers.—k_1 and k_2 have been defined[4] as the first-order rate constants for denaturation and reversal of denaturation, respectively. The dependence of their sum $(k_1 + k_2)$—which is the quantity actually measured in rate experiments—on pH is shown in Fig. 2. It is evident that acetate, monochloroacetate and lactate buffers give approximately the same results, and that denaturation of ferrihemoglobin at a given pH occurs more rapidly with these buffers than with formate. The slope of the curve for log $(k_1 + k_2)$ at low pH is 2.5 in all the buffers, and the general shape of the curve and the range of velocities is unchanged. Formate seems merely to exert a stabilizing effect which shifts the denaturation region toward lower pH by approximately 0.35 unit. This effect is demonstrably not due to the relative basic strength of formate ion, since specific anion effects are absent in the buffers of three other acids which differ widely in pK.

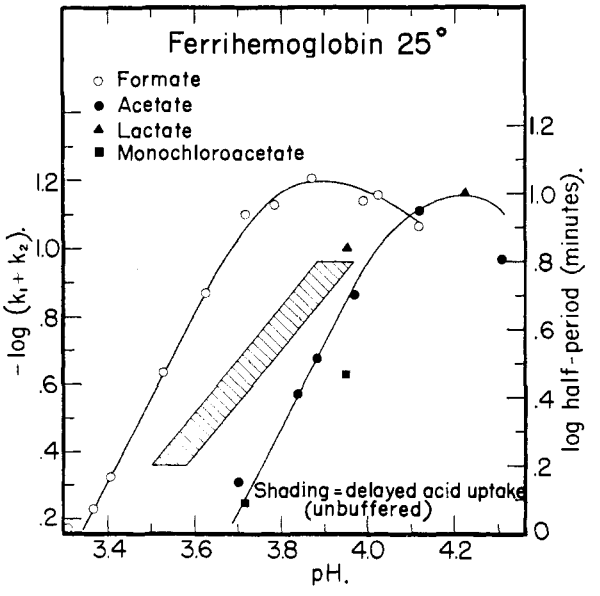

Fig. 2.—Dependence of log $(k_1 + k_2)$ or log half-period on pH in buffers, ionic strength 0.02 (from spectrophotometry at 4060 Å.).

Figure 2 also reproduces an estimate (shaded area) of log $(k_1 + k_2)$ from the effect of pH on the rate of release of extra basic groups when ferrihemoglobin is titrated with HCl.[4] Comparison of this rough estimate with the data for buffers *other than formate* shows that the discrepancy in rates of denaturation and of increase in acid-binding groups is much reduced; the rates of the latter no longer appear *faster* than the rates of the former, at a given pH. The residual discrepancy, if significant, is in the other direction. As will be shown later, the possibility that the rates are identical, and that, therefore, the 36 acid-binding groups appear *as a unit* in denaturation,[3] cannot be ignored.[6]

Dependence of Equilibrium Point on pH in Buffers and HCl.—Figure 3 shows the variation with pH of the fraction of protein denatured at equilibrium, X_∞/A. With the buffers, Guggenheim's method of calculating X_∞ from the initial stages of the time course of the reaction was used as before.[4] The close similarity of acetate, monochloroacetate and lactate is again evident, while the equilibrium curve in formate, of similar form, occupies a place 0.35 unit lower on the pH scale. Equilibrium points (measured at 2.5 hours after mixing) which were measured spectroscopically in solutions of unbuffered HCl and KCl of the same protein concentration (0.06%) and ionic strength (0.02) as the buffered solutions are also shown in Fig. 3.

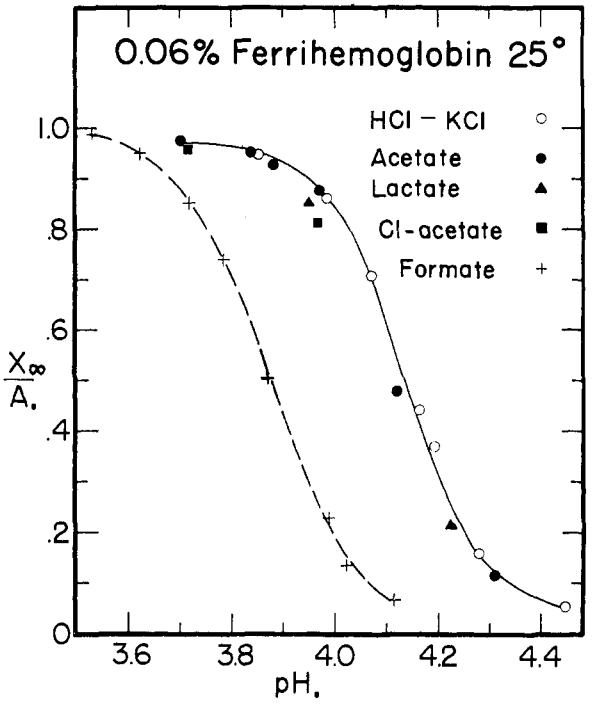

Fig. 3.—Fraction of protein denatured at equilibrium as a function of pH in buffers and HCl–KCl, ionic strength 0.02 (spectrophotometry at 4060 Å.).

Guggenheim's method cannot be applied in HCl solutions because of the small pH drift during the course of the reaction. However, direct comparison beween buffered and unbuffered solutions is possible, since pH drifts in the latter have ceased at equilibrium and the ionic strength has remained sensibly constant. It is apparent that the equilibrium curve determined spectrophotometrically in HCl is identical with that in acetate and other buffers except formate, *i.e.*, formate rather than chlo-

(6) A direct comparison *at equal concentrations* of the rate of extra acid uptake and the rate of spectroscopic change in unbuffered HCl solutions is difficult because precise titration data are obtainable only in solutions too concentrated for spectroscopy at 4060 Å. It is shown in this paper that the effects of concentration, theoretically zero for a first-order reversible equilibrium, are not negligible for this reaction.

ride ion is specific in its effect on the denaturation of ferrihemoglobin. The identity of the spectrophotometric equilibrium curves, as well as the approximate agreement noted above between the rates of appearance of extra basic groups in HCl and of spectral changes in buffers other than formate, further substantiate the earlier concept[3] of the single step character of the unmasking of the 36 acid-binding groups.

If the equilibrium constant, defined as $X_\infty/(A - X_\infty)$, is equal to the ratio of the forward and reverse velocity constants, we may calculate separate values of k_1 and k_2 from the rates and equilibria shown in Figs. 2 and 3. In Fig. 4 values of log k_1 and log k_2 so calculated for the non-specific buffers are presented for comparison with those found earlier for formate. The specific effect of formate is a little greater on denaturation (k_1) than on reregeneration (k_2).

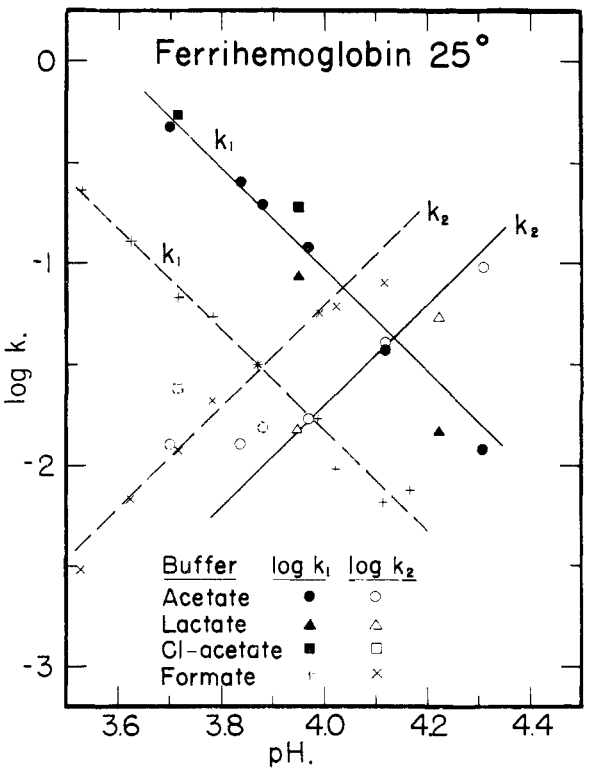

Fig. 4.—Dependence on pH of log k_1 (denaturation) and log k_2 (reversal) calculated from kinetics and equilibria in buffers (slopes of lines ±2.5).

Rates and Equilibria in Mixed Buffers.—Just as it was possible to show that the specific effect of formate is not due to its basic strength, one may demonstrate that the aldehydic function of formic acid is not the determining factor in its specificity. Thus, addition of 0.01 M acetaldehyde does not affect the rate of denaturation of ferrihemoglobin in an acetate buffer. Experiments in mixed acetate and formate buffers (Fig. 5) further show that the stabilizing effect of formate is not catalytic in character (*i.e.*, brought about by a small addition) but is a regular function of the proportion of formate in the mixed buffer. The substitution of chloride ion for half the formate causes a percentage shift in X_∞/A equal to that found when acetate replaces half the formate. This indicates not only that acetate and chloride are interchangeable, but that only the anions and hydrogen ions, and not the undissociated acids in the buffers, affect the denaturation of ferrihemoglobin.

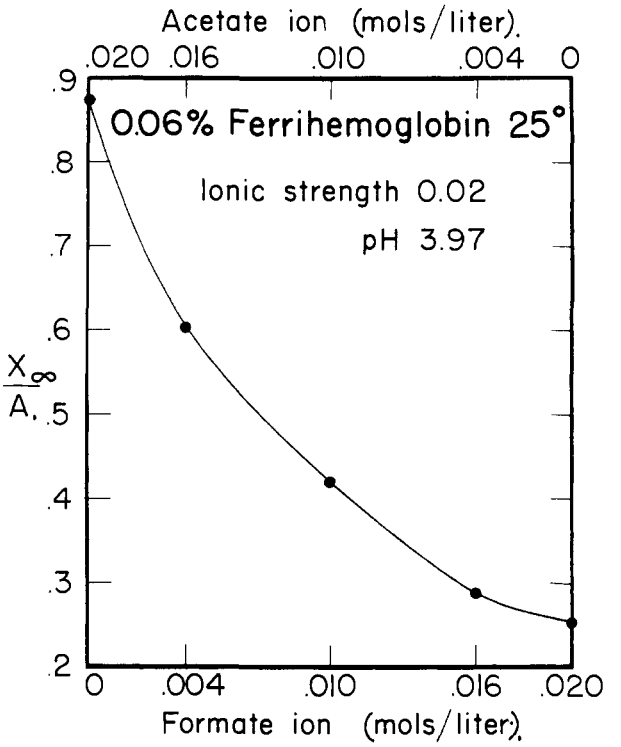

Fig. 5.—Effect on denaturation equilibrium of substitution of formate for acetate ion in mixed buffers at constant pH 3.97 and total ionic strength 0.02.

Comparison of Equilibrium in HCl by Spectrophotometry and Acidimetry.—The fraction of the maximum (0.52 mmole base/g. ferrihemoglobin) increment of basic groups which appear in acid denaturation, and which is *titrated* at any pH is represented by curve A in Fig. 6, which has been calculated from the equilibrium curve for the titration of 0.4% ferrihemoglobin with HCl.[4] This curve may be regarded as the product of two functions, one of which represents the fraction of protein denatured at each pH, and the other of which represents the fraction of the new groups which can combine with acid at the same pH. Curve B in the same figure repeats the values of X_∞/A determined spectrophotometrically on equilibrium mixtures of 0.06% ferrihemoglobin and HCl already discussed above. The two curves should be expected to coincide only if *all* the new groups combine quantitatively with acid at all the pH values represented.

If a difference between curves A and B appears, it is a measure of the extent to which combination of the extra basic groups is incomplete. In fact, the dissociation curve of the liberated groups over the pH range of the experiments may be found by obtaining the quotient of the ordinates of the two curves as a function of pH.

Before this comparison can be made and conclusions drawn, the effect of the difference in ferrihemo-

globin concentration must be taken into account. Although the kinetics of all but the last stages of the denaturation reaction are well described by the use of two first-order rate constants corresponding to rapid denaturation and regeneration reactions, a study of the regeneration process to be given elsewhere has shown that the regeneration reaction is actually complex. At least one *second*-order step is included. The existence of this step causes the values of X_∞/A to decrease with an increase in protein concentration. The following relation would be applicable to correct for the influence of protein concentration if the regeneration reaction were wholly second order

$$\frac{A(y^2)}{1-y} = \frac{B(z^2)}{1-z} \qquad (1)$$

Here A and B are two different protein concentrations and y and z are the corresponding values of X_∞/A. Regeneration experiments to be reported elsewhere show that this equation does indeed apply over moderate concentration differences, but tends to overcorrect for large differences, since the regeneration reaction is not entirely second order. The use of eq. 1 to correct curve A of Fig. 4 from 0.4 to 0.06% protein yields curve C, representing the fraction of extra basic groups titrated as a function of *p*H in 0.06% ferrihemoglobin solutions. The close similarity of curves B and C (which would be closer but for the over-correction of C) indicates that essentially all the extra basic groups broken out at each *p*H combine quantitatively with acid at the *p*H at which they are broken out. Thus their *pK* must lie above 5.

Identity of Basic Groups Liberated.—In an earlier consideration of the identity of the 36 extra acid-binding groups that appear when a molecule of horse hemoglobin is denatured at mild acidity, the hydrolysis of amide or peptide groups was eliminated as a possible source of base.[2] The differential titration of native and fully denatured protein showed that the titration curve of the 36 groups liberated could be ascribed to two sets of groups, equal in number, having *pK*'s of about 4.4 and 6.1. Since all of the 34 to 36 histidine groups[7] are titrated in the native protein,[2,8] both sets of liberated groups were tentatively identified as carboxylates. The results just presented show that the groups liberated are considerably more basic than carboxylate groups. This suggests that they may be ϵ-amino groups of lysine, of which there are about 38 in the molecule (or possibly, in part guandino groups of arginine of which there are

(7) H. T. Macpherson, *Biochem. J.*, **40**, 470 (1946).
(8) J. Wyman, Jr., *J. Biol. Chem.*, **127**, 1 (1939).

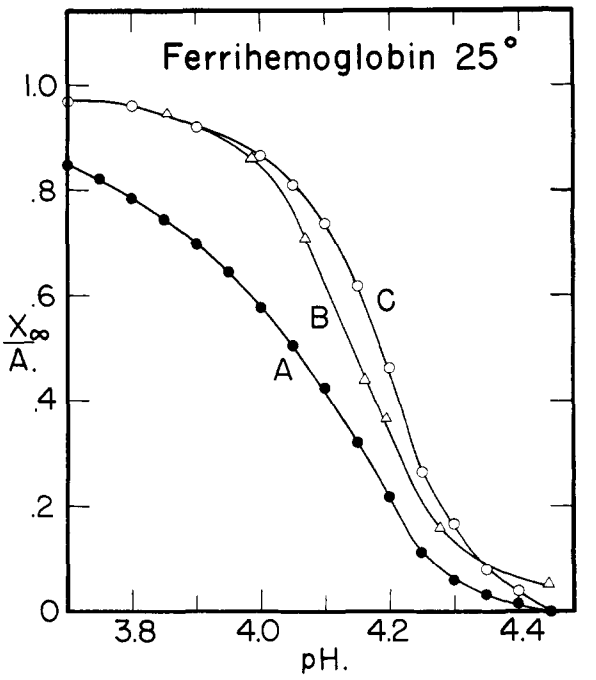

Fig. 6.—Dependence on *p*H of equilibria in HCl–KCl solutions, ionic strength 0.02: A = fraction of maximum number of extra basic groups titrated in 0.4% ferrihemoglobin; B = fraction denatured by spectroscopic criterion in 0.06% ferrihemoglobin; C = result of correction of curve A to 0.06% protein, using eq. 1.

14).[7] At the *p*H's at which denaturation occurs such groups take up hydrogen ion quantitatively. In unbuffered solutions this would cause the dissociation of a corresponding number of carboxyl (or imidazolonium) groups, and the net effect would be the appearance of extra carboxylate(or imidazole) groups, simulating the *pK* values previously reported.

The groups freed must of course be distinguished from the small number of groups per molecule (trigger groups) whose reaction with acid initiates the liberation; these have been considered to be carboxyls.[2] The combination with acid of an average of 2.5 COO^- groups per molecule seems to precede the unfolding process which results in the appearance as a single step of all 36 extra basic groups.

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