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Masked Imidazole Groups in Cyanoferrihemoglobin and Carbonylhemoglobin*

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Earlier papers have shown that the reversible acid denaturation of ferrihemoglobin and carbonylhemoglobin at both 0° and 25° liberates approximately 22 acid-binding groups (presumably imidazole) which do not combine with acid in the native protein. The demonstration for ferrihemoglobin has depended (a) on rapid-flow measurements (2–3 seconds) of pH in solutions of sufficiently high ionic strength (0.3 M chloride) to suppress differences in acid-binding between native and denatured protein which are due only to changes in the electrostatic interaction of their charged groups; and (b) on the fact that the difference in the titration curves does not pass through a well-marked narrow maximum as the pH is lowered (as it would if only electrostatic effects were involved), but remains at a nearly constant level over a wide range of pH. At an ionic strength of 0.3 the difference is not sustained below pH 4, presumably because at lower pH values partial denaturation occurs within the 2 to 3 seconds required for pH measurement. In the present paper recourse is had to titrating cyanoferrihemoglobin (CN-hemoglobin) and carbonylhemoglobin (CO-hemoglobin)—both of which are much more stable to acid than is ferrihemoglobin—to extend the constant difference between the titration curves of native and denatured protein in solutions of high ionic strength to considerably lower pH, and thus to strengthen the interpretation of actual unmasking. The results show an extension of the pH region of constant difference up to 2 pH units, and confirm the earlier conclusions that the basic function of the imidazole groups in 22 histidines is masked in native horse hemoglobin, and that other less basic groups, such as carboxylate, are completely reactive. The results also show that the rate of unmasking in cyanohemoglobin is approximately the same as the rate of denaturation of this protein measured spectrophotometrically.

In acid-denatured carbonylhemoglobin (CO-hemoglobin) (Steinhardt and Zaiser, 1951) or ferrihemoglobin (Steinhardt and Zaiser, 1953) the number of prototropic groups titrating between pH 4.5 and 7.5 exceeds by about thirty-six the number titrating between these limits of pH in the corresponding native proteins. In the earliest report (Steinhardt and Zaiser, 1951) the possibility was advanced that at least part of these "masked" groups were in histidine residues, but weak carboxyls were also considered a strong possibility. In subsequent papers other possibilities were put forward (Zaiser and Steinhardt, 1956; for summary of earlier work, see Steinhardt and Zaiser, 1955). More recently Tanford (1955) suggested that no actual unmasking occurred, but that the effects observed could be attributed quantitatively to changes brought about by denaturation in the electrostatic interaction term w in the familiar titration equation:

$$\mathrm{pH} = \mathrm{pK_{int}} + \log \frac{h}{n - h} - 0.868wz \qquad (1)$$

in which h represents the number of sites of a set n having intrinsic dissociation constant K_{int}, which have dissociated a hydrogen ion at each pH, and z is the net charge at that pH. However, the validity

* A brief account of part of this work was included in a paper presented at the 138th meeting of the American Chemical Society in Chicago in April, 1960.

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of the unmasking concept for twenty-two of the thirty-six groups reported earlier was demonstrated conclusively when it was shown that the difference in the amounts of hydrogen ion bound consistent with theory persisted when titrations were performed at high salt concentrations (Beychok and Steinhardt, 1959). Such an environment should have suppressed practically all effects due to differences in w. This paper also established a strong presumption that the twenty-two groups masked in the native protein are in histidine residues, and thus represent the prototropic function of imidazole. Recently Breslow and Gurd (in press) have demonstrated by a more direct means (catalytic effect on hydrolysis of *p*-nitrophenol acetate) that in sperm whale myoglobin six histidines which are catalytically inactive in native protein become normally reactive on denaturation. This figure of six per heme unit is half the histidine in whale myoglobin, while the hemoglobin figure twenty-two is 61% of the thirty-six groups present in this protein.

The experimental distinction between Tanford's alternative and the masking concept depended on the demonstration that the difference between the amounts of acid bound by native and denatured protein rose to a maximum with decreasing pH, and then remained constant over a substantial pH interval, forming a plateau in the curve obtained by subtracting ordinates of the titration curve of native protein from those of acid-denatured protein (Beychok and Steinhardt, 1959). Typical results with ferrihemogloblin are shown in Figure 1, in which the upper broken line in the inset represents the theoretical prediction for twenty-two masked groups. The alternative Tanford concept would require the bell-shaped difference curve with a well-marked maximum, also shown in the figure. Neither "theoretical" curve is to be taken as more than an approximation, but the distinction between them is clear, and is reinforced by other experimental tests and considerations (Beychok and Steinhardt, 1959).

It will be noted, however, that the demonstration is less than perfect, since the experimental difference curve drops at low pH values instead of remaining on a plateau. It has been assumed in earlier papers that this departure from the simple theoretical expectation is due to the practical impossibility of getting equilibrium pH data on the protein at pH values less than about 4.2 before the protein is partially denatured. The 2 or 3 seconds of flow time to the end of the glass electrode systems (Steinhardt and Zaiser, 1951) was assumed to permit an appreciable amount of denaturation to occur during the measurement. However, subsequent direct measurement of the rate of denaturation of ferrihemoglobin in 0.3 M KCl solutions has indicated that the midpoint in the drop at the low pH end of the plateau in the difference curve should be found at about 3.45, whereas it is actually found at about 3.95. The drop from the plateau thus should not be discernible above pH 3.7, whereas the difference curve in Figure 1 has a discernible drop at pH about 4.2.

The existence of this small but real anomaly,

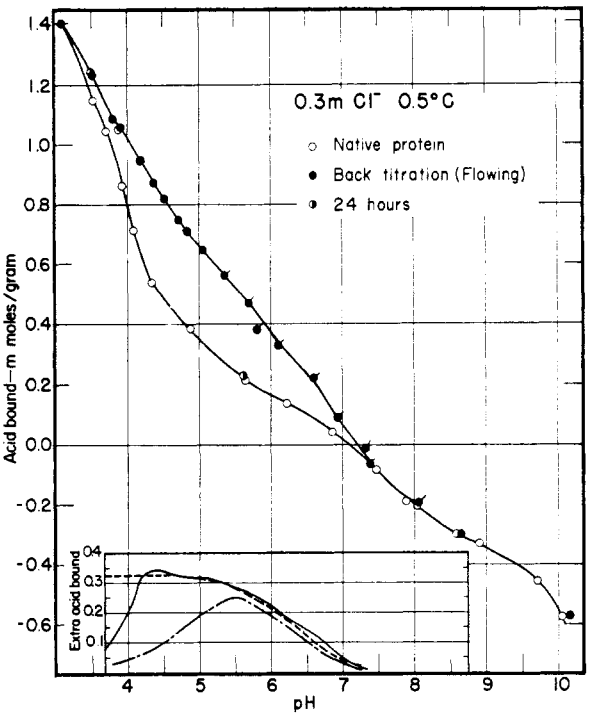

FIG. 1.—Previously published data for the rapid-flow titration and back-titration of ferrihemoglobin at 0.5° in the presence of 0.3 M chloride. The difference between the amounts of acid combined by the native and denatured proteins at each pH are shown in the inset at the bottom of the figure (solid line), together with the results to be expected if twenty-two imidazole groups are unmasked in denaturation (upper broken line). The small differences to be expected, in the absence of unmasking, from postulated effects on the electrostatic interaction factor w are also included for comparison (lower broken line).

plus the desirability of extending the plateau as far as possible into more acid pH regions to make its existence and meaning most clear, has suggested titrating hemoglobin solutions that have been stabilized against acid denaturation by means other than cooling to 0° (already done) and reduction of ionic strength (the latter recourse is eliminated by the need to minimize the electrostatic effect). The fact that CO-hemoglobin is far more stable than ferrihemoglobin (Steinhardt and Zaiser, 1951) suggested its reinvestigation at high salt concentrations. This has been done and is reported here. Fortunately, a number of ligands are effective in stabilizing ferrihemoglobin itself to a degree comparable to CO-hemoglobin.[1] Cyanide ion has been selected as a stabilizing ligand because the dissociation constant of HCN is sufficiently small (*ca.* 10^{-10}) to cause no interference with the titration curve over the pH range

[1] The remarkable effects of numerous ligands on the resistance to acid denaturation of both ferrohemoglobin and ferrihemoglobin have been reported (138th meeting of the American Chemical Society, 1960) and are to be presented in detail elsewhere. Among the most effective stabilizers for ferrihemoglobin are cyanide and azide ions. Most ferrohemoglobin compounds, including reduced hemoglobin, are very stable relative to ferrihemoglobin. The specific effect of formate ion reported earlier (Zaiser and Steinhardt, 1956; Steinhardt and Zaiser, 1955) is a particular case of ligand stabilization, since there is evidence that formate is a ligand also.

of principal interest, and because it is highly effective in very low concentration. In 0.3 M KCl at 0°, cyanide ion reduces the velocity of acid denaturation at constant pH by a factor of about 600. On the simple interpretation given earlier it should thus be effective in extending the plateau by about 1.1 pH units toward the acid side unless other processes (such as cyanide dissociation or heme-separation accompanied by denaturation) occur. The results reported here with both CN-hemoglobin and CO-hemoglobin show that a very substantial extension has been obtained, and the existence of masking in the native proteins has been made even clearer than before.

Experimental

All materials, methods, and procedures (including the rapid-flow pH measurements which give values for 2 seconds after mixing) were the same as previously described (Steinhardt and Zaiser, 1951, 1953; Beychok and Steinhardt, 1959) except as stated below or elsewhere in this paper.

Protein Preparations.—Stock solutions of horse ferrihemoglobin were prepared by air oxidation of CO-hemoglobin (thrice crystallized) dissolved in 0.2 M KCl at pH 5. The solutions were stored in the cold at pH 6.6 after dialysis. Spectrophotometric analysis (Zaiser and Steinhardt, 1951) immediately after dialysis showed that less than 3% of the absorbing protein was still present as CO-hemoglobin or oxyhemoglobin. When the solutions were kept for several weeks, however, a very slight apparent decrease in the fraction present as ferrihemoglobin occurred. Assays made by dry-weight determinations (heating to constant weight at 105° at 1 atmosphere, or at 60° in vacuum) did not change with time, but they did show, with various preparations and after various times, between 5 and 12% more solids than did spectroscopic analysis. These solutes contain nitrogen and iron in almost the same proportions as hemoglobin, as is shown by analysis (17.8% N and 0.348% Fe, based on dry weight) and are assumed to be products related to hemoglobin itself. For this reason, all amounts of acid combined are given on the basis of total dry weight. This is essentially equivalent to reporting them on the basis of iron content as is done usually.

When applied to ferrihemoglobin itself, this mode of calculation leads to titration curves which agree in detail with those obtained earlier with other batches, when the amounts combined are adjusted by a small constant additive correction (in the case of the two batches used +0.021 and 0.051 mmole/g respectively), comparable in magnitude to similar terms applied to bring earlier batches, initially at different pH values, into agreement. The largest discrepancies in any part of the curves do not exceed 0.03 mmole/g and are usually much smaller.

Stocks of CO-hemoglobin were kept at higher pH values (about 7.3); calculations of acid bound were therefore subject to larger corrections (*ca.* 0.14 mmole/g) than in the case of ferrihemoglobin. No discrepancy existed between assays by dry weight and by spectrophotometry. Only 2% of the protein was present as ferrihemoglobin. Two batches gave exactly comparable data. Care was taken at every step of handling to minimize exposure to air, and fresh CO was introduced into the solutions frequently. This precaution is important, since in acid solutions traces of oxygen convert CO-hemoglobin quite rapidly to ferrihemoglobin, which is denatured rapidly.

Cyanoferrihemoglobin Titrations.—The protein solutions used in the titration experiments at 0.01 M cyanide were prepared (prior to introduction into the titration apparatus) by mixing the following in the order given: 15.0 ml of 2 N KCl, 10.0 ml of 0.099 N HCl, 10.0 ml of 0.099 N KCN, and 18.0 ml of nearly isoelectric stock solution of ferrihemoglobin, plus enough water to bring the volume to 100 ml. Transient exposure of the protein to high or low pH levels was thus avoided. For the back-titration experiments the reagents were added in the same order; an additional 10 ml of 0.099 N HCl was added with gentle stirring prior to dilution to 100 ml, to denature the protein at pH 2.95–3.1 at 0° for 30 minutes before introduction of the protein into the titration equipment.

If the amounts of acid combined by ferrihemoglobin in the presence of 0.01 M HCN solutions (made by mixing solutions of HCl and KCN) are calculated in the simple fashion which would be appropriate in the absence of this mixture the results require additional interpretation. Since cyanide is bound more strongly to ferrihemoglobin than to hydrogen ion (Coryell *et al.*, 1937), a small excess of hydrogen ion appears in HCN solutions as soon as even small amounts of ferrihemoglobin are added, because cyanide ion is bound and leaves an equivalent amount of hydrogen ion either free or bound by the protein. Thus even without excess HCl (over the amount required to neutralize the cyanide ion), it is to be expected that about 0.06 mmole H^+/g will be bound by the protein (assuming one cyanide is bound by each heme). If such a correction is not added, titration curves in the presence of cyanide at pH values not far below neutrality will be lower by this amount than those of ferrihemoglobin alone. If cyanide is *not* bound by denatured protein, this effect will disappear at the low pH values at which denaturation occurs, and it will be absent in the back-titration curve. The back-titration data in this paper suggest that cyanide is bound by denatured protein (as it is by hemin itself), and no part of the slope of the titration curve need therefore be attributed to dissociation of bound cyanide. At very acid pH, however, there is a possibility that $[H^+]$ will be large enough to displace the equilibrium in this direction for both native and denatured proteins. The equilibrium of ferrihemoglobin with cyanohemoglobin reported in the literature[2] suggest that this might happen at pH values below about 3.2 *at 25°*. However, CN-hemoglobin appears

[2] Coryell *et al.* (1937) report the equilibrium constant of the reaction ferrihemoglobin$^+$ + HCN $\rightleftharpoons$ CN-hemoglobin + H^+ to be 18 at 22–26°, when measured by a magnetic method, but Coryell concedes in a later paper (Coryell, 1939) that the data are not accurate enough to exclude interaction between the hemes; if this is the case the constant cannot be considered to have high precision, but it is accurate enough to support this conclusion.

to exist in our solutions at 0° as determined spectroscopically and by its effect on the stability of the protein, to pH values down to at least 3.

Carbonylhemoglobin Titrations—In the titration of CO-hemoglobin additional precautions were necessary to assure exclusion of oxygen, which in acid solutions speeds denaturation (Zaiser and Steinhardt, 1951). Since the maintenance of a small constant pressure head is required for the rapid flow measurements, and since the location of the apparatus precluded the use of CO for this purpose, nitrogen was used both to provide the pressure and to keep the apparatus oxygen-free (the CO combined with the protein is not appreciably freed under these conditions in the short time involved). All reagents were thoroughly equilibrated with nitrogen immediately preceding their introduction into the titration apparatus. CO-hemoglobin solutions were resaturated with carbon monoxide, then bubbled briefly with nitrogen as they were introduced. Once in the apparatus, all solutions were exposed only to nitrogen and were never in contact with air until their discharge into waste jars.

Results and Discussion

Cyanoferrihemoglobin.—The hydrogen-ion titration curve of ferrihemoglobin in the presence of 0.01 M HCN and 0.3 M KCl is shown in Figure 2. The back-titration of protein denatured by exposure of ferrihemoglobin solutions containing 0.01 M HCN and 0.3 M KCl for 30 minutes to pH 2.95–3.09 for 30 minutes is also shown.[3] For comparison, previously published data for ferrihemoglobin itself at the same temperature and ionic strength are included.

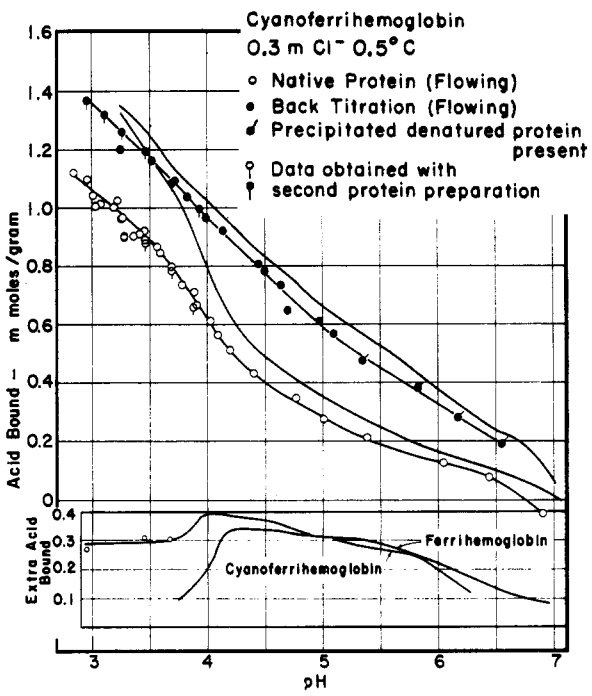

FIG. 2.—Titration data for native and denatured (back-titration) cyanoferrihemoglobin at 0.5° in the presence of 0.3 M chloride. The difference in acid bound is shown as a function of pH in the inset at the bottom of the figure. Similar data for ferrihemoglobin are included as smooth curves for comparison.

The salient features of the data may be summarized as follows:

(1) All of the back-titration curve and half of the titration curve (at pH above 4.2) of cyanoferrihemoglobin lie below the curves for ferrihemoglobin by approximately 0.06 mmole/g. This difference is only apparent, as explained in the Experimental section, and is caused by failure to include the extra acid liberated by the binding of 0.06 mole/g cyanide ion from HCN into the calculation of acid bound. Its presence demonstrates that both native and denatured ferrihemoglobin bind one cyanide ion for each atom of iron within the pH range of the data.

(2) At pH values below about 4.2 the titration curve of cyanoferrihemoglobin falls increasingly below that for ferrihemoglobin. In doing so, it remains closely parallel with the curve for the corresponding back-titration. With ferrihemoglobin both curves converge at these low pH levels, because denaturation (and unmasking) occurs to an increasing extent during the 2–3 second interval in which the pH is measured. Thus, at pH below 4.2, the titration curve of native ferrihemoglobin is not really known, whereas with CN-hemoglobin the data represent the true titration curve of the native protein to considerably lower pH regions.

(3) The differences in amounts bound at each pH by native and denatured protein (plotted at the bottom of Figure 2) rise to a maximum at pH 4.8, very much as in the case of ferrihemoglobin, which is also shown. This maximum of about 0.36 mmole/g, corresponding to twenty-four masked groups per 67,000 molecular weight unit, is very close to the value 0.33–0.34 found with ferrihemoglobin. However, unlike the plateau for ferrihemoglobin, the plateau for CN-hemoglobin at 0.36 mmole/g is not short, and does not start to drop sharply at pH just below 4.2; the plateau for cyanoferrihemoglobin drops only slightly at pH about 4 and then remains level at about 0.32 mmole/g to pH values below 3. In order to show that this conclusion is not the result of arbitrary curve-fitting at the lower pH value, three points have been plotted on the difference curve in Figure 2. These points represent differences in acid bound at identical pH values (±0.005 pH) in forward and back-titrations with the same protein preparation. The small dip at pH 4 is possibly the effect of a small residual electrostatic effect of the kind postulated by Tanford.

Since all three of these features are in accordance with the expectation that led to these measurements, it may be concluded that:

(1) Ferrihemoglobin exists as cyanoferrihemoglobin, containing one cyanide per iron atom, in the solutions used in these experiments, at pH

[3] In such solutions the half-period of the denaturation reaction is less than 1 minute.

values as low as, or lower than, 3.0.[4]

(2) Cyanoferrihemoglobin is far more resistant to acid denaturation than is ferrihemoglobin itself; this conclusion is further supported by direct rate measurements to be reported elsewhere.

(3) Cyanide is bound by denatured ferrihemoglobin at all pH values within the range of those shown in Figure 2. Thus, it is valid to refer to denatured cyanoferrihemoglobin, so long as there is no implication that the protein-cyanoheme link is the same as in the native protein.

(4) The concept of masked histidines in ferrihemoglobin is supported by the predicted extension of the difference curve plateau in CN-hemoglobin. Conversely, the previous identification (Beychok and Steinhardt, 1959) as histidine imidazole of the groups which are unreactive (masked) in native ferrihemoglobin and reactive (unmasked) in denatured protein appears fully applicable to CN-hemoglobin.

(5) There is no evidence that any other acidic groups, such as carboxyl, are masked prior to denaturation in horse CN-hemoglobin. In fact, the difference curve in Figure 2 makes such unmasking quite improbable.

Carbonylhemoglobin.—The data for CO-hemoglobin shown in Figure 3, show marked similarities to the data for CN-hemoglobin just described. For purposes of establishing unmasking, the present measurements are considerably more significant than those published earlier. This is true not only because of the high salt concentration but also because of the more stringent precautions taken to avoid exposure of the protein to air, especially when at acid pH.

The data themselves and the important features of the curves are practically identical with those already enumerated for CN-hemoglobin (when the latter are corrected for combination with cyanide ions) and permit the same conclusions to be drawn. However, there are minor differences: (a) the back-titration curve is slightly lower than that of ferrihemoglobin and of the adjusted curve for CN-hemoglobin, although there is reason to expect the denatured protein to be at least partially oxidized by residual air; (b) at pH below 4, CO-hemoglobin appears to be slightly more resistant to acid denaturation. The latter effect is seen most clearly in the difference curve at the bottom of Figure 3. Although both proteins give the same maximum difference, the plateau for CO-hemoglobin is flatter than that for CN-hemoglobin and there is no sign of the slight drop in the CN-hemoglobin difference curve at the same pH (4.2) at which the curve for ferrihemoglobin dropped sharply. These differences, however, are small.

It will be observed that all three proteins show distinct difference curves in the descending portion (pH 5.0–7.2), which represents the titration range of the groups which are masked in the native form.

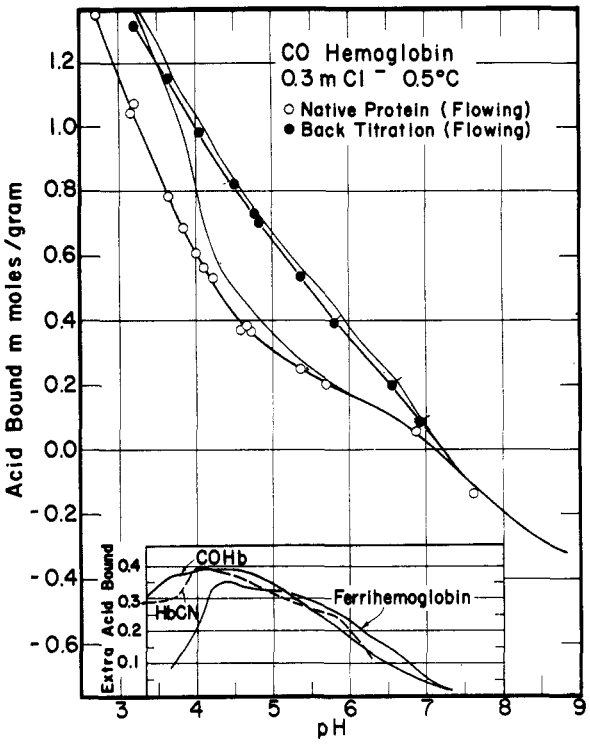

FIG. 3.—Titration data for native and denatured (back-titration) carbonylhemoglobin at 0.5° in the presence of 0.3 M chloride and with precautions taken to exclude air. The difference in acid bound is shown as a function of pH in the inset at the bottom of the figure, together with the difference curves for ferrihemoglobin and CN-hemoglobin. Similar titration data for ferrihemoglobin are included as smooth curves.

This is the region in which hemoglobin ligands or changes in the oxidation state of the iron have been shown to affect the dissociation of a small number of acid groups, presumably imidazole (Wyman and Ingalls, 1951; German and Wyman, 1937).

Comparison with Kinetic Data.—Previously published kinetic data on the acid denaturation of ferrihemoglobin and of carbonylhemoglobin have been obtained at low salt concentrations (0.02 M) (Steinhardt and Zaiser, 1953, 1955; Steinhardt *et al.*, 1958; Zaiser and Steinhardt, 1951, 1954, 1956); in addition the results obtained with CO-hemoglobin are known to depend on the presence of traces of air (Zaiser and Steinhardt, 1951). Therefore, they are not available for analyses of the drop in the level of the difference curve at low pH values, which has been attributed to partial denaturation within the 2–3 seconds required for measurement of pH. A very thorough investigation of the effects of various ligands on the stability of both ferro- and ferrihemoglobins as affected by temperature, ionic environment, and pH will be published elsewhere. However, for the purpose of the present paper, half-periods of denaturation (measured spectrophotometrically) of both ferrihemoglobin and CN-hemoglobin in 0.3 M KCl will be cited from this work:

[4] This conclusion is compatible with the equilibrium constant (18) reported in footnote 2. Neglecting the effects of interaction and the difference in temperature, only 10% of the protein exists as free ferrihemoglobin at pH 3.2. With the equilibrium constant (10.5) reported by Havemann (1943), this pH is reduced to 3.0.

TABLE I

COMPARISON OF ACTUAL AND EXPECTED pH VALUES AT WHICH EXTRA ACID BOUND DECLINES TO HALF ITS MAXIMUM VALUE

Protein	pH at Which Difference Curve in 0.3 M KCl Falls to Half Its Maximum Value	pH (Extrapolated) at Which Half-Period is 3 sec
Ferrihemoglobin, 0.3 M KCl	3.92	3.43[a]
Cyanoferrihemoglobin, 0.3 M KCl	Not reached ($\ll$2.95)	2.38[a]
Ferrihemoglobin, 0.02 KCl	2.95	3.24[a] 2.88[b]

[a] Determined spectrophotometrically. [b] Determined by analysis of pH drifts (Beychok and Steinhardt, 1959).

With ferrihemoglobin at 0.3 M KCl the drop in the difference curve occurs at about 0.5 pH unit higher than would be expected from extrapolations of denaturation rates measured spectrophotometrically. With low KCl concentrations the discrepancy is smaller and in the other direction (it practically disappears if the rates are calculated from the timed pH changes that occur when the flow through the cylindrical glass electrode is stopped) (Beychok and Steinhardt, 1959). The results obtained in low KCl concentrations make it appear that unmasking is slower than denaturation. With high KCl concentrations the opposite might suggest itself.

Table I shows that with CN-hemoglobin there may be no discrepancy at 0.3 M KCl. Analysis of rates of pH change have therefore been made with cyanoferrihemoglobin at 0.3 M KCl (Beychok and Steinhardt, 1959). The results (Fig. 4) are not characterized by high precision, but give approximately the same pH slope (−2.5) that is found in the kinetics of denaturation of this protein at lower KCl concentrations and in the absence of cyanide. The rates are lower than in the absence of ligand, even when the data obtained with the ligand at high KCl concentration are compared with those obtained in its absence at 0.02 M KCl. For example, at pH 3.5 the initial unmasking rate of CN-hemoglobin is about 0.014 mEq per g of protein per minute, while the same rate for ferrihemoglobin in 0.02 M KCl is about 0.05.[5]

Such changes in stability are to be expected from the results shown in Table I. Equally consistent is the finding that the rates of denaturation measured spectrophotometrically over the pH range 3.1–3.7 differ very little if at all from those calculated from the pH drifts—*i.e.*, the rate of unmasking is not appreciably faster (or slower) than the rate at which the optical changes occur in denaturation. This result supports the tentative conclusion, reached on the basis of Table I, that the stability shown by CN-hemoglobin in titration experiments (the long plateau in the difference curve) is wholly consistent with its kinetic behavior measured spectrophotometrically. A discrepancy is found only with ferrihemoglobin, and then only at high KCl concentrations.

[5] Although increasing the ionic strength from 0.02 to 0.3 raises the rate of denaturation of ferrihemoglobin (measured spectrophotometrically) about twenty times, the same change increases the rate for cyanoferrihemoglobin less than twofold.

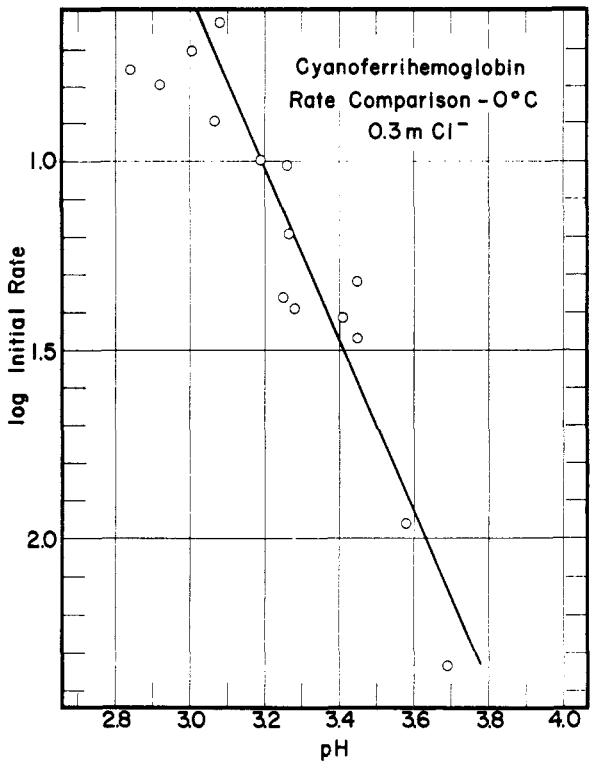

FIG. 4.—Logarithm of the initial rate of denaturation as a function of pH. The values given are calculated from changes in pH occurring in the first 30 seconds, and are directly comparable with velocity constants determined spectrophotometrically at the same ionic strength. The latter are shown as a straight line on the graph.

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