The Stabilization of Horse Ferrihemoglobin to Acid Denaturation by Combination with Ligands*

J. STEINHARDT,† R. ONA-PASCUAL, S. BEYCHOK,‡ AND CHIEN HO

From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge

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The increase in resistance to acid denaturation of ferrihemoglobin when bound to cyanide, azide, fluoride, thiocyanate, or nitric oxide has been studied as a function of *p*H at three temperatures, and the results have been related to earlier work on the stabilizing effect of formate and on the titration with acid of cyanoferrihemoglobin. A reduction in denaturation velocity of up to 50-fold has been demonstrated with both cyanide and azide at low (0.004 M) concentrations at 0.02 ionic strength. At 0.3 ionic strength the same ligand concentration produces an approximately 250–300-fold effect. Formate and fluoride give lesser effects (the latter only at 0°), and thiocyanate does not stabilize at all. Stabilization, when present, diminishes at higher temperatures. Similar but smaller effects are found on the velocity of regeneration of denatured protein. By making use of measured values of the tendency of each ligand to dissociate from the protein, it has been possible to distinguish the effects of various fractions bound, which depend on *p*H, from the intrinsic stabilizing effect of each ligand when bound. The increased stabilities found appear to be consistent with an approximately equal reduction in velocity of the time-dependent component of denaturation by those strong negatively charged ligands (cyanide and azide) which form low-spin (covalent) complexes with the protein-heme, independent of their affinity for the protein. Weaker ligands (fluoride and thiocyanate) which form high-spin complexes stabilize the bound forms to a lesser extent if at all. Nitric oxide, which is electrically neutral, appears to be without effect, although it is probably bonded covalently. The increased stability of the covalent ferri-complexes (approximately equal to that of ferro-complexes, which do not differ among themselves) may be associated with the absence in the latter of a positive charge in the vicinity of the iron, rather than with the induction of a strong covalent iron-protein bond in place of the "ionic" bond of ferrihemoglobin. An explanation is proposed for this charge effect in terms of differences in protein conformation between ferro- and ferri-hemoglobin, ferrihemoglobin bound to ligands, and globin. Regardless of the validity of this mechanism, it is shown that heme or the protoporphyrin nucleus plays an essential part in stabilizing the structure of the protein moiety of native hemoglobin.

In the course of a series of investigations of the increase in acid-binding groups that occurs in various compounds of horse hemoglobin when they are denatured by dilute acid (Steinhardt *et al.*, 1962)[1] we have shown that the various forms (carbonmonoxyhemoglobin, HbCO; ferrihemoglobin, Hb^+; cyanoferrihemoglobin, HbCN; and others) differ very greatly in respect to the rates at which they are denatured by dilute acid at temperatures between 0° and 25°. For example, ferrihemoglobin denatures in 0.02 M lactate buffers at 0.3 ionic strength about 250 times faster than when 0.01 M cyanide is present in the same solutions (Steinhardt *et al.*, 1962).

The difference in stability between ferrihemoglobin and carbonmonoxyhemoglobin may be even greater, but exact comparisons have not been made since the rate of acid denaturation of very dilute solutions (0.06%) of the latter is extremely sensitive to traces of oxygen (Zaiser and Steinhardt, 1951). Advantage has been taken of the stabilizing effect of cyanide and CO on hemoglobin to avoid ambiguities in differential titrations of native and denatured hemoglobins which would be introduced by partial denaturation of the native protein at low *p*H during the few seconds required for measuring the *p*H after mixing (Steinhardt *et al.*, 1962).

These remarkable differences in stability of the globin moiety which are brought about by changes in the immediate environment of the iron atom in the prosthetic group of this conjugated protein are attested to not only by differences in rates of unmasking but also by differences in rates of loss of solubility at the isoelectric point and in rates of change in the characteristic light absorption (Soret band)—all of which run closely parallel, not only in denaturation but in regeneration as well. In the belief that such differences may indicate the direct involvement of the iron of heme, or of heme itself, in the stabilization of the folded form (tertiary structure) of horse hemoglobin, a detailed investigation of the effect of various ligands on the stability of horse hemoglobin,[2] and the way in which these effects depend on concentration, temperature, *p*H, and ionic strength, has been made and is, in part, reported here. Because it is much easier to work with ferrihemoglobin derivatives than with ferrohemoglobin or its derivatives, and the data are consequently less ambiguous, the present paper is mainly concerned with the effect of ligands on protein having the iron in the oxidized form. Results with HbCO, HbO_2, and other derivatives of ferrohemoglobin, under anaerobic conditions, and with ferrohemoglobin itself, will be reported elsewhere.

Experimental

All materials, methods, and procedures were the same as those previously described (Steinhardt *et al.*,

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† Present address: Department of Chemistry, Georgetown University, Washington 7, D. C.

‡ Present address: Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York.

[1] Work prior to 1955 is summarized in Steinhardt and Zaiser (1955).

[2] Keilin and Hartree (1952), and Keilin (1960) have cited earlier evidence that ligands of the heme proteins such as oxygen, carbon monoxide, and azide, and the state of oxidation of the iron, affect the resistance of these proteins to denaturation by acid, alkali, heat, and chloroform.

1958; Zaiser and Steinhardt, 1951) except as stated elsewhere. The reagents used as ligands were potassium cyanide (Mallinckrodt Analytical Reagent), sodium azide (Fisher Practical or Purified grade), potassium fluoride (General Chemical Anhydrous Reagent), and potassium thiocyanate (Fisher Certified Reagent). Acetate and lactate buffers were prepared by adding KOH (Baker Analytical Reagent) to the acids (reagent grade). For reasons cited later, two different batches of lactic acid were used (Merck and Mallinckrodt). For denaturation rate measurements freshly prepared solutions of protein (final concentration 0.06%) containing the desired ligand were mixed with buffers (lactate or acetate 0.02 M with respect to the anion) to give the desired *p*H at the time of initiation of the measurements. In some experiments the ligand salt plus an equivalent quantity of HCl was used instead of the ligand acid alone. The kinetic results obtained over a period of up to 8 years with both acetate and formate buffers with six different protein preparations have consistently agreed.

Except where otherwise stated, the protein was thus initially at a *p*H different from that of the denaturation run (in the extreme case of cyanide at *p*H near 8) and at a higher concentration of ligand than after mixing. Since we have observed that, with cyanide, combination of protein and ligand or dissociation occurs as a time-dependent bimolecular reaction (particularly slow at 0°), it cannot be assumed that the fraction combined with ligand at zero time represents the fraction characterizing an equilibrium at the *p*H of the experiment. However, in the majority of the experiments with cyanide, in which large excesses of ligand were used, essentially all the protein is combined with ligand except at the lowest range of *p*H. With azide and other ligands equilibrium is reached so rapidly that the reservation mentioned above does not apply even at low concentration. Even with low cyanide concentrations (7.5×10^{-5} M), at which the rate of association is slow, less than 10% difference in the velocity measured resulted from varying the procedure by introducing the cyanide with the buffer instead of adding it to the protein initially. However, when the ligand was introduced with the buffer, a very fast component of the reaction, representing denaturation of the free protein, was over before any experimental observations were made. The ionic strength was always brought to 0.02 with lactate or acetate buffers in the experiments with cyanide, or very slightly higher (up to 0.021) with azide, fluoride, and thiocyanate (since a portion of these added anions are free in the *p*H range of interest).

The equilibria between combined and uncombined protein were studied spectrophotometrically (at 406 mμ or 419–420 mμ or both) at a number of low ligand concentrations at each temperature. To assure that the equilibrium constants do not depend on the total charge, *z*, of the protein (*i.e.*, do not depend on *p*H) measurements of the equilibria were made at two or three *p*H values. The range of *p*H that can be covered is unfortunately limited; at *p*H values below about 4.3 denaturation is fast enough to prevent attainment of equilibrium, and at *p*H values over 5.5 the protein exists primarily in the familiar undissociated four-heme form which is characterized by a high degree of interaction between the hemes (Coryell, 1938, and Wyman, 1951). Thus, at *p*H 6.36 the equilibrium of ferrihemoglobin with cyanide is characterized by a Hill exponent, *n*, of 2.8. At *p*H values below 5.5, however, none of the ligands used gave appreciable evidence of any interaction, *i.e.*, the equilibria all conformed, within the experimental error, to the general reaction:

$$Hb^+ + HA = HbA + H^+ \qquad (1)$$

and also obeyed the Hill equation with values of *n* very close to unity. This result is consistent with the reported dissociation of horse hemoglobin into half-molecules at acid *p*H (Gralen, 1939; Gutter *et al.*, 1957).

The values of the equilibrium constant K_{eq} cited in this paper are based on equilibrium concentrations approached by both association and dissociation. The assumptions made are that the thermodynamic activity coefficient of the protein is not affected by binding ligand, and that the activity coefficient of the ligand acid is unity. Attempts were also made in the case of cyanide to determine K_{eq} by measuring the velocity constants, k_1 and k_2, for association and dissociation reactions. The association velocity constant was determined experimentally in the presence of a large excess of cyanide as the pseudo first-order velocity constant divided by the concentration of cyanide. The dissociation velocity constant was determined, by diluting an initially 10-fold more concentrated solution, as the pseudo first-order velocity constant divided by the hydrogen-ion concentration of the buffer. With 0.06% protein (iron equivalent 3.6×10^{-5} M) and 0.0005 M HCN the average half period for association at 0° was 1.6 minutes, independent of *p*H; the half-period for dissociation, which should depend on *p*H, was approximately 75 minutes *at all pH values* at this temperature. Thus, the initially more rapid reaction paths (possibly association of Hb^+ with HCN rather than with the very dilute CN^-, and the dissociation of CN^- from HbCN) are not those that determine the final equilibrium. The latter may be governed by a slower reaction of Hb^+ with CN^- and of HbCN with the very low concentrations of H^+. The ratio of the faster rates gave a fairly constant apparent K_{eq} of 0.15 at 0° rather than the average true value, 0.81, cited below.[3]

The values of K_{eq} ($= [Hb^+][HA]/[HbA][H^+]$), presumably independent of ionic strength, used throughout this paper are given in Table I. Values in parentheses are taken from Scheler and Jung (1954), who worked with horse ferrihemoglobin at *p*H near 6, and whose values for azide are consistent with those we have determined at *p*H 4-5. No use has been made of the earlier values (Coryell *et al.*, 1937) obtained with bovine ferrihemoglobin at *p*H 4.77 (partly because they are considerably higher, and because an incorrect value of K_{HCN} appears to have been used in computing K_{diss}). Neither have we used a value at *p*H 6.08 and 38° (Havemann, 1943), because our own measurements at *p*H 6.36 showed that even with our air-oxidized protein strong heme-heme interaction exists at this *p*H (the Hill exponent is 2.8, close to that found with oxyhemoglobin; the Hill pK is 5.23).[4]

[3] The values found were 0.72 at *p*H 4.55, 0.62 at *p*H 4.85, and 1.10 at *p*H 5.55. The average value for the interaction exponent (in the Hill equation) was 1.04. Four concentrations of cyanide over a 5- to 10-fold range of concentrations were used in determining these values.

[4] Our dissociation-equilibrium measurements with azide show no evidence of heme-heme interaction in this *p*H interval. The cited value of K_{eq} for azide is much lower than the value calculated by Coryell and Stitt (1940) from Keilin's data at higher temperatures (1.2×10^{-5}) but are in close agreement with the results of Scheler and Jung (1954) at *p*H 6.2–6.5. The values given here for 25° are also those of the latter investigations.

TABLE I
HEMOGLOBIN-LIGAND BINDING CONSTANTS

	K_{eq}	
	0.2°	24.8°
HCN	0.81 (*p*H range 4.55–5.55)	5.1 (*p*H 5.02)
HN_3	0.024 (*p*H 4.29) 0.048 (*p*H 5.31)	(0.23)[a]
HF	—	(0.09)[a]
	K_{diss}	
	0.2°	24.8°
CN^-	4.8×10^{-11}	2.5×10^{-9}
N_3^-	5.3×10^{-7} (6.3×10^{-7})	(4×10^{-6})
F^-	(1.2×10^{-2})	(1.7×10^{-2})[b]
CNS^-	(2.1×10^{-3})	(7.4×10^{-3}) *p*H 6.7 (2.24×10^{-3})[c] *p*H 4.92

[a] These values of K_{eq} are derived from the K_{diss} values of Scheler and Jung by dividing the latter by the dissociation constants of HN_3 (pK = 4.75 at 25°) and of HF (pK = 3.3) respectively. The latter value is too high by a factor approaching 2, since another equilibrium ($HF + F^- \rightleftarrows HF_2^-$; K = 0.027) reduces the fluoride ion concentration by such a factor. However, the true value of K_{diss} for fluoride at *p*H 3–4 is undoubtedly much lower than at the *p*H of Scheler and Jung's measurements. See footnote *b*. [b] There is some doubt concerning the validity of this value in the *p*H range (3–4) of the experiments in this paper. The authors are indebted to C. D. Coryell of Massachusetts Institute of Technology for the opportunity to examine an unpublished thesis (1942) by W. B. Lewis, in which the fluoride-ferrihemoglobin (bovine) equilibrium is investigated and compared with the recalculated results of Lipmann (1929) (pig), which are in close agreement. They find a drop in K_{diss} from 1.55×10^{-2} at *p*H 4.7 to 5.6×10^{-4} at *p*H 3.8. Lewis refers to this trend as a "Bohr effect" and attributes it to a dissociable hydrogen (pK *ca.* 3 in the free protein and 4.5 in the bound protein). Such a trend can also be due to an effect of the increase in electrostatic charge on K_{diss} as the protein takes up protons. It is noteworthy that the effect is small in the *p*H range 4.5–5.5, in which such an effect was unsuccessfully sought in the measurements with HCN and HN_3 summarized in Table I, increasing rapidly at lower *p*H, below the range of the measurements reported here. [c] This value is given in an unpublished thesis (1942) by T. Vermeulen, made available to us by C. D. Coryell.

The values of K_{diss} ($= [Hb^+][A^-]/[HbA]$) depend on ionic strength; the values given are for 0.02.

RESULTS

No Ligand Present.—The choice of a buffer system, and therefore of a standard for comparing the velocities of denaturation in the presence of various ligands, presents difficulties. Zaiser and Steinhardt (1954) reported that, at 25° and 0.02 ionic strength, velocities of denaturation are unaffected by the choice of buffer when any of the following acids are used: hydrochloric, acetic, lactic, or monochloracetic; they are slower (but have the same dependence on *p*H) when formic acid buffers are used. It was assumed therefore that the velocities in the buffers enumerated (except formate) represent the rate at which ferrihemoglobin itself (or aquoferrihemoglobin) denatures. Since it will be shown that many ligands stabilize the protein, it is likely that at the extreme dilutions of protein employed (3.6×10^{-5} M iron equivalent) even very small traces of ligand impurities may affect the results obtained when no ligands are deliberately added. Thus, results with acetate buffers are somewhat more variable than those obtained with formate. The latter, which is a ligand, gives more highly reproducible results.[5]

Among the nonspecific acids just enumerated lactic acid has the most suitable pK to act as a buffer in the *p*H range of interest, and therefore it has been used in many of the experiments with added ligands. This choice, although logical, has two disadvantages: (*a*) unlike acetic acid, samples of commercial reagent-grade lactic acid have been encountered which give lower velocities than those obtained with acetic acid or in the earlier experiments with lactic acid; and (*b*) the later experiments show at low temperatures a lesser degree of dependence of denaturation velocity on *p*H than at high, *i.e.*, the slope of the linear log k *vs.* *p*H relation varies considerably with temperature. These two effects could be the result of traces of an impurity (X) which is itself a ligand; the concentration of X would necessarily increase rapidly with decreasing *p*H. Thus, addition of small quantities (0.004 M) of strong ligands (cyanide or azide) might displace X and restore the slope of the relation between log k and *p*H to the value (roughly 2.5) found with other buffers, with or without ligand, or with some of the samples of lactic acid. So simple an *ad hoc* explanation, however, is not fully adequate: thus velocities of denaturation in some lactate buffers with added ligands present are *always* lower than in acetate buffers with the same ligands, *i.e.*, the stabilizing effects of known ligand and of lactate are not fully competitive but have an additive component as well. These anomalies will be made clearer in the sections that follow.

As a practical matter, results have been obtained both with acetate buffers, which require rather high concentrations of free acid but which appear to be free of variability or of specific effects, and with lactate buffers, which are not far from their optimum buffering range but which are subject to the variability and to the specific effects described in the preceding paragraph. The lactate buffers, when ligands are present, appear to give quite reproducible results, variability appearing only when they are used alone. The degree of stabilization produced by the ligand is given for both buffers as a factor obtained by dividing the velocities obtained at a given *p*H for the same buffer in the absence of ligand by the velocity in its presence. At high temperatures (24.8°), where the results with lactate alone are not far different from those with acetate, these stabilization factors do not differ appreciably in the two buffer systems.

Comparison of All Ligands at 0.2°.—In order to permit examination of the full range of phenomena, free of the complexities introduced by lactate buffers and by changes in temperature, Figure 1 presents the results obtained at a single temperature, 0.2°, in acetate buffers alone and in acetate plus fixed concentrations (0.004 M) of the ligand—cyanide, azide, fluoride, and thiocyanate—all at the same total ionic strength (0.02). For comparison, results obtained with an early lactate buffer and data obtained earlier with formate buffers (Steinhardt *et al.*, 1958) are included. The concentra-

[5] Brill and Williams (1961), following Scheler *et al.* (1957), consider that both acetate and formate form ferrihemoglobin complexes. This conclusion depends on the existence of similar very small differences in extinction coefficients and in magnetic moments as compared with ferrihemoglobin in water at *p*H 6.4. However, at the *p*H of our own experiments, the Soret extinction in formate buffers is lower than in acetate buffers (Zaiser and Steinhardt, 1954) and the protein in formate buffers is much more stable than in acetate. Data obtained with ferrihemoglobin that has been oxidized by ferricyanide may be affected by the residual bound ferricyanide that does not dialyze at neutral *p*H.

tion of ligand used is over a hundred times the stoichiometric equivalent to protein iron.

The most obvious and remarkable feature of these data is that the velocity of denaturation at a particular *p*H is far less in the presence of certain ligands, notably cyanide and azide, than in their absence. It is also clear, since the slope relating the logarithm of the denaturation velocity to *p*H is nearly the same in each of the series (except in the presence of azide), that the presence of most of the ligands does not greatly affect the mechanism by which *p*H changes the stability (possibly ionization of certain "trigger groups"), but that the effect is either on the *p*H of their ionization range or on the velocity of the time-dependent reaction that follows the ionization. The slopes as drawn are for acetate alone (and one set of lactate buffers) 2.68, for fluoride 2.78, for formate 2.44, for cyanide 2.03, and for azides 3.36. The slope for thiocyanate, which does not appear to stabilize at all, is about the same as for acetate alone.

In attempting to understand these effects, it is well to start with equation (1), disregarding at first that each protein unit contains two hemes (which do not, however, show appreciable interaction). Then the fraction of protein which has bound ligands is related to *p*H as shown in equation (2).

$$\frac{[HbA]}{[HbA] + [Hb^+]} = \frac{[HA]}{[HA] + K_{eq}[H^+]} \qquad (2)$$

However, in the case of formate the fraction of protein binding formate ion acting as ligand does not depend on *p*H at all, but is constant at $0.02/(0.02 + K_{diss})$, since $(H^+) = K_{HCOOH}(HCOOH)/(HCOO^-)$ and $(HCOO^-)$ is constant at 0.02. The fact that all the slopes found (except for azide) are close to the slope given by formate furnishes a strong indication that the slopes are not determined by the effect of *p*H in dissociating ligand from its combination with protein. They are therefore presumably determined by the equilibrium involving the ionization of the "trigger groups" postulated in earlier papers.

Further support is lent to this tentative conclusion from a calculation (based on values of K_{eq} given in Table I) of the fraction of protein hemes bound by each ligand at a number of *p*H values. The quantities given in Table II do not necessarily give the fraction of protein molecules which are stabilized, since no evidence has been offered to distinguish between the stabilities of protein with one or two hemes bound to ligand.

It should be noted that in a *p*H interval in which the velocity changes some 15 to 30 times the fraction of free protein changes 3-fold or less, and with thiocyanate as well as formate it does not change at all.[6]

The fact that cyanide dissociates slowly from its fully combined form which prevails before mixing with acid, as well as the exceedingly small fractions of uncombined ferrihemoglobin in the azide experiments (Table II), permit the conclusion that the denaturation velocities measured with both azide and cyanide are essentially those characterizing the fully combined protein. With cyanide, doubling the ligand concentration (in lactate buffers) causes no further reduction in velocity.[7]

The extent of stabilization brought about by the more effective ligands, cyanide and azide, is not independent of *p*H, *i.e.*, the slopes in Figure 1 for these ligands differ from the slope for acetate alone and from one another. Thus azide at *p*H 3.8 reduces the denaturation velocity by a factor of 47 and cyanide reduces it by a factor of 19. At *p*H 3.3 the corresponding factors (extrapolating the curve for ace-

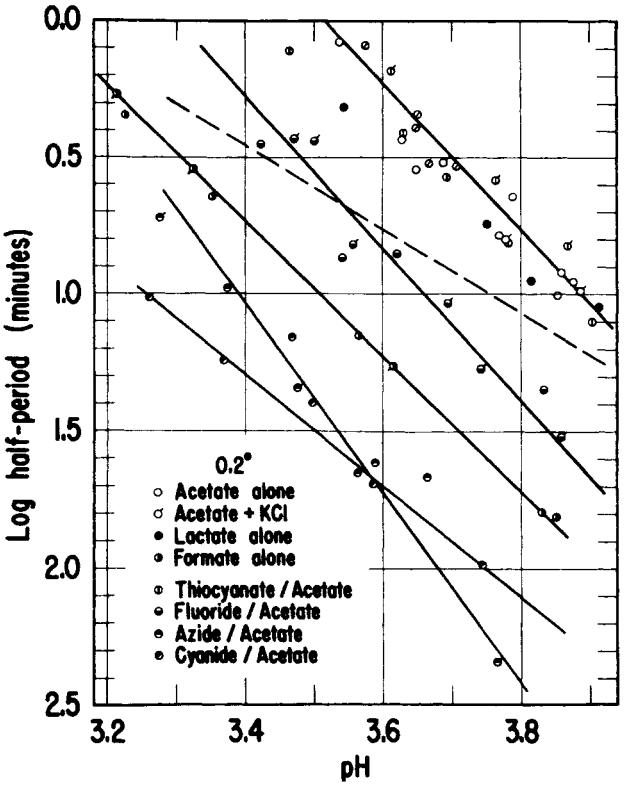

FIG. 1.—Logarithm of the half-period of denaturation (minutes) in acetate buffers of 0.02 ionic strength as a function of *p*H at 0.2°. Results obtained in "normal" lactate buffers (without ligand) are also shown. Results obtained with another sample of lactate alone are represented by a broken line. Tagged circles indicate experiments in which half the acetate was replaced by KCl. In the results with acetate alone, experiments with two different protein preparations are represented.

TABLE II

CALCULATED FRACTION OF PROTEIN HEMES NOT BOUND TO LIGAND

(Ligand concentration 0.004 M, 0.2°)

The values for fluoride are based on the data cited in footnotes *a* and *b* of Table I. They are probably high, since they apply to 25° rather than to 0.2°. The thiocyanate values are calculated from K_{diss}, since HSCN is a strong acid.

	*p*H 3.3	*p*H 3.8
Cyanide	0.092	0.031
Azide	0.0030	0.0010
Fluoride	0.31	0.12
Thiocyanate	0.344	0.344
Thiocyanate	0.174 (0.1 M)	0.174 (0.1 M)

[6] The cyanide data cannot be stressed in arriving at this conclusion, since dissociation of the cyanide complex at 0°, on lowering the *p*H, is so slow that it must be considered that *all* the cyanide results have been obtained with fully combined protein produced at the higher *p*H of the cyanide-containing protein solutions before the acid buffers were added. With azide, however, equilibrium is obtained extremely rapidly.

[7] With azide, doubling the concentration reduces the velocity about 20%. A part of this effect can be accounted for by the suppression of the contribution to the total velocity by the much greater (40-fold) denaturation rate of the free protein. Since the azide equilibrium is continuously reestablished during the course of the reaction, about 12% of the total velocity at *p*H 3.3 and 4% of the total at *p*H 3.8 is contributed by the denaturation of free protein.

tate) are 19.4 for azide and 49 for cyanide (their relative effectiveness is reversed). Fluoride stabilizes by a much smaller factor (4.5) that changes very little with *p*H, and thiocyanate does not stabilize. The stabilization brought about by formate (factors of 9.4 at *p*H 3.8 and 15.2 at *p*H 3.3) is not strictly comparable since the stabilization is brought about by higher concentrations of ligand (0.02). With fluoride it is plain that the protein is not fully bound, and the velocities found may represent combinations of velocities for both free and bound proteins.

Such combinations of velocities may be investigated with the strongly bound anion, azide, by reducing its concentration. At concentrations of ligand which are much lower than those represented in the data of Figure 1, and with such a ligand as azide which re-equilibrates very rapidly after mixing with buffer, the protein is not predominantly in the bound form. The denaturation velocities observed should then, like those for fluoride, represent the addition of velocities for the denaturation of both free and bound protein respectively—the *p*H slope becomes steeper and all the velocities are higher than those shown in Figure 1.

The effect of varying the concentration of azide has been investigated in detail at a single *p*H (3.78) at 0.2°. Table III gives the effect of a number of concentrations on the velocity of denaturation (expressed as half-periods) in a lactate buffer. All of the kinetics appear first-order (except for an anomaly noted in a later section) for at least one half-period and, at the higher ligand concentrations, for considerably longer.

It is apparent from the last two columns of Table III that small amounts of azide (up to about stoichiometric equivalence with the heme present) have a much larger effect on the velocity than can be explained on the basis of equation (2), even if it is assumed that only one heme per molecule need combine with ligand to account for a large gain in resistance to acid denaturation. Thus, the combination with azide of less than one-fifth the hemes present (last entry in last column of Table III) suffices to double the half-period. A more elaborate form of equation (2), derived to predict the velocity in mixtures of bound and free protein, fails to describe the data.[8]

A Peculiarity of Azide.—When ferrihemoglobin solutions containing azide ion are mixed with either acetate or lactate buffers at 0.2°, a phenomenon occurs which is not observed with other ligands. During an initial period which may last as long as 10 minutes (at *p*H above 3.9) little if any change in light absorption at 419 mμ occurs. Thereafter normal first-order kinetics are observed. This initial lag or induction period is greatly reduced at higher temperatures and lower *p*H values and therefore may not be observed at all. Its occurrence does not depend on whether azide is added to the protein before mixing with the acid buffer or whether it is added with the buffer itself; thus, the lag cannot be due to a time-dependent dissociation of azide from the protein (the latter is known to be fast). It may be formally explained by postulating the successive reactions shown in equation (3), in which

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \qquad (3)$$

(native protein) (intermediate) (denatured protein)

A and *B* have identical absorptions, and k_1 and k_2 have suitable relative magnitudes and are both functions of *p*H. This hypothesis has not been subjected to any critical test. In Figure 1 and the accompanying text, the reaction velocities referred to are those of the first-order process that follows the lag.

Effects of Ligands at 24.8°.—Conclusions concerning effects of ligands must be reserved until the effects obtained at more than a single temperature are examined. Not only are the affinities of the various ligands temperature dependent, but the products of denaturation at 0° with at least one ligand, formate, differ from the products at room temperature (Beychok and Steinhardt, 1960).

Figure 2 resembles Figure 1 in giving the results obtained in acetate buffers, but includes a few results with certain of the ligands in lactate buffers, obtained with the same protein preparation. The results shown are qualitatively similar to those shown in Figure 1 (the average slope is very slightly higher, but the sequence of the magnitude of the slopes is, within experimental error, the same). The results, however, differ quantitatively in a number of significant respects:

(*a*) As would be expected, all the reaction rates are greater at 24.8° than at 0.2°, but the factor by which they are increased is higher with the stabilizing ligands than with acetate buffers alone or with the systems that resemble acetate.

(*b*) As a result of (*a*), the stabilization factors at a given *p*H are all smaller at the higher temperature, and fluoride can hardly be said to stabilize at all. These factors are summarized in Table IV.

The calculated fractions of unbound protein are higher at 24.8° than those given in Table II (at *p*H 3.7, 0.203 for cyanide, 0.011 for azide, 0.65 for thiocyanate—the value given in Table II for fluoride was

TABLE III

VELOCITY OF DENATURATION OF FERRIHEMOGLOBIN IN LACTATE BUFFERS AS AFFECTED BY AZIDE CONCENTRATION, AT *p*H 3.78 AT 0.2° AND 0.02 IONIC STRENGTH

(Initial $[Hb^+] = 3.6 \times 10^{-5}$ M [iron equivalent])

Total Azide (M)	Half-Period (min.)	$\frac{[Hb^+]}{[HbN_3]}$ Calcd. From Velocities	$\frac{[Hb^+]}{[HbN_3]}$ Calcd. From Optical Density at Time Zero
8×10^{-3}	225	—	Assumed 0.0001
4×10^{-4}	127	0.0472	*ca.* 0.03
7.5×10^{-5}	93	0.0759	0.036
4.0×10^{-5}	73.5	0.1370	0.137
2.0×10^{-5}	59.4	0.195	0.98
7.5×10^{-6}	24.2	0.943	5.22
None	12.4	—	—

TABLE IV

STABILIZATION FACTORS AT *p*H 3.8

Ligand (0.004 M)	Temperature 0.2°	Temperature 24.8°
Thiocyanate	*ca.* 1.0	*ca.* 1.0
Fluoride	4.5	1.2
Formate (0.02 M)	9.4	7.1 (*p*H 3.7)
Cyanide	19	10.5
Azide	47	17.3

[8] The values of $[Hb^+]/[HbN_3]$ given in the last column of Table III are not far from those found in equilibrium experiments at higher *p*H, at which denaturation did not occur. They yield an average value of K_{eq} at 0° of 0.0071 at *p*H 3.75 instead of the values of 0.024 obtained at *p*H 4.29 and 0.048 at *p*H 5.31, and suggest the possibility of a small charge effect on K_{eq} of the type to be expected on the basis of the prevailing electrostatic theory of macromolecular ions. There is no clear evidence of such a charge effect with cyanide, but a similar charge effect has been reported in an unpublished thesis by Elliott L. Albers (1941), made available to us by C. D. Coryell.

actually for 25°). Note that thiocyanate, at all *p*H values, binds less than half the hemes present.

The results with lactic acid in Figure 2 are noteworthy in three respects:

(*a*) Lactate buffers appear to stabilize appreciably relative to acetate (the stabilization factor is 1.8 at *p*H 3.8).

(*b*) This extra stabilization persists in the presence of the strong ligands, cyanide and azide; additional stabilization factors of about 1.4 and 1.9, respectively, are found at *p*H 3.8. This additive stabilization is not found when the strong ligand, azide, is added to formate, which stabilizes much more than lactate. The velocity in formate when azide is present is actually *greater* than with azide in lactate.

(*c*) The data shown are in good agreement with those obtained several years earlier with other hemoglobin preparations and other batches of lactic acid represented by the broken lines.

As at 0.2°, at concentrations of ligands below those represented in Figure 2 or Table IV, or at high *p*H, the protein may not be predominantly in the bound form. With such concentrations the velocities of denaturation should represent a combination of velocities for both free and combined protein. Thus at these lower concentrations the log half-period–*p*H slope is steeper and all the velocities are higher than those shown in Figure 2. The effect of varying the cyanide concentration has been investigated in detail at 24.8° at a single *p*H (3.59) in lactate buffers, because at this temperature the velocity of dissociation of the cyanide complex, slow at 0.2° (see Experimental), may be fast enough for the rates found to be readily related to the concentrations of bound protein calculated from the equilibrium constant. Thus at concentrations of HCN barely higher than the equivalent concentration of the protein (3.6×10^{-5} M), the kinetics remain first-order, although the ligand dissociation constant (see Table I) indicates that almost half the protein is uncombined with ligand. The half-periods for a number of concentrations are shown in Table V. At lower concentrations than those shown in the table, the kinetics are no longer first-order, but even the fast initial phase (half-period about 2.65 minutes) is slower than in the total absence of cyanide. At 1.5×10^{-5} M, the fast initial phase accounts for about 80% of the total reaction.

TABLE V

HALF-PERIODS OF DENATURATION OF FERRIHEMOGLOBIN AT 24.8° AT *p*H 3.59 IN LACTATE BUFFERS (IONIC STRENGTH 0.02) AS AFFECTED BY HCN ADDED

Total Cyanide (M)	Half-Period (min.)
0.002	11.9
7.5×10^{-4}	5.8
7.5×10^{-5}	3.22
3.75×10^{-5}	2.65[a]
None	1.1

[a] A slight break appears after about 50% reduction in optical density. The remainder of the reaction is characterized by a half-period of 3.22 minutes.

Attempts to interpret these results quantitatively must take into account (1) differences in denaturation velocity for free and bound forms; (2) rate of dissociation of protein bound to cyanide to replace the free protein which is denatured more rapidly—and consequent changes in concentration of unbound ligand; (3) uncertainties concerning the equilibria between denatured protein and ligand; and (4) the possibility that the binding of cyanide by a single heme in each two-heme unit may produce stabilization to an unknown extent.

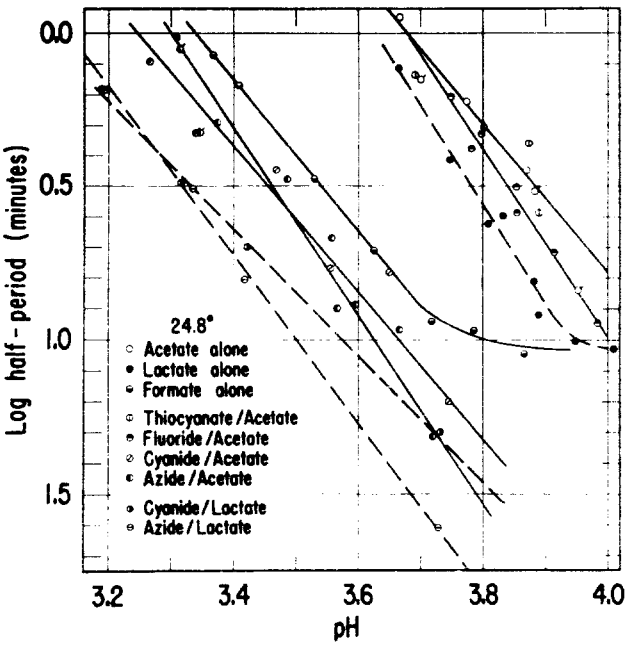

FIG. 2.—Logarithm of the half-period of denaturation (minutes) in acetate buffers of 0.02 ionic strength at 24.8°. Results obtained with lactate buffers alone are also shown. The results obtained with lactate buffers with certain ligands are indicated by broken lines.

The first item involves an ascertainable quantity; the second involves uncertainty only with respect to the rate at which equilibrium is restored. Only in two special cases has it been possible to obtain an easily tested mathematical model for the dependence of the combined velocity on cyanide concentration. These are, respectively, for the cases: (*A*) equilibrium with ligand restored instantaneously as protein denatures; (*B*) equilibrium concentrations present at outset of experiment but not restored at all.

Application of the kinetic equations derived for both of these limiting cases has failed to yield good equilibrium constants. In general (with both cases) the velocities obtained with the lowest concentrations indicate a smaller K_{eq} than those obtained with the higher concentrations or by direct measurement by spectrophotometric means (see Experimental). Similar results with azide at 0.2° have already been discussed. The assumption that stabilization can occur by combination with ligand by a single heme, in each two-heme protein unit, improves the results, but the calculated K_{eq} still decreases as $[CN^-]$ increases.

The Effect of Temperature on Denaturation Velocity and on Stabilization.—Zaiser and Steinhardt (1954) and Steinhardt *et al.* (1958) have shown that with formate buffers there is only a small increase in velocity between 0° and 15.5°, but a much larger one in the smaller temperature interval 15.5°–25°. On the basis of this finding it was postulated that the reaction products were different above and below 15.5°. This difference was subsequently demonstrated directly by Beychok and Steinhardt (1960). Determination of temperature effects in ferrihemoglobin denaturation must therefore be based on more than two widely spaced temperatures.

In the case of formate the effects observed were practically independent of *p*H because the log *k* *vs.* *p*H

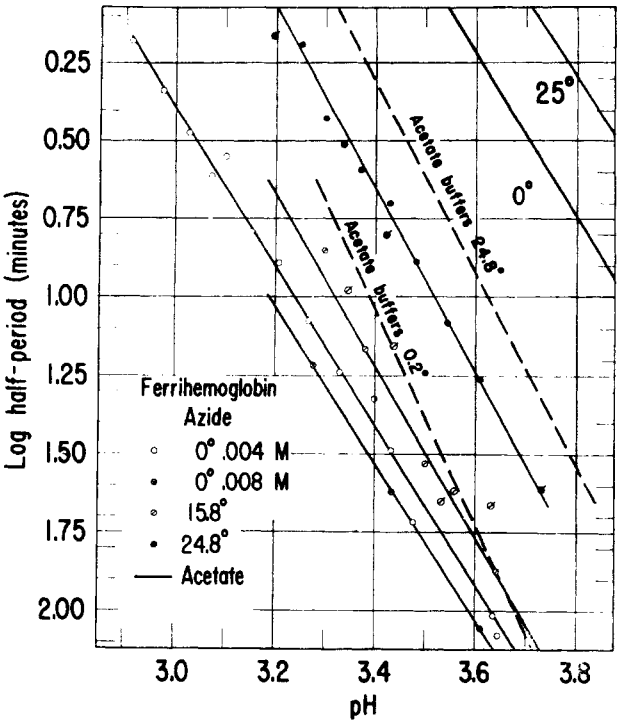

FIG. 3.—Logarithm of the half-period of denaturation (minutes) of cyanoferrihemoglobin in lactate buffers at three temperatures. Results obtained with acetate buffers with and without added ligand at the same temperature are included for comparison. Tagged circles represent data obtained with two later samples of lactic acid.

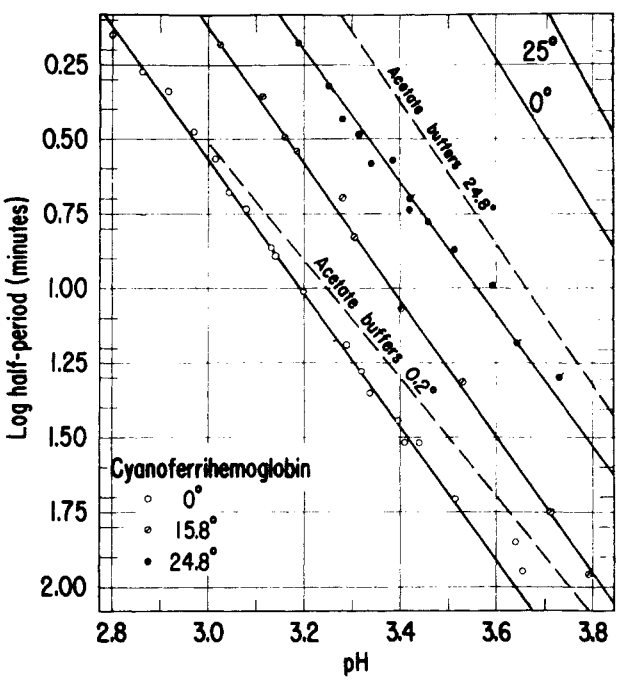

FIG. 4.—Logarithm of the half-period of denaturation (minutes) of ferrihemoglobin azide in lactate buffers at three temperatures. Results obtained with acetate buffers with and without added ligand at the same temperatures are included for comparison. Filled circles represent data obtained with two later samples of lactic acid.

slope did not change with temperature. In the present work with ligands this is not always true, and the temperature effects vary with *p*H. For this reason temperature effects are reported here as the change in log velocity per reciprocal degree

$$\left(\frac{T_1T_2}{T_2 - T_1} \log \frac{k_2}{k_1}\right)$$

rather than as activation energies, since it is not obvious how to interpret an activation energy that depends strongly on *p*H. The ordinates in Figures 5 and 6, which summarize some of the effects of temperature, may be converted into "energies of activation" by multiplying them by 4.606.

The results shown depend critically on the accuracy with which the log half-period *vs.* *p*H slopes (Figs. 1 and 2) represent the experimental data. Our data for cyanide and azide in lactate buffers, (Figs. 3 and 4) are more suitable for these comparisons than are the more meager data for acetate shown in Figures 1 and 2, and the additional data for acetate which are available for 15.8°. The data in Figures 3 and 4 differ principally from those in Figures 1 and 2 by showing additional stabilization due to lactate and, in the case of azide, in having a slightly lower dependence on *p*H.

Figure 5 represents the effects of temperature for azide and cyanide in two temperature intervals and compares them with the earlier results with formate and the present results with lactate and acetate alone. It shows clearly that azide and cyanide in lactate are characterized by the higher temperature coefficient in the higher temperature interval which characterized the results with formate. The temperature effects with the tightly bound ligands are either as large as, or larger than, those with formate in both temperature intervals.[9]

The results in acetate buffers (Fig. 6) are less reliable because the log half-period *p*H slopes (Figs. 1 and 2) are less well defined, but they suffice to show that the temperature effects in the two temperature intervals are not sharply distinguishable as they were with lactate and formate. It is possible, therefore, that in acetate buffers there is no change in denaturation product between 0° and 25°. However, it must be remembered that the difference in product has been demonstrated with HCl as well as with formic acid. Measurements of the intrinsic viscosity of the protein denatured in acetate buffers should serve to confirm or reject this possibility (Beychok and Steinhardt, 1960).

The unique case of cyanide requires further comment. At 0.2°, and possibly at 15.8°, the slow dissociation of the preformed cyanide complex resulted in a fraction of bound protein close to unity and independent of *p*H. At 25° the dissociation should be fast enough to result in a free fraction close to that calculated from K_{eq} (0.21 at *p*H 3.7; 0.39 at *p*H 3.3). A large part of the rate observed at 24.8° is therefore that of the free protein. This should be almost twice as great at *p*H 3.3 as at *p*H 3.7 and accounts, in part, for the higher slope observed in acetate buffers at 25° (about 0.7 of the 2.4 total slope—this is about the difference in slope observed between 0.2° and 24.8°). This increase in slope at 24.8° is in large part responsible for the strong negative slope in the cyanide data in Figure 6 for the higher temperature interval.

With azide, the free fraction of protein remains too low to have a great effect on the observed velocity even at 24.8° and low *p*H. *The relatively large temperature effect shown with azide must represent an effect on the denaturation of the hemoglobin azide compound itself.* The same conclusion applies to formate because, as

[9] The reversal of the relative size of the temperature effects for azide and cyanide in the two ranges of temperature may indicate that azide has a lower "critical temperature" than 15° and cyanide a higher one, and that both have somewhat higher energies of activation than formate in both ranges.

explained earlier, the composition of the buffers results in a ratio of bound to total protein which is independent of *p*H. It is significant, therefore, that the moderately large effect of temperature (in the higher temperature range) which can only be an effect on the rates of denaturation of both free and bound forms, is practically independent of *p*H. With thiocyanate, also, the bound fraction does not depend on *p*H, but the very slight effect of this ligand on the denaturation velocity does not permit conclusions concerning the contribution of free and bound forms to the temperature effect to be drawn with certainty.

Nitrosoferrihemoglobin.—In order to determine the effect of an uncharged ligand, efforts were made to prepare and study the acid denaturation of nitrosoferrihemoglobin by adding nitric oxide to ferrihemoglobin solutions. Although a nitroso derivative was readily formed, as attested by a change in spectrum, these experiments failed to produce valid reproducible denaturation-rate data. Even after elaborate precautions were taken to prevent the formation of NO_2 and its introduction into the solutions, enough was formed to cause a decrease in *p*H in every case. In addition, a variably rapid autoreduction to nitrosoferrohemoglobin (Keilin and Hartree, 1937) also occurred before denaturation was complete. Nevertheless, observation of initial denaturation rates indicated that there is little if any difference in denaturation velocity between ferrihemoglobin and its NO complex. The results with nitrosoferrohemoglobin will be reported in another paper which deals with the ferrohemoglobins.

Ineffective Substances.—In an effort to determine the effect of other *uncharged* substances the effect on the ferrohemoglobin spectrum has been observed in the presence of the following substances in concentrations up to 1 M: nitrosophenol, ethyl isocyanate, ethyl thiocyanate, acetamide, methanol, and pyrrole. Ethylene diamine and sulfate ion were also tried. None of these substances changes either the light absorption or the velocity of denaturation in lactate buffers. Methyl cyanide has a discernible effect on the spectrum at moderately high concentrations but has no stabilizing effect. Hydrogen peroxide has an effect on the spectrum at low concentrations (0.01 M) but produces marked and complex color changes as soon as acid is added.

Effects of Ligands on Regeneration Velocity.—The effect of ligands on the velocity of regeneration of denatured protein has been studied only in the case of azide and formate. Two methods have been used: (*a*) determination of the equilibrium (*i.e.*, the fraction denatured after very long times, when no further change is occurring); (*b*) regeneration of native protein by raising the *p*H.

Extensive experiments of both these kinds have been reported for ferrihemoglobin in formate buffers (Steinhardt *et al.*, 1958), and the identity of the original and regenerated products has been established.

The fractions of ferrihemoglobin azide denatured at equilibrium at four *p*H values at 0.2° are given in Table VI.

The *p*H dependence of the equilibria can be expressed by a line relating log [native protein]/[denatured protein] to *p*H. This line has a slope of about 5 (identical with the slope for ferrihemoglobin in formate or acetate uncorrected, as here, for irreversible loss). The *p*H of the midpoint of the equilibrium (3.99) is 0.14 *p*H units more acid than the midpoint for nonspecific buffers (4.13) reported by Zaiser and Steinhardt (1954).

The regeneration experiments were carried out on protein denatured in the presence of azide (0.004 M)

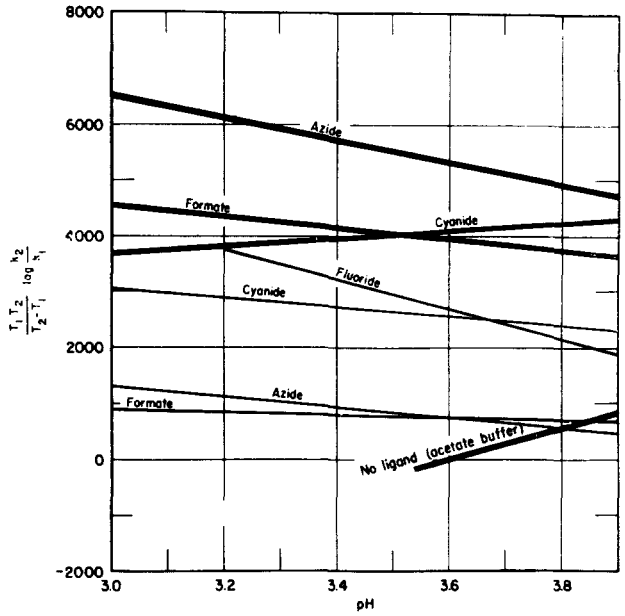

FIG. 5.—Increment in logarithm of rate of denaturation in lactate buffers (and in formate with no added ligand) per reciprocal degree. Results are shown for two temperature intervals. Heavy lines represent the higher interval. The results with formate correspond to the intervals 0.2°–15.5° and 15.5°–25.0°

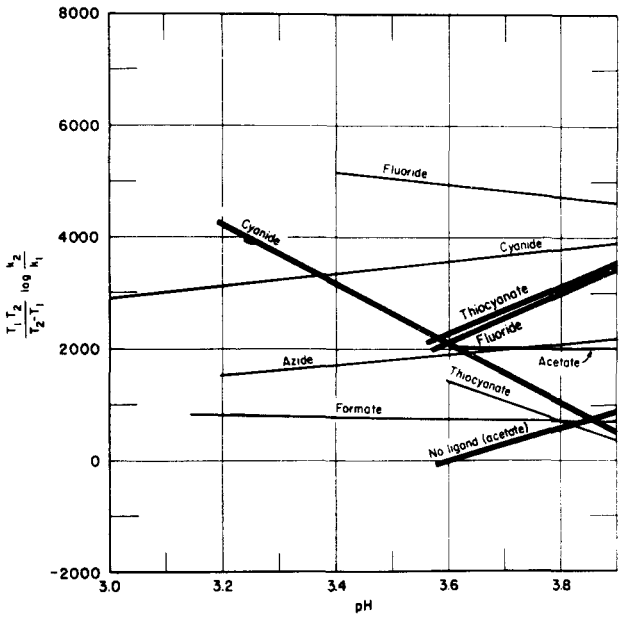

FIG. 6.—Increment in logarithm of rate of denaturation per reciprocal degree in acetate buffers (and in formate with no added ligand). Results are shown for two temperature intervals. Heavy lines represent the higher interval. The results with formate correspond to 0.2°–15.5° and 15.5°–25.0°

at *p*H 3.337 at 0° for eight half-periods. When the *p*H was raised to 5.418, 88% of the protein was regenerated in 47 hours. The half-period was approximately 10 hours, about 20 times as long as when *formate* buffers are used (approximately 30 minutes). At *p*H 4.643 the recovery in 47 hours was 75%, and the half-period was roughly the same as at the higher *p*H.

It appears, therefore, that azide retards not only the rate of denaturation but the rate of regeneration as well, although to a lesser degree. This conclusion is borne out by the equilibrium data cited above, in which

TABLE VI
FRACTION OF PROTEIN DENATURED AT EQUILIBRIUM WITH AZIDE PRESENT (0.004 M)

*p*H	Fraction Denatured in 120 Hours
3.94	0.633
4.10	0.231
4.23	0.058
4.43	0.000

the *p*H of half-denaturation, approximately 3.99, does not differ as much from the value for ferrihemoglobin in acetate or lactate alone, 4.13, as the large effect on denaturation alone, were there no effect on regeneration, would require.

Similar conclusions may be drawn for formate. Previously reported equilibrium experiments with formate (Zaiser and Steinhardt, 1954; Steinhardt *et al.*, 1958) show that there is a *p*H shift of the midpoint (half-denaturation) of 0.26 units at 25° relative to acetic and several other acids. This shift is almost twice as large as with azide. However, with azide, the shift in equilibrium *p*H values is smaller than the *p*H shift for velocity (about 0.35 units with formate at 25°). Thus, as with azide, regeneration is retarded, but less than is denaturation.

Effects of High Salt Concentration.- Only a few measurements have been made with high salt concentrations, but the results show a spectacular effect of a ligand in almost abolishing the great effect of salt concentration on denaturation rate. Raising the ionic strength (KCl) from 0.02 to 0.3 in the absence of ligands (using nonspecific buffers such as acetate) increases the rate of denaturation at least 30-fold (the *p*H at which a given half-period is obtained is raised about 0.50 units). When cyanide (0.01 M) is present, the rates at 0.3 ionic strength *are only about five times as great* as at 0.02 strength.

Large effects of salt on reaction rate (very characteristic of protein denaturations in general) are often associated with a multiply charged state of one of the reactants when the other reactant is hydrogen ion. The very great reduction of this effect by cyanide suggests reduction by the ligand anion of the large charge.

DISCUSSION AND CONCLUSION

It has been reported elsewhere (Steinhardt and Beychok, 1960) and will be reported in greater detail later that *ferrohemoglobins* are all more resistant to acid denaturation than is ferrihemoglobin. Uncombined ferrohemoglobin and ferrihemoglobin are both high-spin iron complexes, but the latter is distinguished from the former by the presence of a positive charge in the vicinity of the heme iron.

All the ligands used which have been effective in protecting ferrihemoglobin against denaturation by acid bear negative charges. The most highly effective of those studied, cyanide and azide, reduce the magnetic moment of ferrihemoglobin to about one-fifth of its value (Coryell *et al.*, 1937). Thus the 3*d*-orbital electrons of the iron atom become involved in low-spin complex formation; the bonds between iron and the four pyrrole nitrogens *plus the nitrogen on the protein-imidazole to which the heme is bound* become essentially covalent rather than ionic-type bonds. A ligand which stabilizes to a lesser extent, fluoride, and one which does not stabilize, thiocyanate, reduce the magnetic moment little if at all, and thus cannot be expected to affect the nature of the heme-protein bond.

Cyanoferrihemoglobin and its azide analog are approximately as stable as the low-spin complexes of ferrohemoglobin such as HbCO. The latter, however, also carry no positive charge on the heme; it is worth distinguishing between the formation of low-spin complexes *per se* and their possible effect on removing the positive charge as the diagnostic sign of whatever mechanism confers the extra stability on these stable complexes. Before attempting to establish such a distinction, it is necessary to show that the effect of the ligands cannot be accounted for by an effect on the pK of the trigger groups whose association with H^+ instantaneously initiates the time-dependent reaction, the velocity of which is affected by the ligands. It will then be desirable to offer a model which can account for the effect either of the type of the iron-protein bond or, conversely, of the presence or absence of a positive charge on the iron on the ease of rupture by H^+ of the native structure. A later step must be the determination of whether all covalently linked ligands stabilize to the same extent or whether their stabilizing effect is graded with the extent of the electrical polarity of the bonds they form with iron. In the latter determination it will be necessary to distinguish between the different *extents* of combinations (determined by K_{eq}) which tend to accompany the differences in polarity themselves. We will find that a comparison of cyanide and azide is well suited to this purpose because of the 10,000-fold difference in the strengths of their conjugate acids and the much smaller (30%) difference in their magnetic moments.

The possibility that ligands have an effect *solely* on the trigger groups can be excluded by the demonstration in a preceding section that the effect of temperature on denaturation velocity is larger with ferrihemoglobin combined with such a ligand as azide than with ferrihemoglobin itself. It may be assumed by analogy with all known strong acids having $pK < 4$ that the effect of temperature on the trigger groups should be negligibly small. The effect of temperature is therefore on the velocity of the time-dependent reaction; the fact that it is larger for protein bound with ligand indicates either that rapidly denaturing free protein is being formed at the higher temperatures in quantities sufficient to affect materially the velocity (unlikely in the case of azide) or, more plausibly, that the activation energy for the denaturation of HbN_3 is itself higher. Both alternatives imply an effect of ligand on the velocity of denaturation of the reactive complex itself, formed by the ionization reactions of the trigger groups. An additional small effect on the trigger groups is not excluded by the reasoning just given. However, it is rendered very unlikely by the fact that ligands reduce the velocity of regeneration, which has no *p*H dependence, as well as the velocity of denaturation, which has.

It would be of great interest to establish whether there are differences in the amounts of stabilization brought about by the binding of anions which result in complexes of widely different spin-coupling when they are compared on the basis of equal fractions bound. For this purpose it is instructive to examine the results in acetate buffers obtained with cyanide, azide, fluoride, and thiocyanate (Fig. 1). Cyanide and azide, which are most effective, stabilize approximately equally well (reduce the velocity 20 to 50 times, depending on the *p*H) although their affinities (Table I) differ by a factor of about 10,000. In both cases the compounds formed are low-spin complexes, *i.e.*, the bonds formed have a high degree of covalent character. For reasons already given, these reduced velocities at 0° may be taken as representing predominantly the velocities of denaturation of the two ligand complexes. In the case

of thiocyanate, which is lower in affinity by an additional factor of some 20,000 and which forms a highly polar ("ionic") bond, there is no detectable stabilization. In our experiments, although a third of the protein molecules may be free of ligand, a change in velocity by a factor of less than 2, caused by stabilization of the two-thirds which are bound, could be detected with certainty.

The case of fluoride is more uncertain at 0° than at 25°; at the latter temperature it behaves like thiocyanate. Less of the protein is free than with thiocyanate, but little if any stabilization results. At 0°, however, stabilization (4 to 5 fold) is found. Unfortunately the free and bound fractions cannot be calculated with certainty at this temperature, so the amount of stabilization characteristic of the protein-fluoride complex itself can only be given within wide limits of uncertainty. If one-tenth of the protein is free at 0° (and this seems a reasonable upper limit for *p*H values above 3.5) the protein ligand compound is calculated to be stabilized relative to ferrihemoglobin by a factor of less than seven. Only if more than 20% of the protein is free do the calculated stabilization factors lie in the range characteristic of cyanide and azide.

The evidence suggests therefore that negatively charged ligands which form low-spin ("covalent") complexes with ferrihemoglobin may all be approximately equally stable (and as stable as such ferrohemoglobin compounds as HbCO). The fluoride data at 0° suggest that *at this temperature* the iron-fluoride bond is mixed in character (as is the case with the iron-hydroxyl bond in HbOH) and confers an intermediate degree of stability. Unfortunately no measurements of the magnetic moment of HbF have been made at low temperature. Such measurements would furnish a critical test of this hypothesis.

The foregoing discussion leads to one of two alternative possibilities: (*a*) the greater instability of ferrihemoglobin as compared to HbCO and HbCN may be due to the ionic character in the former of the bond between the heme iron and a protein imidazole[10,11]; or (*b*) the greater instability may be associated in some as yet undefined manner with the presence of a positive charge on the iron of ferrihemoglobin when it is not bound covalently to negative ligands.

Two experimental observations tend to favor the second of these alternatives because they are inconsistent with the first. These are (*a*) indications that a neutral ligand (NO), which presumably forms a low-spin complex,[12] fails to stabilize; and (*b*) preliminary experiments which appear to establish that ferrohemoglobin ("reduced hemoglobin"), which has highly polar bonds between the heme iron and its surrounding five nitrogens (including the bond to protein), is very stable to acid (Steinhardt and Beychok, 1960).[13] If confirmed, this observation would appear to show that a covalent iron-protein bond is not required for high resistance to acid, although globin is known to be less stable than hemoglobin (Rossi-Fanelli *et al.*, 1958).

The extension of some ideas of Wang (1961) permits us to hypothesize a mechanism for the instability of hemoglobin compounds which carry a positively charged iron atom. Wang *et al.* (1958) and Wang (1961) argued, from the nearly million-fold difference in affinity for cyanide between ferrohemoglobin and free heme, that the heme in ferrohemoglobin is situated in a hydrophobic region of the molecule. There is no correspondingly large difference between the affinity for cyanide of ferrihemoglobin and hematin. We may postulate that the charged iron atom of ferrihemoglobin is drawn (together with the attached porphyrin) toward the outer regions of the molecule, where it is exposed to the high dielectric solvent medium. This postulated displacement, or merely increased exposure, corresponds to a difference in conformation between oxidized and reduced hemoglobin. When cyanide is bound the formation of a covalent bond removes the positive charge[14] and may thus permit the iron (and heme) to return to the less completely exposed position it occupies in the reduced form. The difference in stability between ferrihemoglobin and its cyanide complex may then depend on consequences of this difference in the position of the hematin, when covalently or ionically bound, rather than on the greater strength of the protein-heme bond in the protein-ligand compounds. The more infolded heme stabilizes the native configuration of the molecule by its position relative to particular parts of the polypeptide chains, as Perutz and others have suggested.

According to this view the stabilizing effect of strongly bound ligands would be to remove the charge and reduce the work required to return the iron from an environment of higher dielectric constant to the less exposed low-dielectric environment. The failure of thiocyanate to stabilize may then be a consequence of the high electric asymmetry of its bond with the iron, which tends to keep the heme in the region of higher dielectric constant. The intermediate degree of stabilization conferred by relatively high concentrations of formate cannot be explained without more knowledge of the nature of its bonding to heme, but the effect of formate at 0° finds a parallel in the behavior of fluoride at 0°.

The distinction between this view and the hypothesis that strong covalent iron-imidazole bonds are responsible for stabilization depends critically on the observation that nitrosoferrihemoglobin is not stabilized relative to ferrihemoglobin and that reduced hemoglobin is, in spite of ionic-type bonds of iron to nitrogen, very stable.[13] It is therefore important to attempt to obtain additional data to confirm these observations. Such additional data will be reported later, together with other information on ferrohemoglobin compounds.

Recent experiments of Rossi-Fanelli *et al.* (1959) show that human protoporphyrin-globin (reconstituted

[10] Although the binding of cyanide and azide changes the character of the iron-porphyrin bonds from high to low spin, there is no unambiguous evidence that the bond to protein, *trans* to the added ligand, is also strengthened (Dwyer, 1961). However, there is ample evidence that a ligand which forms low-spin complexes with heme *can* increase the attraction of the ligand field for another ligand (Nakahara and Wang, 1958).

[11] O'Hagan (1959a,b) has advanced several arguments in favor of a heme link to protein carboxylic side-chains rather than to imidazole. These rest, in part, on "*p*H stability" curves of horse ferrihemoglobin, from which a *pK* of 4.7 for the ionization of these groups is inferred. Actually the equilibrium relation between native and denatured protein is far too complex to support this simple inference. Steinhardt and Zaiser (1955) and Beychok and Steinhardt (1959) have presented evidence that the *pK* of the controlling ionizations is much lower than the value inferred by O'Hagan.

[12] The evidence that Hb$^+$NO is a low spin complex has been summarized by Sancier *et al.* (1962).

[13] The apparent results of these experiments are contrary to prevailing views. See, for example, Keilin and Hartree (1952).

[14] It has been shown by Itano and Robinson (1958) that, although ferrihemoglobin has a different electrokinetic mobility from that of cyanoferrihemoglobin, cyanoferrihemoglobin and carboxyhemoglobin migrate with the same velocity.

iron-free hemoglobin) is qualitatively much more stable than globin. Protoporphyrin as well as heme, therefore, appears to provide at least part of the stability of the natural conjugated protein, possibly because of an interior location similar to that of heme, such as has been postulated to account for the results of the experiments reported here. Should this be the case, the presence of an iron-protein bond, essential for combination with oxygen, may not be essential for a high degree of stability against acid denaturation of the protein. These experiments, as well as those reported here, render it unlikely that a "crevice" structure involving more than one bond between iron and protein is responsible for the stabilization. The "crevice" structure for heme proteins has also been criticized by others (see Nichols, 1962). Measurements of the rates of denaturation of globin (which must lack either the iron-imidazole bond or the physical presence of protoporphyrin as sources of stability) should therefore be highly relevant and will be undertaken.

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REFERENCES

Beychok, S., and Steinhardt, J. (1959), *J. Am. Chem. Soc. 81*, 5679.
Beychok, S., and Steinhardt, J. (1960), *J. Am. Chem. Soc. 82*, 2756.
Brill, A. S., and Williams, R. J. P. (1961), *Biochem. J. 73*, 246.
Coryell, C. D. (1938), *J. Phys. Chem. 43*, 841.
Coryell, C. D., and Stitt, F. (1940), *J. Am. Chem. Soc. 62*, 2942.
Coryell, C. D., Stitt, F., and Pauling, L. (1937), *J. Am. Chem. Soc. 59*, 633.
Dwyer, F. P. (1961), *in* Haematin Enzymes, vol. I, Falk, J. E., Lemberg, R., and Morton, R. K., editors, London, Pergamon Press, p. 19.
Gralen, N. (1939), *Biochem. J. 33*, 1907.
Gutter, F. J., Sober, H. A., and Peterson, E. A. (1957), *Arch. Biochem. Biophys. 71*, 342.
Havemann, R. (1943), *Biochem. Ztschr. 316*, 138.
Itano, H. A., and Robinson, E. (1958), *Biochim. Biophys. Acta 29*, 545.
Keilin, D., and Hartree, E. F. (1937), *Nature 139*, 548.
Keilin, D., and Hartree, E. F. (1952), *Nature 170*, 161.
Keilin, J. (1960), *Nature 187*, 365.
Lipmann, F. (1929), *Biochem. Ztschr. 206*, 171.
Nakahara, A., and Wang, J. H. J. (1958), *J. Am. Chem. Soc. 80*, 656.
Nichols, P. (1962), *Biochim. et Biophys. Acta 60*, 217.
O'Hagan, J. E. (1959a), *Nature 183*, 393; (1959b), *Nature 184*, 1652.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochim. Biophys. Acta 30*, 608.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1959), *Biochim. Biophys. Acta 35*, 93.
Sancier, K. M., Freeman, G., and Mills, J. S. (1962), *Science 137*, 752.
Scheler, W., and Jung, F. (1954), *Biochem. Ztschr. 325*, 515.
Scheler, W., Schoffa, G., and Jung, F. (1957), *Biochem. Ztschr. 329*, 232.
Steinhardt, J., and Beychok, S. (1960), Oral presentation at the meeting of American Chemical Society in April, 1960, at Chicago.
Steinhardt, J., Ona, R., and Beychok, S. (1962), *Biochemistry 1*, 29.
Steinhardt, J., and Zaiser, E. M. (1955), *Advan. Protein Chem. 10*, 151.
Steinhardt, J., Zaiser, E. M., and Beychok, S. (1958), *J. Am. Chem. Soc. 80*, 4634.
Wang, J. H. (1961), *in* Haematin Enzymes, Vol. I, Falk, J. E., Lemberg, R., and Morton, R. K., editors, London, Pergamon Press, p. 98.
Wang, J. H., Nakahara, A., and Fleischer, E. B. (1958), *J. Am. Chem. Soc. 80*, 1109.
Wyman, J., Jr. (1951), *Advan. Protein Chem. 6*, 407.
Zaiser, E. M., and Steinhardt, J. (1951), *J. Am. Chem. Soc. 73*, 5568.
Zaiser, E. M., and Steinhardt, J. (1954), *J. Am. Chem. Soc. 76*, 1788.

Interaction Between Phosvitin and Iron and Its Effect on a Rearrangement of Phosvitin Structure*

GEORGE TABORSKY

From the Department of Biochemistry, Yale University, New Haven, Connecticut
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Earlier work showed that some amino groups of phosvitin become masked as a result of a rearrangement at alkaline *p*H. This paper reports that metal-free phosvitin does not rearrange and that the reaction recurs upon the addition of iron to the protein. Phosvitin promotes a rapid oxidation of ferrous ion, and it binds ferric ion strongly and extensively. The earlier hypothesis that phosphoryl groups may be shifted from hydroxyl groups to amino groups of the protein is amended by the suggestion that metals, such as ferric ion, may play a catalytic or stabilizing role in the transfer process. An alternative hypothesis that a direct substitution of some ligand of iron by amino groups of the protein is responsible for the observations made on alkaline solutions of the protein is rejected on the basis of a consideration of the probable nature and rate of such a ligand substitution reaction.

Phosvitin, one of the egg yolk phosphoproteins, liberates acid when incubated in alkaline solution. This acid formation is accompanied by a masking of amino groups and is not accompanied by the appearance of inorganic phosphate. This reaction was suggested to be related to structural changes, possibly of the nature of group migrations or changes in metal-protein interactions. Titrimetric evidence supported the hypothesis that, in alkaline solution, phosphoryl residues may migrate from hydroxyl oxygen to amino nitrogen

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