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Effects of pH on the Rate of Acid Denaturation of Horse Oxy-, Deoxy-, and Other Ferrohemoglobins*

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ABSTRACT: The pH profiles for acid denaturation of oxyhemoglobin (O_2Hb) and deoxyhemoglobin (Hb^0) have been measured by working at 0° and low pH to minimize oxidation (with O_2Hb) and by working under strictly anaerobic conditions (with Hb^0). Under certain conditions, O_2Hb is kinetically even more stable than carbonylhemoglobin (COHb). Plots of the log rate constant against pH give parallel straight lines with slopes of 2.4 at pH 2.4–3.6. Hb^0 is denatured about 100 times faster than O_2Hb. To test an earlier hypothesis that large differences in stability between Hb^0 and O_2Hb are due to differences in the electronic configuration of the heme iron, we measured the rates of denaturation of nitric oxide hemoglobin (NOHb), nitrosobenzene hemoglobin (C_6H_5NOHb), and hemoglobin complexes of various alkyl isocyanides (RNCHb), all of which are diamagnetic. In comparison with O_2Hb and COHb, they have a low kinetic stability – about the same as Hb^0 and ferrihemoglobin (Hb^+), which are paramagnetic. It is suggested that the relatively large size of the ligands may counteract any stability conferred on the protein by formation of covalent bonds. Spectroscopic data for one of the isocyanide complexes (ethyl, EtNCHb) indicates that although denaturation (unfolding), as determined by changes in the ultraviolet spectrum, is a one-step process, formation of the final product is a multistep process, possibly involving formation of multiligated species. Optical rotatory dispersion spectra show that the pH of half-denaturation for EtNCHb at equilibrium is 4.2. Perhaps more striking than the differences in kinetic stability is the fact that only two slopes are observed for the various pH profiles: 3.3 for $N_3^-Hb^+$, COHb, NOHb, and *i*-PrHb; 2.4 for all other complexes for which data are available. Thus, the difference in slopes appears to be unrelated to either the electronic configuration or to the charge of the heme iron. Stability at any given pH and slope of the pH profiles are also uncorrelated.

Extensive investigations on the kinetics and thermodynamics of acid denaturation of hemoglobin have previously been carried out largely with ferrihemoglobin (Hb^+) because the ferrohemoglobins undergo oxidation to Hb^+ in denaturation experiments which are not painstakingly anaerobic (Steinhardt *et al.*, 1966). When low-spin complexes of Hb^+, namely with N_3^- and CN^-, were used, it was found that they are kinetically stabilized toward acid denaturation (Steinhardt *et al.*, 1963; Molday and Steinhardt, 1969). CNS^- and F^-, ligands which form high-spin complexes, have only a small stabilizing effect. More recently, extensive anaerobic investigations of ferrohemoglobin as carbonylhemoglobin (COHb) have been carried out (Steinhardt *et al.*, 1966; Geddes and Steinhardt, 1968; Allis and Steinhardt, 1970). It has been found that COHb is about 100 times more stable than Hb^+, and is about as stable as $N_3^-Hb^+$ and CN^-Hb^+. In contrast to the case of Hb^+ where breaking of the heme imidazole bond and denaturation are simultaneous events, with COHb the heme bond persists in the denatured protein above pH 3 (Allis and Steinhardt, 1970). Below pH 3 the heme is removed at a rate slower than the denaturation; hence, the two reactions can be separated. This separation is facilitated by the fact that separate isosbestic points exist in the ultraviolet spectrum for each reaction.

Little work has been done on the stability of other ferrohemoglobins, especially those which are most important physiologically, *i.e.*, oxyhemoglobin (O_2Hb) and deoxyhemoglobin (Hb^0). In this paper we report the effect of pH on the rate of the acid denaturation of O_2Hb and Hb^0. The purpose of this more extended investigation was an attempt to confirm the hypothesis of Steinhardt *et al.* (1963) that the stability of hemoglobins toward acid denaturation may depend on either (a) the formation of a low-spin (covalent) bond between the heme iron and imidazole nitrogen; or (b) the absence of a net positive charge on the heme group; or both. We also measured the pH profiles of acid denaturation for nitric oxide ferri- and ferrohemoglobins ($NOHb^+$ and NOHb, respectively) and those of various alkyl isocyanide complexes of ferrohemo-

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globin (methyl, MeNCHb; ethyl, EtNCHb; *n*-propyl, *n*-PrNCHb; and *i*-propyl, *i*-PrNCHb). Also included are spectral and equilibrium data for EtNCHb.

Experimental Section

Materials. Horse COHb was prepared from blood of a single animal as described previously (Steinhardt *et al.*, 1966) and stored frozen in 5% solutions. Hb^+ was prepared by oxidizing COHb with 2 equiv of $K_3Fe(CN)_6$ and afterward dialyzing against first 0.2 M phosphate buffer (pH 6.8) and then distilled water. O_2Hb was prepared from COHb by passing O_2 over 5–10 ml of a 5% solution of stirred COHb in a 250-ml erlenmeyer flask for 2 hr. The visible spectrum showed complete conversion into O_2Hb. Ethyl, *n*-propyl, and *i*-propyl isocyanides were prepared by the method of Jackson and McKussik (1963). Methyl isocyanide was prepared by the method of Casanova *et al.* (1963). Solutions (0.1 M) of the isocyanides were prepared just before using. The hemoglobin complexes of the isocyanides were formed by mixing O_2Hb of the desired concentration with the volume of stock solution necessary to form a 10^{-2} M solution. The concentration of isocyanide was large enough to ensure complete complex formation (St. George and Pauling, 1951). The solutions were then deaerated with prepurified N_2 (Baker). Control experiments in which O_2Hb was converted into Hb^0 before addition of the isocyanides gave the same results. Nitrosobenezene (Aldrich) was recrystallized from absolute ethanol. Solutions of known concentration were added to the O_2Hb solution prior to deaeration. Nitric oxide, 99.0%, (Air Products) was passed through a 30% KOH solution to remove NO_2. $NOHb^+$ was prepared by mixing a deaerated solution of Hb^- with an acid (or buffer) solution which had previously been deaerated and then saturated with NO (removal of O_2 is necessary because of its reactivity toward NO). Examination of the spectrum at zero time showed that $NOHb^+$ was formed rapidly prior to denaturation. NOHb was also formed by deaerating and saturating an Hb^0 solution with NO. Hb^0 was always prepared by deaerating O_2Hb with N_2 in the manner described above. With O_2Hb, the rapid autoxidation in acid solution presented complications which were overcome by working only with fast reaction rates at low pH and low temperature (see Results). Ionic strength was maintained at either 0.05 or 0.02 M with sodium acetate or potassium chloride.

Methods. Kinetic runs were performed by following the changes in optical density at particular wavelengths on either a Cary 14 spectrophotometer, equipped with thermostated cell holders, or on a Gibson-Durham stopped-flow apparatus, equipped with gas-tight drive syringes. The temperature was controlled to ±0.1°. pH measurements were made on a Radiometer 25 pH meter, standardized by National Bureau of Standards potassium acid phthalate or potassium tetroxalate buffers. When using the Cary instrument, an apparatus which permitted the mixing of solutions and the filling of a cuvet without exposure to air was employed. For the stopped-flow runs, solutions were deaerated by slowly bubbling N_2 through them. The reservoir syringes were filled through an attached serum cap. The changes in optical density with time were usually measured at the following wavelengths: R-NCHb, 427 nm; NOHb, 420 nm; O_2Hb, 413 nm; Hb^0, 421 and 430 nm; C_6H_5NOHb, 422 nm; and $NOHb^+$, 420 nm.

Optical rotatory dispersion measurements were performed on a JASCO Model UV 5 spectropolarimeter. Solutions were mixed at least 6 hr (10 half-lives) before measurements were made.

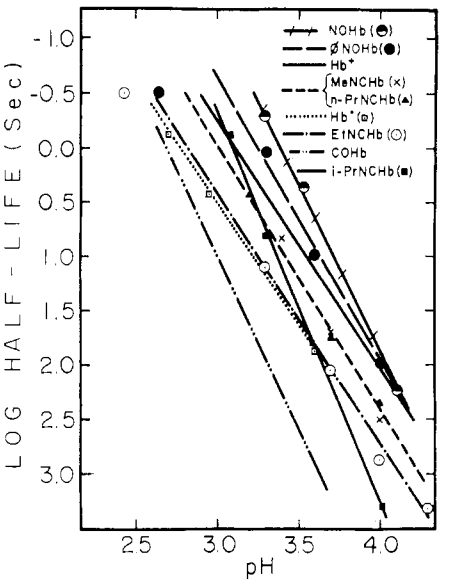

FIGURE 1: Log $t_{1/2}$ as a function of pH for various hemoglobin complexes at 25° and an ionic strength of 0.05 M.

Results

The denaturation reactions of the various hemoglobin complexes were followed spectrophotometrically in the region of the Soret maximum (see Experimental Section). Either acetate buffers or HCl were used to acidify. The results with both were compatible (see Sebring and Steinhardt, 1970). The reactions were first order for at least two half-lives. In those cases in which there were subsequent reactions, the rates of the latter were slow enough so as not to interfere with the determination of the denaturation kinetics. Some of the denaturation experiments for each complex in the pH range 2.5–2.9 were observed at 285 nm. At this wavelength, Allis and Steinhardt (1970) have shown that the unfolding process (denaturation), uncomplicated by heme interactions, is all that is observed. Our results at 285 nm were the same as those obtained at the Soret region, where larger optical density changes made the measurements more convenient. Allis and Steinhardt (1970) found that the half-lives obtained for COHb were not the same at the two wavelengths because heme separation dominated the kinetics in the Soret region. Since our results at the two wavelengths are the same, as with Hb^+, separation of heme from the denatured protein is much more rapid with the ferro complexes investigated here than the unfolding reaction.

The collected results are shown in Table I. The log of the various half-lives are plotted as a function of pH in Figures 1 and 2. Results previously obtained with Hb^+ and COHb are included. The pH profiles for Hb^+ and COHb at an ionic strength of 0.05 M and a temperature of 25° were determined for comparison purposes, and these plots are shown in the appropriate figures. From these plots, the slopes have been determined and are tabulated in Table II. Examination of the data shows that the values of the slopes fall into two groups—2.4 and 3.3. Although the difference in slopes precludes direct comparison of kinetic stabilities on the basis of half-lives, it is apparent that of all the ligands tried, only O_2 and CO appreciably stabilized Hb^0 toward denaturation. It is noteworthy that the stable complexes of Hb^+, $N_3^-Hb^+$, and CN^-Hb^+, are almost exactly as stable as O_2Hb and COHb.

O_2Hb. Brooks (1931, 1935) has shown that the iron in hemo-

TABLE I: Kinetic Date For Denaturation Reactions.

Complex	pH	$c_{protein}$ (%)	c_{ligand} (M)	Temp (°C)	Ionic Strength (M)	$t_{1/2}$ (sec)
O_2Hb	1.90	0.010	*a*	2	0.020	3.40
	2.21	0.010	*a*	2	0.020	9.50
	2.60	0.010	*a*	2	0.020	22.3
	2.80	0.010	*a*	2	0.020	88.0
	3.10	0.040	*a*	2	0.020	380
	3.42	0.040	*a*	2	0.020	1500
	3.62	0.10	*a*	2	0.020	6400
Hb^0	1.65	0.045		25	0.050	0.092
	2.69	0.045		25	0.050	0.755
	2.95	0.045		25	0.050	3.00
	3.19	0.090		25	0.050	6.25
	3.60	0.090		25	0.050	72.0
EtNCHb	2.40	0.090	1.0×10^{-2}	25	0.050	0.30
	3.30	0.090	1.0×10^{-2}	25	0.050	13.3
	3.69	0.75	1.0×10^{-2}	25	0.050	116
	3.99	0.75	1.0×10^{-2}	25	0.050	799
	4.29	0.75	1.0×10^{-2}	25	0.050	2210
MeNCHb	2.69	0.090	1.0×10^{-2}	25	0.050	0.332
	3.42	0.75	1.0×10^{-2}	25	0.050	6.63
	3.69	0.75	1.0×10^{-2}	25	0.050	49.0
	3.98	0.49	1.0×10^{-2}	25	0.050	344
i-PrNCHb	2.25	0.090	1.0×10^{-2}	25	0.050	0.214
	3.10	0.090	1.0×10^{-2}	25	0.050	0.725
	3.32	0.090	5.0×10^{-3}	25	0.050	5.20
	3.61	0.77	5.0×10^{-3}	25	0.050	72.0
	3.70	0.77	2.0×10^{-3}	25	0.050	81.0
	3.98	0.77	5.0×10^{-3}	25	0.050	1610
n-PrNCHb	3.20	0.090	1.0×10^{-2}	25	0.050	2.72
	3.69	0.114	1.0×10^{-2}	25	0.050	56.0
	4.0	0.66	1.0×10^{-2}	25	0.050	222
C_6H_5NOHb	2.65	0.075	1.0×10^{-3}	25	0.050	0.280
	3.29	0.075	1.0×10^{-3}	25	0.050	1.18
	3.59	0.077	1.0×10^{-3}	25	0.050	10.7
	3.98	0.87	1.0×10^{-3}	25	0.050	100
NOHb	3.30	0.050	*b*	25	0.050	0.470
	3.55	0.050	*b*	25	0.050	2.20
	4.10	0.050	*b*	25	0.050	170
$NOHb^+$	2.60	0.040	*b*	2	0.020	2.20
	2.72	0.040	*b*	2	0.020	3.30
	2.89	0.040	*b*	2	0.020	6.50
	3.58	0.060	*b*	2	0.020	180

[a] Solution in equilibrium with air. [b] Solution saturated with NO.

globin is oxidized by molecular oxygen in acid solution fairly quickly at room temperature. Even at pH 4.9 oxidation is nearly complete in 24 hr (Holden, 1936). This oxidation has a higher temperature coefficient than that observed for the denaturation of Hb^+, which is almost equal to zero below 15° (Steinhardt *et al.*, 1963). The pH dependence for the oxidation appears to be much less than for the denaturation. Thus, by working O_2Hb below pH 3.3 and at low temperature (2°), oxidation is too slow to affect the data. The fact that there is no optical density change at 630 nm on acidification shows that the starting material is O_2Hb, not Hb^+. However, an alternate mechanism for denaturation is conceivable in which oxidation is the rate-determining step, followed by the comparatively rapid denaturation of Hb^+. The observed pH dependence, however, rules out any substantial role for this mechanism: extrapolation of the Brooks data leads to an expected slope of unity for the pH profile if the alternate mechanism were to prevail. In fact, a slope of 2.3 is observed.

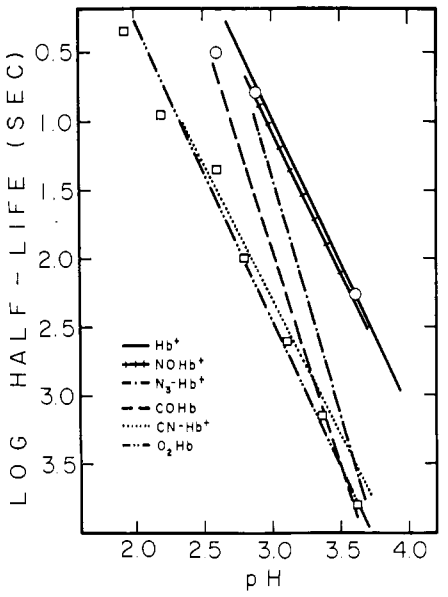

FIGURE 2: Log $t_{1/2}$ as a function of pH for various hemoglobin complexes at 0° and an ionic strength of 0.02 M. O_2Hb is represented by the open squares and $NOHb^+$ by the open circles.

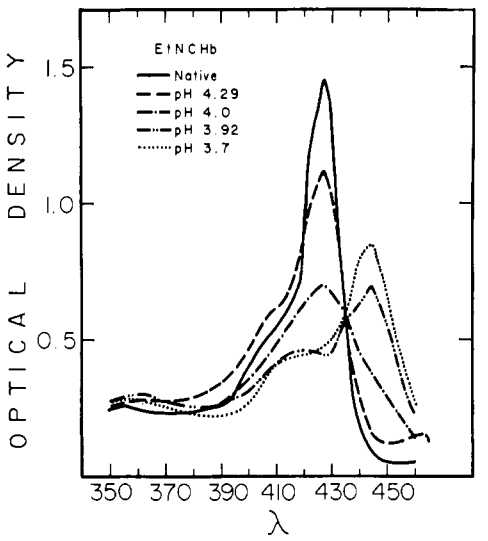

FIGURE 3: Spectra of EtNCHb as a function of pH: c_{EtNCHb} 0.77%; path length = 0.2 mm.

Examination of the kinetics show further that only one process is involved. The spectrum of the product is identical with that observed when the starting material is Hb^+. Thus, oxidation does occur, but only after denaturation has occurred. It had been shown previously that acid-denatured COHb is oxidized extremely rapidly (Steinhardt *et al.*, 1966).

Hb^0. When Hb^0 was acidified, a shift of the Soret peak from 430 to 421 nm occurred prior to denaturation. This shift was not prevented by the presence of sodium dithionite and, therefore, cannot be attributed to either oxidation or oxygenation. The spectrum of the final product, both in the presence and absence of sodium dithionite, has a fairly broad peak with a maximum at 380 nm, rather than at 370 nm, the wavelength observed when Hb^+ is denatured (Polet and Steinhardt, 1969).

Isocyanide Complexes. Both equilibrium and kinetic data were collected for EtNCHb. It has been shown previously that the fraction of protein in the native state is equal to $([m]_{233} - [m]_{233}^{denat})/([m]_{233}^{nat} - [m]_{233}^{denat})$, where $[m]_{223}^{denat}$ and $[m]_{233}^{nat}$ refer to the mean residue rotation at 233 nm for the protein in the fully denatured (pH 3.6) and native states, respectively (Geddes and Steinhardt, 1968). Values of $[m]_{233}$ from optical rotatory dispersion measurements are presented in Table III. They are equilibrium values obtained after waiting at least 6 hr (>10 half-periods). They show that the protein is half-denatured at about pH 4.1–4.2. The data in Table III also show quite clearly that when EtNC is present in large excess, the extent of denaturation is independent of the ligand concentration, *i.e.*, the denaturation reaction is stoichiometric.

The effect of pH on the spectra is shown in Figure 3. The peak in the spectrum characteristic of the products first appears at 460 nm at pH 4.3, but shifts to 445 nm at lower pH and to 440 nm at very low pH (see also Figure 4). In the visible region of the spectrum, the peak positions shift from 559 nm and 530 nm in the native protein, to 565 nm and 540 nm in the denatured product. These latter maxima are close to those reported by St. George and Pauling (1951) for heme complexed to two ethyl isocyanide molecules located in the axial positions. In one experiment with hemin chloride reduced by sodium dithionite at pH 7 (in the presence of 4000-fold excess of ethyl isocyanide), the spectrum in the Soret region showed two sharp peaks, one at 456 nm (ϵ 1.20×10^5) and the other at 410 nm (ϵ 0.54×10^5). No change occurred when the pH was reduced to 3.0. These data suggest that the final products may be denatured globin and the bisisocyanide heme complex.

TABLE II: Slopes of pH Profiles for the Acid Denaturation of Various Hemoglobin Complexes.

Hemoglobin Complex (Temp, °C; Concn, M)	Slope
O_2Hb (0, 0.02)	2.3
COHB (0, 0.02)	3.3
COHb (25, 0.05)	3.3
Hb^+ (0, 0.02)	2.4
Hb^+ (25, 0.05)	2.4
Hb^0 (25, 0.05)	2.4
MeNCHb (25, 0.05)	2.8
EtNCHb (25, 0.05)	2.4
n-PrNCHb (25, 0.05)	2.5
i-PrNCHb (25, 0.05)	3.3
$NOHb^+$ (0, 0.02)	2.4
NOHb (25, 0.05)	3.3
ϕNOHb (25, 0.05)	2.5

TABLE III: Mean Residue Rotation Measured at 233 nm For EtNCHb.

pH	c_{ligand}	$[m]_{233}$
Native	1.0×10^{-2}	−89
4.32	2.5×10^{-3}	−72.5
4.29	1.0×10^{-2}	−69
4.0	1.0×10^{-2}	−54.5
4.0	2.5×10^{-3}	−51
3.92	1.0×10^{-2}	−42.5
3.6	1.0×10^{-2}	−40

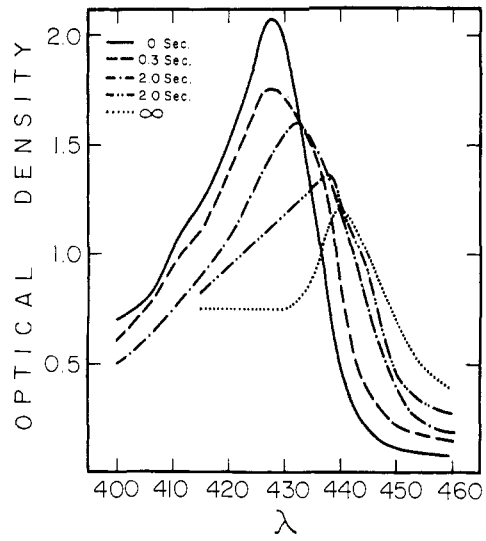

FIGURE 4: Spectra of EtNCHb measured as a function of time at pH 2.3: c_{EtNCHb} 0.055%; path length = 2 mm.

Free heme itself precipitates rapidly in acid solution. Since the detached heme or heme complex is held in solution, it is, therefore, probably attached to the protein at a site other than the heme pocket. To further elucidate the nature of this process, spectra were determined as a function of time at pH 2.3 by measuring the isotherms at a number of closely spaced wavelengths on the stopped-flow apparatus, and by plotting values at each wavelength for a number of fixed times (Polet and Steinhardt, 1969). The results are shown in Figure 4. Absence of any clearly defined isosbestic point, however, shows that the mechanism for formation of the end product described above is not a single transition in a two-state system.

NOHb and NOHb$^+$. With NOHb$^+$, autoreduction is fairly rapid (Kon, 1968) and complicates the spectral data. For this reason, only the acid solution was saturated with NO. As a further precaution, reactions were carried out at 2°. The NOHb$^+$ complex was formed rapidly ($t_{1/2} \sim$ 5 msec) as shown by an optical density increase at 420 nm and a simultaneous decrease at 406 nm. NOHb was formed in like manner from Hb^0- and NO-saturated acid. Since there is little spectral difference between NOHb and NOHb$^+$ (Keilin and Hartree, 1937), extrapolation of the rates to zero time proved inconclusive in determining whether autoreduction of NOHb$^+$ preceded denaturation. The fact that the results are significantly different when the starting materials are Hb^0 or Hb$^+$ suggests that the denaturation of NOHb is measured when NO dissolved in acid is added to Hb^0.

Discussion

The most important result of this work is the determination of the kinetic stabilities of HbO_2, Hb^0, and other ferrohemoglobin complexes. Two features stand out: (1) only O_2 and CO stabilized Hb^0 appreciably toward acid denaturation; and (2) the slopes of the pH profiles appear to fall into two groups, those with slopes ~2.4 and those with slopes of ~3.3. Both parts are discussed in detail below.

Significance of the Slopes. In the region in which the pH profiles are linear, the following rate law is valid, where

$$\frac{-\mathrm{d(protein)}}{dt} = k'(\mathrm{H}^+)^m(\mathrm{X} - \mathrm{Hb}) \qquad (1)$$

X − Hb represents the concentration of native hemoglobin, and k' represents a composite rate constant. A plot of log $t_{1/2}$ as a function of pH will be linear only if the rate of denaturation of the most highly protonated states is much larger than that of the other protonated states. Under such circumstances the exponent m, obtained from the slope of the pH profile, represents the average number of hydrogen ions present in the transition state and not in the starting materials (the protonation of certain groups ("trigger groups") is necessary to effect denaturation). At sufficiently low pH, these groups are fully protonated, and the rate of denaturation becomes independent of pH. Thus, if experiments are carried out at sufficiently low pH, a value for the association constant of these trigger groups can be calculated. From the pH profiles presented here and elsewhere (Allis and Steinhardt, 1970; Polet and Steinhardt, 1969), a maximum value of 2.5 for log of the association constants can be estimated.

The trigger groups are probably heme-linked imidazole groups since their protonation has been invoked as a major driving force toward acid denaturation (Steinhardt and Hiremath, 1967). The abnormally low K could result from the fact that for protonation to occur at all, the protein must undergo a thermodynamically unfavorable conformation change which exposes some sites to the solvent. Once protonation does occur, the equilibrium shifts to favor the denatured state (Allis and Steinhardt, 1969).

The difference in slopes (see Table II) then possibly represents a difference of about one in the average number of sites which must be protonated for denaturation to occur. That group most likely to be affected by a change of the ligand coordinated to the iron is the proximal imidazole because of its position trans to the coordinated ligand. Allis and Steinhardt (1970) have shown that with COHb, the heme is dissociated from the protein at a rate which is slower than the unfolding reaction. With Hb$^+$, however, breaking of the heme–imidazole bond occurs simultaneously with the unfolding reaction (Polet and Steinhardt, 1969). This results in the generation of an additional positive charge *prior* to denaturation. Thus, it may be that in the absence of this positive charge in COHb, an additional positive charge must be generated elsewhere in the molecule. As a result, one more group would have to be protonated to effect denaturation.

It is important here to consider the state of aggregation of the hemoglobin molecule. The fact that the kinetics are first order throughout the pH range studied shows that (1) the species which is denatured has the same state of aggregation as the stable species at the highest pH studied, and (2) that either the state of aggregation does not change over the pH range studied (4.3–1.9), or if it does, all states of aggregation are denatured at the same rate. It has been shown that human O_2Hb and COHb exist as dimers at pH 4 (Briehl, 1970; Field and O'Brien, 1955). Hence, it seems likely that at least with COHb, O_2Hb, and other Hb^0 complexes, the species denatured is the dimer. Thus, the slope refers to the number of protons on a dimer basis, and if the difference of about one refers to a difference of one in the number of trigger groups, then that one group is in only one subunit. It follows, then, if this interpretation is correct, that denaturation of one-half is slow and rate determining, while denaturation of the second occurs rapidly, or possibly that the transition between native and denatured hemoglobin may occur within a single chain.[1]

Kinetic Stabilities. Previous workers (Steinhardt *et al.*, 1963) have ascribed differences in kinetic stability of various

[1] For further discussion, see Steinhardt and Reynolds (1969).

Hb^+ complexes to the differences in the electronic configuration of the heme iron or to neutralization of the positive charge on the heme iron. For Hb^0 complexes it is readily apparent that no such simple correlation can be invoked. Only O_2 and CO have a large stabilizing influence. In fact, it is apparent that O_2Hb and COHb bear a marked resemblance to CN^-Hb^+ and $N_3^-Hb^+$, respectively (see Figure 1). Thus, it appears that with these complexes a state of maximum stability is achieved.

In addition to differences in charge and the spin state of the heme iron, an additional factor which was not present in the Hb^+ work must affect the results. Many of the ligands are large compared with the small ligands employed previously. In fact, without exception, the alkyl isocyanides and nitrosobenzene form unstable complexes. Thus it appears that the large size of the ligands, which probably causes spatial distortion of the heme pocket through nonbonded interactions, counteracts the stability which arises from the electronic configuration. The fact that Hb^0 is somewhat more stable than Hb^+ may be due to the neutralization of the positive charge on the heme iron. Anomalous behavior is exhibited by the NO complexes of both Hb^0 and Hb^+. As noted previously (Steinhardt *et al.*, 1963) and confirmed in this work, NO does not stabilize Hb^-. Even more surprising is the fact that NOHb is less stable than Hb^0 or Hb^+. The primary difference between NO and the other ligands studied is the fact that NO is a strong reducing agent. In fact, most nitrosyl complexes can be regarded as being formed from NO^+ and the metal in the reduced oxidation state (Cotton and Wilkinson, 1966). In such cases, considerable charge separation could contribute to the lack of stability. van Voorst and Henmerick (1966) have shown that $Fe(CN)_5NO^{-2}$ is protonated below pH 6 and that the site of protonation is the NO group. If this occurs with NOHb and $NOHb^+$, the anomolously low stability could be accounted for. The hypothesis of Steinhardt *et al.* (1963) that hemoglobin complexes, where the positive charge on the iron is neutralized and the iron is low spin, are stabilized with respect to high-spin positively charged complexes may be preserved with the modification that large ligands cause spatial distortion of the heme pocket and thus counteract the stability contributed by the formation of covalent bonds.

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