A Comparison of the Resistance of Human and Horse Ferrihemoglobin to Acid Denaturation*

(Received for publication, September 27, 1966)

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SUMMARY

Many of the stability characteristics of horse ferrihemoglobin (Hb+) in acid solutions, such as pH dependence and susceptibility to stabilization by iron ligands, are shared by human ferrihemoglobin, but striking differences between the two proteins exist. The most noticeable is the much greater rate of denaturation of the human protein at all pH values. Other differences include a shift to higher pH in the equilibrium between native and acid-denatured forms, differences in the temperature at which the temperature effect on the equilibrium-pH curve reverses, a complete absence in human Hb+ of the dependence of regeneration rate on extent of denaturation that is found with horse Hb+, and a related failure of the regeneration kinetics of human Hb+ to follow the inhibited regeneration model developed for horse Hb+. pH-stat experiments, and rapid flow titration curves of both native and denatured forms, indicate that human Hb+ has about four more masked groups (presumably imidazoles) than horse Hb+, although both proteins contain the same number of histidine residues. Although regeneration as measured by reappearance of the Soret band occurs readily, there is very little remasking of the liberated groups unless a stabilizing ligand (cyanide) is present; even then recovery is far from complete. It has been observed with both proteins that the band at 370 nm, characteristic of denatured oxidized heme-protein complexes, disappears much more rapidly during regeneration than the Soret band, characteristic of native protein, reappears. The discrepancy is more marked with human than with horse Hb+.

A long series of kinetic and equilibrium studies of the denaturation by acid of horse ferrihemoglobin (Hb+), as well as studies of its subsequent regeneration at higher pH, have established the following conclusions.

1. Denaturation follows a first order isotherm at constant pH with a rate that depends on (H+)^2 (1). There is a normal effect of temperature on rate between 15° and 25°, but almost no effect between 0° and 15° (2).

2. This transformation is almost fully reversible when the pH is raised to about 5; at 0°, an intermediate of short lifetime appears to be formed in the regeneration that complicates the regeneration kinetics. The half-transformation equilibrium pH in formate buffers at both 15° and 25° is about 3.8, and, at 0°, 4.2 (3).

3. Distinct denatured products are formed at 25° and 0°, recognizable by viscosimetric differences and differences in electrostatic interaction factors, although the spectra are indistinguishable (4).

4. Out of 38 histidine residues in this protein, 22 are inaccessible to protonation until the protein is denatured in the pH range from 3.1 to 4.6 at temperatures between 0° and 25° (5, 6). The rate of unmasking of the hidden imidazoles exactly parallels the spectrophotometric changes accompanying denaturation (7). The unmasking is an all-or-none cooperative phenomenon.

5. The protein is stabilized to an important extent (50- to 250-fold) against acid denaturation at 0.02 to 0.3 ionic strength by certain ionic ligands; formate, cyanide, and azide are the most effective ones of those tried. A tightly bound neutral ligand, NO, did not stabilize. All buffers tried, except formate, gave the same results as those given by HCl alone. The stabilized protein has a resistance to acid denaturation approximately equal to that of COHb and O2Hb, in which the iron is divalent instead of trivalent (8).

These findings lead to views as to the role of the prosthetic group, and the presence or absence of charge on the iron, in stabilizing the native structure, which have been discussed elsewhere (8).

The amino acid sequences of human hemoglobin are completely known (9), and those of horse hemoglobin, almost so. It has therefore seemed desirable to determine to what extent the two proteins are alike in the respects just described. Both contain 38 histidines. In the α chains, 20 histidines occupy identical positions in the two proteins. The only differences in histidine positions are in the β chains, in which there is a gap in the known sequence between residues 82 and 104.

Quantitative determination of similarities or differences between the two closely similar proteins should be useful in interpreting the effects of amino acid sequence on conformation and conformational stability, and the results of studies of the tertiary structure of human hemoglobin, such as x-ray analyses.

The present paper reports differences in the behavior of human and horse ferrihemoglobin with respect to rates and...
equilibria involved in denaturation or regeneration of native protein as functions of pH and temperature, titration curves, numbers of masked groups, and changes in absorption spectra. No attempt will be made here to relate the differences to known differences in amino acid composition between the two apoproteins. In a later paper a comparison will be made of the stabilizing effects of various ligands on the two proteins, and of the effects of ligands on the titration curves of the two proteins. The greater stability of the ferro form is still under investigation (10).

**EXPERIMENTAL PROCEDURE**

**Preparation of Human Ferrihemoglobin**—Human carboxyhemoglobin, COHb, was prepared by adapting the procedure of Drabkin (11) to the preparation of COHb instead of oxyhemoglobin. Thanks are due to the local Red Cross for providing several batches of fresh pooled red cells. The protein was crystallized by salting out with 2.8 M phosphate buffer (pH 6.8) while allowing the temperature to fall gradually from 37° to 2°. It was twice recrystallized by dissolving the crystals in a minimum amount of CO-saturated water at room temperature, and repeating the salting-out procedure after dissolving insoluble material. The final crystals were dissolved in CO-saturated water and dialyzed until free of phosphate. The concentration was then reduced from about 8% to about 3%, and the protein was oxidized to Hb+ with approximately 1.1 eq of dissolved K3Fe(CN)6. After complete oxidation (monitored by the shift of the Soret band from 420 nm to 408 nm) and initial dialysis against 0.2 M KCl, the solution was dialyzed against water until free of chloride. It has been established in this laboratory that Hb+ produced by ferricyanide oxidation has the same spectroscopic and kinetic properties as the Hb+ oxidized by air at pH constant. Regeneration experiments were carried out at 0° and 25° as in earlier experiments with horse Hb+ (8), except for the use of the Cary spectrophotometer with temperature control at 25° described above. With KCN the concentration was 0.01 M.

**pH-stat Experiments**—The course of denaturation and regeneration of human Hb+ was also followed by measuring the increase in accessible acid-binding groups incident to denaturation, which has already been described for horse Hb+ (1, 14). A Radiometer Titrigraph and SI2U recorder were used as in recent similar work with horse COHb (10). The uptake of H+ as a function of time in 0.2% protein solutions, brought quickly to various acid pH values, was measured recording the amounts of HCl added from an automatic syringe microburette to maintain the pH at the values designated. Sets of measurements were made at 25° at two constant chloride concentrations, 0.02 M and 0.2 M. The latter concentration was used in efforts to determine the total amounts of masked acid-binding groups, uncomplicated by differences which might be caused by changes in the acid titration curves of native and acid-denatured protein brought about solely by changes in the electrostatic interaction factor, w, in the Linderström-Lang equation (5, 14); at 0.2 ionic strength, the value of w for globular native protein is small, with a correspondingly small effect on the titration curve, which therefore cannot change greatly on denaturation. At this high ionic strength, the denaturation rate is so great that pH-stat experiments are impracticable unless the rate is decreased. Advantage has been taken of the stabilizing effect of combining ferrihemoglobins with cyanide to observe the full course of the reaction at an ionic strength of 0.2 (8).

In regeneration, acid-binding groups are remasked, and base rather than acid must be delivered by the pH-stat to keep the pH constant. Regeneration experiments were carried out at 0.02 M chloride in the same manner as in the spectrophotometric experiments, except that the protein concentration during regeneration was 0.2%. Regeneration as measured in the pH-stat was less complete than when measured by spectrophotometric criteria in buffered solutions.

**Rapid Flow Titration Experiments**—The contents of two 50-ml Luer syringes, one containing 0.4% protein, the other a predetermined concentration of HCl, both at 0.02 or 0.2 ionic strength, were emptied within 1 min by a synchronous motor into a machined Lucite four-jet mixing chamber connected directly to a flow-through tubular combined glass and reference electrode (Jena, No. 2220C). The fast flow (about 100 ml per min) and the short connection between mixing chamber and glass electrode provided a steady pH reading (Radiometer model PHA 925 with expanded scale) at 0.5 sec after mixing. The completeness of mixing was tested optically with colored fluids, with alkaline phenolphthalein and HCl, and by observing the independence of pH readings of the length of the connection between mixing chamber and cylindrical electrode when it was altered. Standardizations of the electrode system with National Bureau of Standards phthalate and phosphate buffers were made both before and after determining each point on the titration curve. Each determination involved refilling and running the syringes repeatedly until attainment of a constant reading assured complete rinsing out of previous solutions, and attainment of electrode equilibrium. Room temperature was maintained at 25° ± 1°. The glass electrode was cleaned overnight frequently with a pepsin-HCl solution; however, when the electrode was exposed to very acidic protein solutions or to suspensions of precipitated

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1 J. Steinhardt and C. de J. Stephens, unpublished experiments.
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Fig. 1. Kinetics of disappearance of the Soret band at 4060 A for 0.06% human ferrihemoglobin (Hu+) in formate buffers of ionic strength 0.02 at 15.5°. D represents optical density.

Fig. 2. Dependence of rate of disappearance of the Soret band on pH and temperature for both human and horse ferrihemoglobin.

protein, a colored protein deposit formed which could be removed with pepsin-HCl only if treated immediately. It has been shown with horse Hb+ that the presence of suspensions does not invalidate data (back-titration curve) (5).

The back-titration curve was determined with protein that had been kept at pH 3.15 at 25° for 30 min before being titrated with KOH as described above. No longer exposure to strong acid was permitted, in order to avoid the possibility of irreversible effects.

RESULTS AND DISCUSSION

Fig. 1 shows the results of representative spectrophotometric kinetics experiments at 15.5° in formate buffer of ionic strength 0.02. As in the case of horse Hb+, the reactions are clearly first order for three half-periods or longer, close to 90% completion. The rates, however, proved to be 4 to 6 times as fast as with horse Hb+ under the same conditions. As a result, the pH dependence was studied at slightly higher pH values than had been used with the horse protein.

Analysis of individual kinetics experiments shows that a very slow side reaction occurs in human Hb+ which is much less prominent in horse Hb+. We have taken this into account in our calculations. Fig. 2 shows how the rate of reaction (plotted logarithmically) depends on pH; it brings out clearly how the human protein is denatured more rapidly than the horse protein at all three temperatures investigated. In the linear region human Hb+ is denatured about 4.2 times as fast as horse Hb+ at 25°, and about 4.5 times as fast at both 15.5° and 0.2°. The functional dependence on pH (slope of the straight line about 2.5) is almost exactly the same with the two proteins. The departure from linearity at high pH at 25° sets in at a slightly higher pH with human than with horse Hb+.

The difference in denaturation velocity cannot, however, be completely accounted for by postulating a difference in the pH dependence of some triggering process; i.e. to superimpose the curves at 25° for human and horse ferrihemoglobin a shift in the horizontal axis will not suffice; a shift in the vertical axis is required as well. Thus it would appear that at least part of the difference between the hemoglobins of horse and man is a difference in the activation energy required for the reaction step

\[ \text{Hb+H}_n \rightarrow \text{denatured Hb+} \]

where Hb+H_n stands for the acid-combined native protein species, which is unstable. The activation energy for this step in human hemoglobin must be lower than the 16,000 cal found in horse hemoglobin (2).

Fig. 2 also shows that the proteins of both species exhibit the same unresolved anomaly at low temperatures; i.e. with both proteins there is much less difference in rate at the same pH between 0° and 15° than there is in the smaller interval from 15° to 25°. Other experiments show that the effect of temperature disappears almost completely in the close vicinity of 0°. It has been shown, in the case of horse Hb+, that different reaction products or mixtures of reaction products are produced at 0° and 25° (4). It cannot be said categorically that the reaction which predominates at 0° requires no activation energy (this conclusion would be incompatible with its finite rate), since simultaneous changes in absolute rate of reaction of Hb+H_n and in the pK of the acid-base reaction which forms Hb+H_n might fortuitously simulate a very low value of the activation energy, E, as calculated from rates at two temperatures at a given pH. Experiments at much lower pH values, with the use of a Gibson stopped flow device to cope with half-periods of as little as 10 msec, are in progress to assist in resolving this issue.

Fig. 3 shows that the equilibrium between native and denatured forms lies at higher pH values in human than in horse Hb+. It may be concluded, therefore, that the regeneration reaction differs in velocity from the regeneration rate in horse Hb+ by a smaller factor than applies to the denaturation velocities (there is no dependence of regeneration velocity on pH in

![Fig. 3. The dependence of the equilibrium between native and acid-denatured ferrihemoglobin of man and horse on pH and temperature at ionic strength 0.02.](http://www.jbc.org/)
horse Hb+). The equilibrium curves differ in two other important respects: (a) the pH span over which the equilibrium principally changes is considerably wider with human than with horse Hb+; (b) with human Hb+ there is a different equilibrium curve at each temperature, whereas with horse Hb+ the results at 15° and 25° are identical within experimental error.

In attempting to explain the first of these effects (broader pH dependence), one must take into account that the functional dependence of denaturation rate on pH is essentially the same in the two proteins; it might appear, therefore, that the difference must lie in the rates of regeneration. Thus, since the rate of regeneration of horse Hb+ is independent of pH, it would appear that the rate of regeneration of human Hb+ has at least a small pH dependence, becoming faster at low pH. It will be shown later that this paradoxical pH dependence does not, in fact, exist (Table I). Alternatively, one may seek an explanation analogous to the one demonstrated by Foster, Sogami, Petersen, and Leonard (15) for the fact that the N → F equilibrium in serum albumin has a greater pH span in human protein than in bovine protein. These authors have shown by means of fractional denaturation experiments that the difference lies in the greater "microheterogeneity" of the former.

The effect of temperature on the equilibrium is complex; thus, the results at 0° and 15° lie on either side of the results at 25°. ΔH changes sign at a temperature near 15°. If a change in sign occurs with horse Hb+, it probably does so at a somewhat higher temperature, between 15° and 25°. The change in sign of ΔH is undoubtedly complex in origin, involving the difference in products formed at 0° and 25° (4), and a balance between the effect of temperature on the pK of the stabilizing acidic dissociations and in the rate of reaction of the unstable protein ion. Because of the difference in pH span of the equilibria in the two proteins, it is not possible to draw definite conclusions as to the relative magnitudes of ΔH for the two proteins, but it may be noted that the pH shift in going from 0° to 15° is somewhat greater with horse than with human Hb+.

All of the experiments reported up to this point were carried out in formate buffers. Since it is known that formate exercises a specific stabilizing effect on horse Hb+ (16), a few experiments were performed at 25° with acetate ion in place of formate. Both kinetic and equilibrium measurements were made. In Fig. 4 the kinetic data for human Hb+ with acetate buffers are compared with the data already presented, obtained with formate buffers, together with data previously reported for horse Hb+. The same reaction rates are obtained with acetate, with either protein, at pH values about 0.35 unit higher than the pH values required with formate for that velocity. There is no appreciable difference between the hemoglobins of the two species in the extent to which they are stabilized by formate. An example of the greater stabilizing effects of another iron ligand is described in a later section. Further work on ligand stabilization of human hemoglobin will be published elsewhere.

In Fig. 5 the equilibrium data at 25° obtained with horse Hb+ in acetate are compared with equilibrium data obtained with human Hb+ in the same buffer. The differences are entirely comparable with those obtained in the formate experiments: (a) more denaturation occurs at any given pH when human

### Table I

**Regeneration of human hemoglobin**

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<th>Denaturation %</th>
<th>Regeneration pH</th>
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<td>82</td>
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</tbody>
</table>

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**FIG. 4.** The specific effect of formate buffers on the rates of acid denaturation of human and horse ferrihemoglobins at 25° and 0.02 ionic strength. The figure includes results obtained without buffers at the same ionic strength in a pH-stat.

**FIG. 5.** The pH dependence of the equilibrium between native and denatured ferrihemoglobins of man and horse in acetate buffers of 0.02 ionic strength. Two new determinations with horse Hb+ are superimposed on the curve previously published for this protein.
The pH dependence of the rate is included in Fig. 4, where it can be seen that the rates calculated from spectrophotometric experiments and pH-stat experiments are in good agreement. Thus, it can be concluded that unmasking of individual prototropic groups and changes affecting the binding of the prosthetic group occur simultaneously; one cannot precede the other. Furthermore, all the masked groups in one protein molecule are made accessible at once; a partially unmasked sample of protein is a mixture of molecules which are fully masked and molecules which have no masked groups at all.

No pH-stat equilibrium data are shown, since it is difficult to determine the total extent of reaction in pH-stat kinetic experiments unless the reaction is slow; it is difficult to fix the time of initiation or the point at which a stable level is attained. It is desirable, therefore, to have a rate sufficiently slow to be little affected by adjustments to the pH-stat setting, which sometimes occupy up to 30 sec when the reaction is initiated. It is also desirable to conduct the experiments at high ionic strengths, to minimize effects which may be due to difference in v, the electrostatic interaction factor, between native and denatured protein (5). These requirements are contradictory, since the rate of reaction is increased by a very large factor when the ionic strength is raised from 0.02 to 0.20. The reaction can be slowed, however, by taking advantage of the stabilizing effect of cyanide ion (8). With 0.3 M ionic strength (0.3 M KCl and 0.01 M KCN), the net combined effect is to halve the reaction velocities obtained at 0.02 M ionic strength in chloride or acetate. It thus becomes possible to measure the total amounts of acid added at pH values between 3.84 and 4.03, where denaturation is essentially complete, in reactions having half-periods between about 1.5 and about 4 min. Three runs at pH 3.84, 3.90, and 4.03 gave almost identical end values at 7 to 10 half periods, equivalent to the unmasking of 26 groups per 67,000 molecular weight unit. This was appreciably higher than the 22 groups obtained for horse Hb+ by analyses of the difference between the titration curve and back-titration curve at the same high ionic strength (5, 6). It will be shown below that the same high value for human Hb+ is obtained by the difference titration method at 0.2 ionic strength. Thus the difference between the two species cannot be due to some obscure effect of the presence of cyanide. Recently published amino acid sequences for the two hemoglobins (17-21) showed that the number of histidine residues, 38, is the same in both proteins.

The recent analysis by Tanford and Nonaki (22) of the human hemoglobin titration data of Antonini et al. (23) for human Hb+ in the region between pH 5 and 8 raises a presumption that not all of the masked groups in either protein are imidazoles. Carboxyl groups, which have anomalously high pK values, as in the 10 cases discussed by Steinhardt and Beychok (14), may account for the discrepancy; however, application of the nitrophenyl acetate hydrolysis method of Breslow and Gurud (24) has been reported by Bozum (25) to indicate that 20 histidine groups in native human hemoglobin are masked (22 to 24 in oxyhemoglobin; 23 to 26 in deoxyhemoglobin). Thus the difference between the two species cannot be due to some obscure effect of the presence of cyanide. Recently published amino acid sequences for the two hemoglobins (17-21) showed that the number of histidine residues, 38, is the same in both proteins.

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or base bound at pH 6.8. It is apparent that there are only small differences in the flow results with the two proteins, except at low pH, where the faster denaturation of human Hb+ results in the inclusion of the effects of partial unmasking at slightly higher pH values than in the case of horse Hb+, in spite of the shorter time constant of the apparatus used in the experiments with human Hb+. The positions of the equilibrium curves differ slightly for the same reason. Within the limits of uncertainty imposed by these considerations, the similarity of the two forward titration curves appears to exclude any great difference in pK of the carboxyl groups in the two native proteins.

The maximum of the difference curve at pH 4.5 (inset in Fig. 7), 0.41 mmole per g, corresponds to 27 masked groups per unit of 67,000 molecular weight. Human Hb+ is so rapidly denatured at pH values below that of the apparent maximum that a higher plateau may be obscured by the intrusion, at lower pH values, of denatured protein into the titration curve for "native" protein. On the other hand, the presence of 0.2 M KCl does not wholly suffice to suppress differences between the titration and back-titration which are due wholly to differences in w, the electrical interaction factor in the Linderström-Lang equation, between native and denatured proteins (26, 5). Small residual differences in w may appear in the difference curve as a slightly exaggerated number of unmasked groups. The two sources of error tend to cancel one another; the fact that the pH of the maximum difference is such that the half-period of denaturation is at least 200 times longer than the time constant (0.5 sec) of the rapid flow titration vessel (little denaturation occurs at equilibrium) leads us to believe that the value found may be slightly high rather than slightly low. Thus human Hb+ seems to be characterized by about four more masked groups than is horse Hb+. Since the groups are "buried" in the basic form, it is easier to conceive of them as uncharged imidazoles than as charged carboxylates. The apparent pK value of the masked groups is consistent with this view. There are no indications that an equal number of cationic groups are also "buried," as might be expected if negatively charged groups (carboxylates) were "masked" (had lower pK values in native than in denatured protein). Čejka and Vodrázka (27) have reported the existence of 14 to 18 masked groups in human COHb, on the basis of pH shifts accompanying denaturation by acid, and titration data on native and denatured protein (denatured by heat or photooxidation).

**Regeneration Kinetics (Spectrophotometric)—**Like horse Hb+, acid-denatured human Hb+ is readily regenerated (both native spectrum and solubility properties are recovered) when the pH is restored to 4.5 or higher, if precipitation is avoided. Unlike horse Hb+, however, the rates of regeneration of human Hb+ do not depend markedly on the extent of the preceding denaturation, or on the concentration of protein—two phenomena in horse Hb+ regeneration which were susceptible to quantitative explanation as the effects of the existence of a transitory inhibitor of regeneration. This inhibitor had the same absorption spectrum as native protein, but retained its property of inhibiting regeneration of other protein for 1 to 2 hours (3). With human Hb+, however, a less clear-cut situation prevails; the data (Fig. 8) clearly are not linear when plotted in terms of the inhibition kinetic equation

\[
\log \frac{z_0}{z} + \frac{x}{x_0} - 1 = \frac{ka}{z_0} t
\]

where k is the first order regeneration velocity constant, a is the total protein (native and denatured and regenerated), x is the denatured protein remaining at time t, and z is the initial concentration of denatured protein. Equation 1 gave a linear fit of the horse Hb+ regeneration data. The human Hb+ data show clearly that with human Hb+ simpler results are obtained, which do not depend as strongly on the extents of denaturation, between limits of about 45 and 95% initially denatured, as did the results with horse Hb+ (3). Fig. 9 shows results at 25° obtained with other pH values for denaturation and regeneration, plotted as both first and second order isotherms. Neither isotherm fits the data (the final stages are too slow), and the rates do not depend strongly on the extent of initial denaturation. The percentages recovered are about the same as with horse Hb+, and the rates, expressed as the time required to regenerate half of the protein ultimately recovered, do not differ greatly (Table I). As in the case of horse Hb+, regeneration rates are somewhat higher when denaturation is brought about rapidly (low pH) than when long periods are required. It is not possible to calculate a temperature coefficient for regeneration from the data given, since the protein was denatured at the temperature of
regeneration; it has been shown for horse Hb\(^+\) that protein samples denatured at different temperatures regenerate at different rates when they are both regenerated at the same temperature.

Regeneration Kinetics (pH-stat Method)—Efforts to measure the rate and extent of regeneration by evolution of acid-binding capacity at constant pH led to observation of an unexpected discrepancy between regeneration as measured by degree of reappearance of the Soret band and regeneration as measured by the disappearance of acid-binding groups. Far less remasking of basic groups occurred than would have been expected from the increase in absorption at 4060 Å. This discrepancy is totally absent with horse Hb\(^+\). The course of the remasking reaction followed Equation 1 quite closely (Fig. 10), although the reaction proceeded only about half as fast as when determined spectrophotometrically.

Since cyanide stabilizes all ferrihemoglobins (any species) and shifts the equilibria between native and denatured forms to lower pH, it should favor the regeneration of denatured human Hb\(^+\) by any criterion of measurement. Addition of cyanide to the solutions in the pH-stat experiments increased the extent of recovery by a substantial factor, so that it became almost equal to the extent indicated by reappearance of the Soret band (80 to 90%).

Thus, with human Hb\(^+\), unlike experience to date with horse Hb\(^+\), it has proved possible to distinguish between two criteria of acid denaturation, at least in subsequent regeneration. The presumption is therefore raised that the close parallelism between the results obtained with these criteria in horse Hb\(^+\) may be fortuitous rather than inherent, as in two manifestations of a single process. The distinction is important because on it rests the present concept that a partially acid-denatured hemoglobin solution contains a mixture of wholly denatured and wholly native protein, i.e., that only averages are being observed, rather than partial unfolding of individual molecules.

There is a second respect in which the present results call for care in interpreting the results with horse hemoglobin reported earlier; i.e., the equilibria shown in Fig. 3 for horse hemoglobin could be arrived at either spectrophotometrically (as they were in this case) or by means of the degree of unmasking attained. In the case of human hemoglobin, this would also be true provided that the unmasking equilibria were approached only in the direction of denaturation. The question then arises whether it is legitimate to compare the equilibria for the two species as has been done earlier in this paper. There are indications, to be described in a later paper, that the discrepancy in regeneration experiments between spectrophotometric and pH-stat experiments arises, in the case of human hemoglobin, because of the greater sensitivity of the latter to prolonged exposure to low pH. The two processes by means of which regeneration may be followed are not separated from one another if the exposure, although sufficient for denaturation, is short. If this is the case, one may legitimately compare in the two species equilibria approached by proceeding from native to denatured protein.

The concentrations of buffer anions, and of ligands, are lower than those at which they might be expected to affect the state of aggregation of either native or denatured protein. The former, at the pH values shown, should consist predominantly of dimer (\(\alpha\beta\)).

Identity of Native and Regenerated Human Ferritemoglobin—Fig. 11 compares the absorption spectrum of native and regenerated human Hb\(^+\) near the Soret band. It may be concluded that the regenerated protein has the same absorption as the native material, but that a small amount of protein has been irreversibly lost. The question arises whether any intermediates arise and later disappear during the course of regeneration which
process in regeneration, other than to point out that it obviously appears practically completely in 2 min or less, although the Soret band may take several hours to reappear completely. The characteristic of hematin adsorption complexes with protein, disappears practically completely in 2 min or less, although the Soret band may take several hours to reappear completely. The denatured protein-hematin complex thus appears to be very rapidly dissociated prior to the appearance of refolded protein with the hematin in its proper place. Direct observation of the 370 nm region shows that most of the change occurs in about 1 min. It is reasonable to hypothesize that the dissociation is due to the change in pH, and that refolding, which is time-dependent, must precede coordination of hematin in the position which gives rise to the Soret band. Since, however, hematin is practically insoluble in water, it is hard to suggest where it is after dissociation and before repositioning takes place. Preliminary work indicates that a similar phenomenon occurs with horse Hb, but that the initial dissociation is less drastic.

The indication that the spectral changes accompanying denaturation and regeneration are the result of more than one concurrent process is not wholly unexpected. The complexities encountered in the study of the regeneration of horse hemoglobin have already been interpreted in terms of the existence of transient intermediate states. We cannot now identify the fast process in regeneration, other than to point out that it obviously involves an alteration in the binding or aggregation of the heme rather than a refolding of the apoprotein.

Acknowledgment—Thanks are due to Hernando T. Veloso, who conducted preliminary experiments on human ferrihemoglobin in this laboratory in connection with his thesis submitted to Georgetown University in partial fulfillment of the requirements for the degree of Master of Science.

Alternatively, the 370 nm band may disappear instantaneously and reversibly when the pH is raised to 5.1.

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