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

Increase in Acid-binding Sites on Denaturation of Horse Ferrihemoglobin at 0°[1]

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RECEIVED APRIL 27, 1959

The reversible acid denaturation of CO-hemoglobin and ferrihemoglobin at 25° has been previously reported to liberate a large number of acid-binding groups which are unreactive in the native protein. Recently Tanford has suggested that the observed increase in acid binding may be accounted for by hypothesizing that denaturation brings about large changes in the electrostatic interaction factor, w, that determines the slope of the titration curve. The present paper shows that only a small part of the increase in acid binding between pH 3 and 5 can be accounted for on this basis and that a real increase in the capacity to bind acid at pH 4 to 8 (equivalent to 22 groups) must occur in denaturation. Data obtained at 0°, especially at high salt concentrations, at which w and hence changes in w must be small, show that the phenomenon persists; the increase in acid binding reaches a flat maximum over an appreciable pH interval. These conclusions are supported by the failure of similar differences (of reversed sign) to appear alkaline to the isoelectric point; by the occurrence of pH displacements between the titration curves of native and denatured protein which are several times too large to be accounted for by changes in w alone; by the fact that denaturation *precedes* rather than parallels the appearance of increase in acid-binding capacity; by detailed analysis of salt and temperature effects; and by observation of smaller viscosity changes under relevant conditions than those on which Tanford's calculations were based. In addition, analysis of the temperature effect on the titration curves adds new support to kinetic evidence presented earlier which indicates that the denatured products formed in acid denaturation at 0 and 25°, although both involve unmasking, are distinct from one another and are probably characterized by different values of w.

Introduction

A series of earlier papers dealing with both carboxyhemoglobin and ferrihemoglobin of the horse[2] have established that the amount of acid which they bind in going from the isoelectric point to pH 3.5 approximately doubles when they are denatured by dilute acid at 25°. This effect is reversed when the denatured protein regenerates at higher pH. Since the denatured and regenerated protein rapidly transform into one another as the acidity is changed, the measurements were made by a rapid-flow method, permitting reproducible readings to be obtained within 2 to 3 seconds of mixing. This reported increase in binding was interpreted as an actual increase in the number of binding sites ("unmasking") brought about as one of the changes in the molecule denoted by the term denaturation. Recently, however, Tanford[3] has pointed out that similar increases in binding are to be expected on the basis solely of inferred changes, as the result of denaturation, in the electrostatic interaction factor w, widely used in describing the titration curves of proteins.[2,4]

The quantitative aspects of Tanford's suggestion depend on interpreting the viscosity change accompanying denaturation, which he reported, as due solely to an enlargement of the protein molecule without large changes in shape, and on the concept of a *permeable* denatured molecule similar to the one he had previously proposed for serum albumin.[5] This concept, combined with the Debye–Hückel Theory, leads to sufficiently different values of w (0.065 and 0.023, respectively) for native and denatured forms, to give a difference in acid binding at pH 3.5, near the result Steinhardt and Zaiser reported.

Although we have not confirmed as large a viscosity change under conditions relevant to the titration measurements as Tanford has reported,[3] the suggestion of Tanford is at least qualitatively plausible; at least part of the effect previously reported is likely to be explained in this way. However, it has been impossible to confirm that the entire increase can be accounted for in this manner. The possibility of occurrence of unreactive acid-binding groups in proteins which become normally reactive (at a given pH) on denaturation would, if corroborated, furnish important clues to the tertiary structure or steric configuration of native proteins. The present investigation was therefore undertaken to determine whether Tanford's suggestion of a wholly electrostatic effect is adequate to account for the experimental basis for the earlier views of Steinhardt and Zaiser. By working near 0° instead of 25° and at 0.3 ionic strength, as well as at 0.02 as in the earlier work, it has been possible to distinguish between the opposing views by means of the effects of temperature and ionic strength on the binding. In addition the possibility was added of confirming an earlier conclusion, reached on kinetic grounds, that the acid-denatured protein formed at 0° is qualitatively different from the similar product formed at 25°.[6]

Experimental

All materials, methods and procedures were the same as previously described,[7,8] except as stated below or elsewhere in the following sections.

All parts of the rapid-flow apparatus[7] were fitted with water jackets and maintained at constant temperature by means of continually circulating water, pumped from a large bath regulated at either 0.5 or 24.6° within ±0.1°. The entire electrode system, including all liquid junctions, was kept at these temperatures; the glass electrode, coated with Desicote, was regulated with air rather than water.

In spite of reports[9] of high electrode resistance at low temperature, no apparent loss of sensitivity at 0.5° was encountered. Voltages were reproducible to ±0.5 mv., corresponding to ±0.01 pH unit. Over the pH range 2.08 to 9.46 the slope of the e.m.f./pH relationship was always 54.1 ± 0.5 mv./pH unit at 0.5°, in good agreement with the theoretical value.

(1) A brief account of part of this work was presented at a meeting of the American Chemical Society in Chicago on September 10, 1958.

(2) These papers are summarized in J. Steinhardt and E. M. Zaiser, *Advances in Protein Chem.*, **10**, 151 (1955).

(3) C. Tanford, THIS JOURNAL, **79**, 3931 (1957).

(4) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, pp. 512–540.

(5) C. Tanford, THIS JOURNAL, **77**, 6421 (1955); *J. Phys. Chem.*, **59**, 788 (1955).

(6) J. Steinhardt, E. M. Zaiser and S. Beychok, THIS JOURNAL, **80**, 4634 (1958).

(7) J. Steinhardt and E. M. Zaiser, *J. Biol. Chem.*, **190**, 191 (1951).

(8) J. Steinhardt and E. M. Zaiser, THIS JOURNAL, **75**, 1599 (1953).

(9) R. G. Bates, "Electrometric pH Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954.

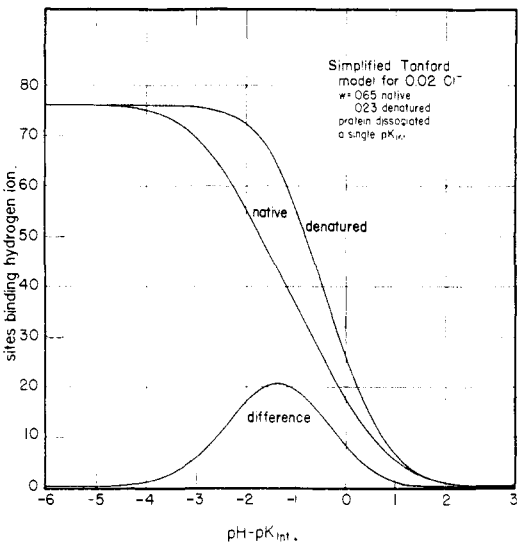

Fig. 1.—Calculations, in accordance with the suggestion of Tanford, of the changes in acid binding due to changes in w which may occur in denaturation. The ordinate represents twice $(38 - h)$ since it refers to a 67,000 molecular weight unit (two half molecules).

Seven different stock batches of ferrihemoglobin, prepared as previously reported, were used in obtaining the data presented here. Results with each batch were brought into consistency by applying small corrections for small residual combination with acid or base after final dialysis. These corrections never exceeded 0.042 mmole/g. and were usually much smaller. All the batches were indistinguishable from one another and from the material used previously, when titrated with acid and readings taken within two seconds (rapid-flow method). A single batch, designated V, showed a consistently slightly higher acid binding when *back-titrated* to pH values more acid than 4, after exposure to pH 2.5–2.9 for from 30 minutes to two hours at the temperature of back-titration before adding KOH. With subsequent batches, protein was fully denatured at higher pH's (3.0 to 3.3) for 20–30 minutes, then back-titrated. Under these conditions, the data of Steinhardt and Zaiser[2] at 25° were reproduced and all remaining batches gave consistent results at 0.5° as well as at 25°. When protein is exposed, initially, to considerably harsher denaturing conditions, some further increase in acid binding is observed. However, this cannot be the full explanation of the aberrant results with batch V, since several equilibrium points (pH measured 24 hr. after exposure to acid) were obtained in which protein had never been exposed to pH below 3 and these, too, exhibited anomalously high acid binding at pH below 4.

Back-titration experiments above pH 6.06 and below 9.09 at 0.02 M chloride and above pH 5.70 to below 8.70 at 0.3 M chloride caused denatured protein to precipitate. At the extremes of these intervals, precipitate appears only in the exit tube after the solution passes through mixing-chamber and glass-electrode. Thus the presence of the solid phase cannot affect these results. However, at pH values close to neutrality, the precipitate appears to form almost immediately on mixing alkali and denatured protein and is thus present in the electrode chamber. Little if any protein remains in solution, and the solutions are so poorly buffered that the electrode chamber must be washed out repeatedly with freshly mixed solution before steady and reproducible potentials are obtained. Back-titration points obtained with precipitate present are so designated in all the figures, without regard to the extent of precipitation, although the solutions are well buffered to within about 0.6 pH unit of the isoelectric point. It must be emphasized, however, that these points are fully reproducible, from batch to batch, and that every indication exists that they represent the same truly reversible acid–base equilibria as when precipitate is absent. Thus, for example, the results obtained do not depend on the protein concentration over wide limits. Therefore, it appears that the amounts combined by both aggregated and dispersed phases are equal and no account need be taken of any slight discontinuity in the binding function which may be brought about by precipitation. At 25° and 0.02 ionic strength it is possible to measure most of the back-titration curve without precipitate forming if the protein concentration does not exceed 0.1%. These results match those with precipitate present. Likewise at pH above about 7.5 back-titration points and ordinary titration points fall on the same curve, and this is true whether precipitates form or do not.

When titrating with acid alone, precipitate formed only when long-time experiments were performed to obtain equilibrium readings, and then only at 0.3 M salt at pH values above 4. Such experiments are not reported here, since the protein–acid equilibrium point is never established while precipitate continues to form, and would not be comparable with the equilibrium point in the absence of solid phase, if it could be measured at all.

Preliminary Discussion

The significance of the experiments presented in this paper will be clearer if they are preceded by the results of greatly simplified model calculations. The purpose of these models is to clarify the distinctions between increased acid binding brought about by an actual increase in the number of acid-binding sites (within a given range of pK) and increase brought about by changes in electrostatic interaction of a fixed number of groups.

Thus, for example, Fig. 1 illustrates the increase in acid binding as a function of pH brought about by the changes in electrostatic interaction factor w postulated by Tanford on the basis of changes in viscosity (Tanford's postulated values for w are used here without questioning their method of derivation or the viscosity measurements on which they are based). The number of available sites (given for simplicity, a single $pK_{\text{intrinsic}}$) is the same in both native and denatured protein. The equation employed in these calculations is the familiar one

$$p\text{H} = pK_{\text{int}} + \log \frac{h}{n - h} - 0.868wz$$

in which h represents the number of sites which have dissociated a hydrogen ion, n is the maximum number of such sites and z is the net charge and has been equated, for simplicity, to $(n - h)$ since it is assumed that the molecule is isoelectric when $h = n$.

The maximum number of sites chosen for this calculation, 76, is actually smaller than the total number of acid-binding sites available in ferrihemoglobin but has been used here because it gives approximately the observed value in the experimental difference curve. In calculating the curves shown, ferrihemoglobin has been taken as wholly dissociated into half-molecules over the entire pH range $(n = 38)$[10]—this assumption is probably not valid for the concentration of protein used at pH values only slightly acid to the isoelectric point, but the effect of this discrepancy is unimportant in the region of small acid binding. The numbers of sites combining acid are, however, all stated in terms of a molecular-weight unit of 67,000, *i.e.*, twice $(38-h)$.

The important feature in Fig. 1 is the well-marked maximum in the difference curve (bottom of figure). The high degree of symmetry of this curve is inherent in any attempt to explain the dif-

(10) E. O. Field and J. R. P. O'Brien, *Biochem. J.*, **60**, 656 (1955).

ference in the titration curves brought about by denaturation entirely in terms of changes in w. If more than a single intrinsic dissociation constant had been used, the difference curve might be bimodal or show a somewhat broader top, but numerous well-selected constants and approximate equality in the fractions of the total groups characterized by each dissociation constant would be required to give a flat plateau at the top of the difference curve over any substantial interval of pH. Increasing the ionic strength reduces the height of the difference curve because it reduces w for both forms, but this change does not affect the shape of the curve. The slopes of both titration curves are increased and the maximum of the difference curve is reduced from 21–22 sites to 16 sites.

The foregoing models may be contrasted with others, in which not only changes in w (if they actually occur) but also changes in the total number of available sites are taken into account. Figures 2a and 2b show such a case, in which the equation and all the parameters employed in obtaining Fig. 1 remains the same, except for the number of available sites which have been assumed to differ in the native and denatured proteins. For reasons which will be apparent later, only sites dissociating in the range of titration of histidine have been assumed to be involved in the change.

The model considers only dissociation of hydrogen ions at pH values above those at which the 51 carboxyl groups of hemoglobin[11] have dissociated, thus contributing 51 negative charges to the total charge (the overlap between carboxyl ionizations and those of other groups is ignored for the sake of simplicity). These 51 charges are almost exactly balanced by 52 positive charges contributed by arginine and lysine residues. Thus the *net* charge at the most acid pH of interest may be attributed wholly to the 50 remaining acid groups which have dissociation constants between those of the aspartic and glutamic acid residues and those of lysine.

These are 36 histidines (all with positive charges), 4 valine end amino-groups[12] (likewise positively charged) and 8 ferrihemic acid groups (neutral).[13] Thus there is a net positive charge of 40 at this acid pH (20 in the half-molecule), and equation 1 becomes

$$p\mathrm{H} - pK_{\mathrm{int}} = \log \frac{h}{25 - h} + 0.868w(h - 20) \quad (1')$$

The isoelectric point falls *inside* the titration range of these intermediate groups (with $h = 20$) but near its alkaline end.

(11) Analytical values, except for valine end-groups, are taken from G. R. Tristram, in "Hemoglobin," F. J. W. Roughton and J. C. Kendrew, Editors, Butterworth, London, Interscience Publishing Co., New York, N. Y., pp. 109–114.

(12) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 4682 (1957).

(13) The dissociation of ferrihemic acid has been assumed to occur close to that of histidine and valine on the basis of the measurements of D. B. Morrison and E. F. Williams, Jr. (*J. Biol. Chem.*, **137**, 461 (1941)), which show that insoluble ferrihemic acid goes into solution on addition of base, with strong buffering occurring at pH near 7.4.

No general agreement exists as to the dissociation constants of the propionic acid groups of heme. If $4FeOH_2$ rather than 8 propionic groups dissociate in this region (see R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience, New York, 1949, pp. 169–170). The values of n used in Eq. (1') and (1'') should be reduced by 2. Such changes would have only negligible effects on the essential characteristics of the curves in Fig. 2.

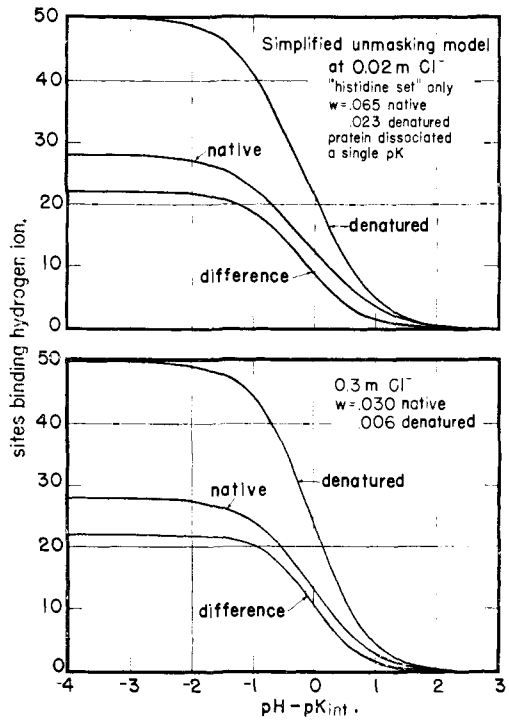

Fig. 2.—Calculations of changes in acid binding brought about by "unmasking" of acid-binding sites in denaturation. The selection of numerical factors for n and for z is explained in the text. In each case the ordinate is twice $(n - h)$ to represent a molecular-weight unit of 67,000 (Part a above and part b below.)

The above choice of parameters applies to the denatured protein in which all sites are reactive. In the native protein evidence which follows indicates that 22 of these groups are unavailable or masked (on a 67,000 weight unit). If all 22 of these groups are positively charged (*i.e.*, the charge does not involve ferrihemic acid) n is reduced to 14 (in the half molecule) and z is reduced to 10, *i.e.*

$$p\mathrm{H} - pK_{\mathrm{int}} = \log \frac{h}{14 - h} + 0.868w(h - 9) \quad (1'')$$

The most important difference between the results in Fig. 2 and those of Fig. 1 is the approach by the difference curve at acid pH to a long flat plateau. Unlike the earlier case, such a plateau obviously does not require diversity in pK_{int}. Changes in ionic strength and the consequent changes in w do not affect the height of this difference curve, although its position and steepness can be shown to be affected by terms containing w and z (net charge) for both native and denatured protein. In fact, the essential features of the titration curves and difference curves do not depend on assuming that any change in w accompanies denaturation. However, it should be noted that the midpoint of the difference curve (apparent pK hereafter) is importantly affected by w or differences in w, but in all cases lies by a greater or lesser amount, to the acid side of pK_{int}. Thus the pH of the midpoint of the differences curve is always smaller than the pK_{int} of the unmasked groups.

Many features of the model just described (*e.g.*, single pK_{int}, disregard of carboxylate combination

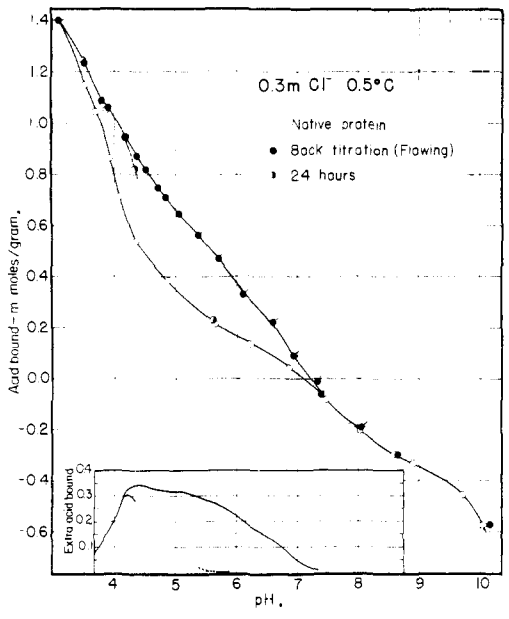

Fig. 3.—Titration data for native and denatured (back-titration) ferrihemoglobin at 0.5° in the presence of 0.3 *m* chloride. The difference in acid bound is shown as a function of *p*H in the insert at the bottom of the figure. The fragments of a second difference curve represent the ends of the equilibrium (24 hour) difference.

with hydrogen ion) are highly artificial, and the artificialities must be expected to affect the calculated titration curves. However, their effect on the difference curve will be small; thus, for example, ignoring the effect of carboxyl ionization in both native and denatured protein should have very little effect on the latter, even in the region of the plateau.

Obviously, the simplest experimental distinction between the two interpretations would be demonstration of the existence or non-existence of an extended plateau. There is a strong indication of the existence of such a plateau with carboxyhemoglobin at *p*H values near 3.3.[14] Unfortunately, this distinction cannot be realized experimentally with ferrihemoglobin at 25° because it has proved impossible at this temperature to obtain the titration curve of the native form at *p*H below about 3.9. Even the 2–3 seconds required by the flow method is too long since ferrihemoglobin denatures more rapidly than carboxyhemoglobin. Working at 0° does not improve matters sufficiently to remove all ambiguity, although denaturation does not set in importantly above *p*H 3 at 0.02 ionic strength. Recourse may be had, however, to making measurements under conditions (high ionic strength) that minimize w, and therefore Δw, so that only small differences between the amounts bound by native and denatured protein should be ascribable to this effect. If large differences persist, they must be real—especially if they behave as functions of *p*H as Fig. 2 indicates such real differences should.

Results and Discussion

Data Obtained at High Ionic Strength.—The results obtained at 0.3 ionic strength at 0.5° are shown in Fig. 3. It will be observed at once that the difference curve shows a distinct plateau between *p*H 5.2 and 4.7. The slight rise just below 4.7 probably reflects a small residual change in w due to denaturation. At still more acid *p*H where denaturation in 2 seconds becomes appreciable, the difference curve falls off sharply, as in earlier work. However, the plateau, which prevails over a half *p*H unit, is (as discussed above) hard to explain on the basis of electrostatic effects alone, especially at so high an ionic strength, and appears to be due to an actual increase in available acid-combining sites.

The difference in acid bound represented by the plateau, about 0.32–0.33 mmole/g., corresponds to the addition of 22 combining sites in the 67,000 molecular weight unit. This is more than a third lower than the earlier estimate of 36 groups, on the basis of work at 0.02 ionic strength and 25° However, it is important to note that the larger difference (0.52 mmole/g.) was found with carboxyhemoglobin and that the largest difference found with ferrihemoglobin was 0.41 mmole/g. (25°). The smaller difference found with ferrihemoglobin was attributed earlier to failure to attain the maximum difference because of the rapid denaturation of the latter protein at *p*H value above those at which the maximum could be demonstrated.

A significant feature of the data in Fig. 3 is that the difference in acid binding persists beyond the isoelectric point for a short distance to the alkaline side. Beyond this, the titration curves of native and denatured protein *merge* rather than cross, as would be predicted from an exclusively electrostatic explanation of differences in acid binding. Failure to find a comparable reverse difference on the alkaline side of the isoelectric point constitutes additional important supporting evidence for the existence of a real difference in the number of available binding sites—*i.e.*, masking in the native protein of about 22 groups.

Since the results just described differ in detail from those published earlier, it is appropriate to consider briefly the evidence they shed on the nature of the 22 groups involved and of the "trigger groups" which on combining acid initiate denaturation itself. The position of the mid-point of the difference curve on the *p*H coördinate indicates that the pK at 0.5° of the liberated groups is somewhat higher than 6.3. This value immediately suggests 22 of the 50 groups, including 36 histidine residues, which dissociate between the carboxyl and ϵ-amino groups (see Preliminary Discussion). If all 22 are alike, they can only be histidine, and the procedure used in the calculations for Fig. 2 has been based on that assumption.

It is obvious, however, that denaturation is initiated only at *p*H values more acid than those at which dissociation of the unmasked groups occurs. At this ionic strength acid denaturation occurs very slowly, if at all, at all *p*H values above 5.3. At this *p*H the difference curve shows that the groups unmasked by denaturation at *p*H 3.3 bind acid practically quantitatively. If the initiation of denaturation by acid involves an acid–base dissociation of trigger groups, as previously discussed,[2] then the trigger groups are characterized

(14) J. Steinhardt and E. M. Zaiser, *J. Biol. Chem.*, **190**, 197 (1951).

by much lower values of pK than are the unmasked groups, indeed probably even lower than those characterizing the carboxyl groups in proteins. The eight propionic acid side-chains of the hemes have been suggested previously as a possibility. It may be concluded that the unmasked groups combine with acid almost quantitatively at the pH values at which they are formed.

At this ionic strength nothing can be said about the pH range of an equilibrium between native and denatured protein, since during the many hours required for the attainment of equilibrium the precipitation of denatured protein continually displaces the equilibrium and causes more protein to be denatured, until practically complete denaturation occurs at all pH's below about 5. This complication does not occur at lower ionic strengths, and consideration of the equilibrium (which bears on the nature of the trigger groups) is therefore deferred to the following section.

Results at 0.02 Ionic Strength.—In spite of broad similarities, there are distinct difference between the results obtained at 0.5° at high ionic strength and those obtained at much lower salt concentrations, as shown in Fig. 4. There is only a

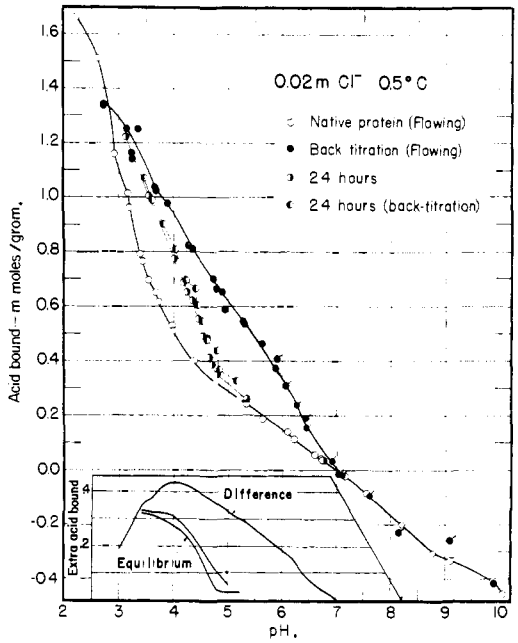

Fig. 4.—Titration data for native and denatured ferrihemoglobin at 0.5° in 0.02 m chloride. The higher difference curves inserted at the bottom of the figure represent 3 second values—the others represent equilibrium (24 hr.) difference curves approached from denaturation and regeneration sides.

hint of a top in the difference curve at 0.32–0.33 mmole/g./bound, obscured by a substantial additional rise with a peaked maximum instead of a plateau, which rises almost to 0.44 mmole/g. It appears reasonable to regard this superimposed additional 0.11 mmole as evidence of an electrostatic effect of the kind postulated by Tanford, due to changes in w brought about in denaturation—visible at 0.02 ionic strength, where w for native protein is large, but vanishingly small at 0.3 ionic strength

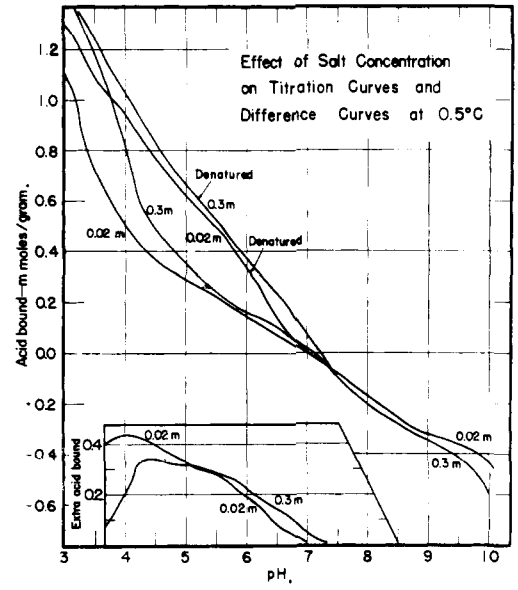

Fig. 5.—Comparison of titration curves and difference curves of native and denatured ferrihemoglobin at 0.5° at two ionic strengths.

where w for both native and denatured protein must be small.

As in Fig. 3, no differences are found in the titration curves of native and denatured protein at alkaline pH. Unlike Fig. 3, however, the curves merge at or very close to the isoelectric point rather than at pH just above it. Since no reverse differences are found in the alkaline region, no special significance attaches to having the point of merging at this ionic strength so close to the isoelectric pH.

Figure 4 also includes equilibrium values, measured after 24 hr. Two sets of data are given, for the approach to equilibrium from the two sides. They are satisfyingly close. To judge from the difference curves which correspond to the equilibrium data, the equilibrium mid-point is near pH 4.5. However, this value must not be identified with the pK of the postulated trigger groups since the equilibrium point is determined not only by this pK, but also by the ratio of rates of denaturation and regeneration as a function of pH. The equilibrium data shown in Fig. 4 will be compared with the equilibrium measured directly by spectrophotometry in a later section.

An important feature of the equilibrium difference curve is that it does not show the additional superimposed maximum of 0.11 mmole/g. present in the back-titration difference curve and which was attributed above to the difference in electrostatic factors postulated by Tanford. Since the only difference between the protein in the equilibrium experiments and in the back-titrations is that the equilibrium solutions were never exposed to the stronger acidity (pH 2.5 to 3.3) used in the initial denaturing before back-titration, the interesting possibility is raised that the change in molecular parameters which produces such a change in the electrostatic interaction factor requires higher acidity (pH 3.3 at 0°) than denaturation itself, which is essentially complete in 24 hours at pH as high as 3.9. This possibility requires further investigation.

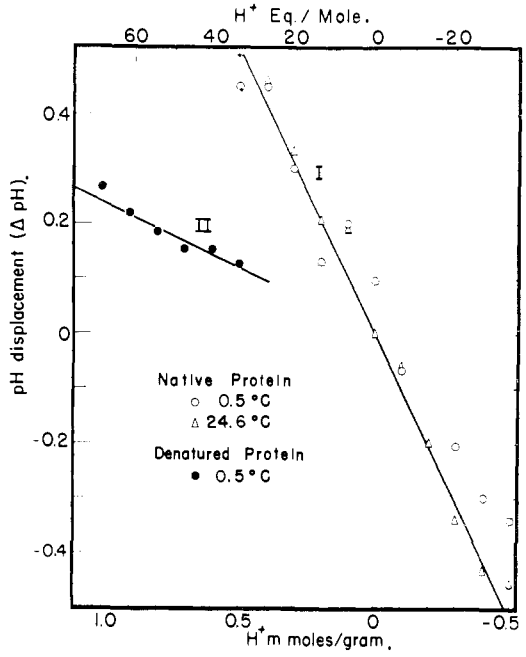

Fig. 6.—pH displacements in the titration curves of native and denatured ferrihemoglobin brought about by differences in ionic strength (0.02 and 0.3 m). Curve I is for native protein at both 0.5 and 24.6°. Curve II is for denatured protein at 0.5°.

The Effect of Ionic Strength on Titration Curves at 0°.—When titration curves for both native and denatured protein are compared separately at both ionic strengths, the displacements found (Fig. 5) are entirely normal and of the magnitude to be expected from electrostatic theory and from a diminution in w for denatured protein, as postulated by Tanford; *i.e.*, when z is large the curves for native protein (w large at low ionic strength) are much farther apart than the curves for denatured protein at the same z (w small at both ionic strengths.) The difference curves almost merge as the "maximum" value of 0.32–0.33 is approached, and diverge importantly only where the superimposed electrostatic effect previously noted occurs at low ionic strength.

A more quantitative examination of the effect of ionic strength is shown in Fig. 6 in which the pH displacement due to ionic strength changes corresponding to a given amount bound is plotted against hydrogen-ion bound. Since there is no appreciable binding of chloride,[15] eq. 1 indicates that such a plot (for native protein) should give a straight line with a slope of 0.868 Δw, or approximately 0.0304, if there is no change in n or pK_{int}. as the ionic strength changes (values for w calculated in the usual way). The linear relation is realized with good approximation, over a very wide range, but the slope found (curve I) is actually 0.0145 if the molecular weight used is 67,000. However, the slope is in almost exact agreement, 0.029, if the molecular weight unit taken is half of 67,000. This agreement appears to imply that the protein is molecularly dissociated on both sides of the isoelectric point at this temperature, whereas data for molecular dissociation is available for the acid side only and at higher temperatures. It should be remarked here that data for native protein at 25° fall on curve I also—gratifying because there should be no temperature coefficient for Δw. On the other hand, curve II in Fig. 6, which is for denatured protein at the same temperature, has a slope only one-quarter as great as that for native if the same molecular weight is used. Thus Δw (possibly an indication that w is small for both forms) must be very small (less than 0.01) unless the large changes have occurred in n,pK_{int} or molecular weight as the result of change in ionic strength. Curve II does not represent the data obtained at 25° with denatured protein, for which Δw depends on temperature.

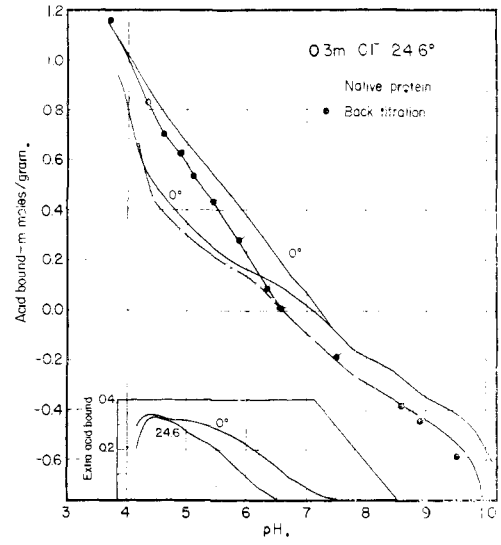

Fig. 7.—Titration data for native and denatured ferrihemoglobin at 24.6° in 0.3 m chloride. The titration curves at 0.5° at the same ionic strength are included for comparison. The insert gives difference curves at both temperatures.

Results at 25°.—Data at 0.3 ionic strength at 24.6° are given in Fig. 7. They show all the essential features of the data obtained at 0.5°, but the plateau is less evident, not only because denaturation at this temperature prevents obtaining data on native protein at pH below 4.4, but also because, as would be expected, the difference curve is displaced to a more acid range of pH values at the higher temperature. The size of this shift, if it is attributed solely to temperature-induced change in pK_{int}, corresponds to a range of heats of ionization from about 7,000 cal. near the top of the difference curve to about 12,000 cal. near the bottom. Only the former of these values is reasonable for the histidine residues to which at least a large part of the difference curve has been attributed. This discrepancy will be considered again later.

Data at 25° at 0.02 ionic strength on the acid side of the isoelectric point have already been published.[16] The older measurements are combined with new ones at 24.6°, in Fig. 8, in order to extend the data to the alkaline side of the isoelectric point as in the other figures. Again the same essential

(15) C. W. Carr, *Arch. Biochem. & Biophys.*, **40**, 286 (1952); **46**, 417 (1953).

(16) J. Steinhardt and E. M. Zaiser, THIS JOURNAL, **75**, 1599 (1953).

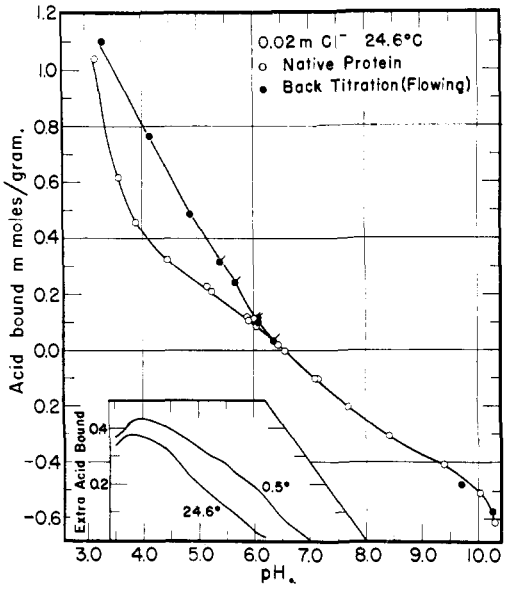

Fig. 8.—Titration data for native and denatured ferrihemoglobin at 24.6° in 0.02 *m* chloride. The insert shows the difference curves at 0.5 and 24.6°.

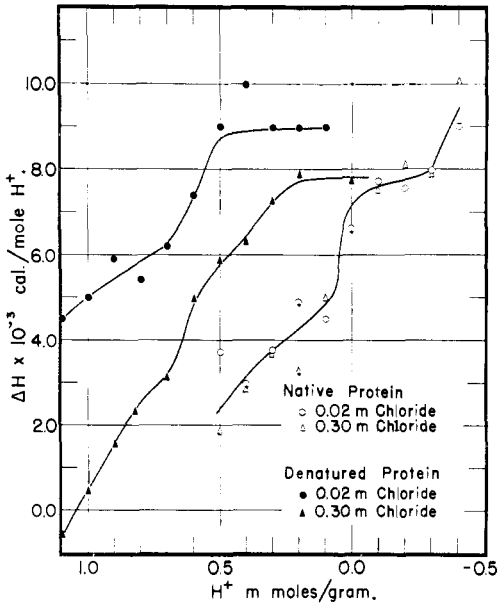

Fig. 9.—Apparent heats of hydrogen-ion dissociation of native and denatured ferrihemoglobin for the temperature interval 0.5–24.6° at two ionic strengths. The heats are calculated in the usual way from the *p*H displacements brought about by changes in temperatures.

features are shown. These data are characterized not only by the same narrowing of the nearly flat section at 0° noted above but also by a reduction in size of the superimposed sharper maximum at about *p*H 4. In fact, no convincing separation of "plateau" and "superimposed maximum" would be possible on the basis of data at this temperature alone. The shift of the difference curve to more acid *p*H is larger than in Fig. 7 and corresponds to heats of ionization of the unmasked groups of from 10,000 cal. to over 14,000 cal. at various levels of the difference curve. The implied dependency of ΔH on ionic strength is quite anomalous and requires explanation. It will be considered again later.

Before going on to consider further the effects of temperature, the data at 25° will be examined for a still more decisive distinction between an electrostatic and an unmasking explanation of the increase in acid binding on denaturation. Such a distinction results from a comparison of the observed *p*H displacement between native and denatured protein at constant acid-bound at the same ionic strength against acid bound, with the displacements which would be predicted on the basis of Tanford's suggestion. If neither n nor K_{int} change in denaturation, these displacements should be equal to 0.87 Δw. Table I gives the observed *p*H displacements at several values of hydrogen ion bound at 25°. The values of acid bound used in the table are restricted to those in which no measurable denaturation occurs in two seconds. Shown also in the last column are displacements calculated with Tanford's proposed values of w for native and denatured protein.

It will be noted that at ionic strength of 0.02 the observed displacements are more than twice those calculated. At 0.3 salt, the actual displacements are more than three times the calculated displacements. At the high ionic strength, no reasonable values of Δw could account for as much as one-half the observed *p*H shifts. Changes in electrostatic interaction, alone, therefore cannot account for the differences in titration behavior.

TABLE I

25°

Ionic strength	H⁺, mmoles/g.	pH$_{den}$	pH$_{nat}$	ΔpH$_{obsd.}$	ΔpH$_{pre}$
0.02	0.4	5.15	4.09	1.06	0.48
	.3	5.37	4.57	0.80	.36
	.2	5.77	5.27	.50	.24
0.30	.4	5.54	4.58	.96	.30
	.3	5.80	4.98	.82	.23
	.2	6.05	5.55	.50	.15

Finally, it should be noted that the observed *p*H displacements (next to last column) are essentially independent of ionic strength. If they depended, to any appreciable extent, on decreases in w brought about by denaturation, they should be substantially smaller at the higher ionic strength. The calculations in this paper have all assumed that native ferrihemoglobin is completely dissociated into half-molecules at all *p*H values of interest. It is reasonable to inquire, here, whether the validity of this assumption is critical to the predicted *p*H displacements considered above. If only denatured protein is completely dissociated and native protein is completely *undissociated* at, *e.g.*, *p*H 5.0, then the simple titration equation predicts at constant H^+ ion bound of 0.30 mmole/g. that the dissociation concurrent with conversion to denatured protein gives rise to a *p*H displacement of 0.10, or less than 12% of the observed displacement. The calculated displacements in Table I are rather insensitive to the exact extent of dissociation at the *p*H values which are involved.

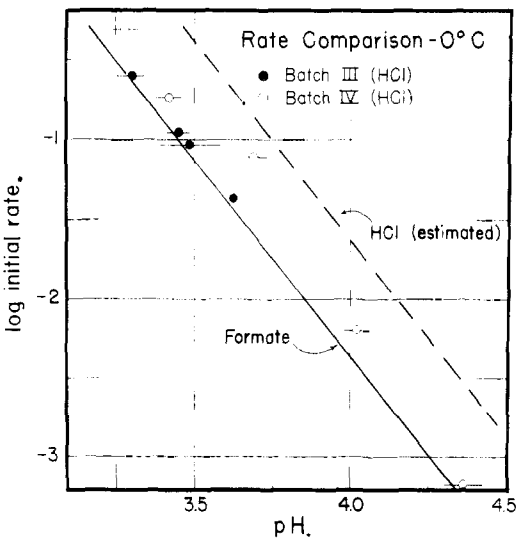

Fig. 10.—Logarithm of the rate of denaturation as a function of *p*H inferred from *p*H drifts. The values given are calculated from the drift in the first 15 seconds, in milliequivalents of H^+ bound per g. of ferrihemoglobin per minute, divided by the maximum attainable increment of acid bound at the same ionic strength. Results are shown for two batches of protein. Footnote 19 explains the position of the line given for spectrophotometric results with HCl.

Apparent and True Heats of Ionization.—Details of the variation of apparent $\Delta H_{\text{ionization}}$ with amounts combined are shown for both native and denatured protein in Fig. 9. The results with native protein are quite normal and qualitatively similar to the data of Wyman[17] for oxyhemoglobin. As expected, they do not depend on ionic strength. The results with denatured protein, however, are entirely different at the two ionic strengths used. They are anomalously large at the lower ionic strength. At high ionic strength, they are only slightly higher than for native protein. The discrepancies already noted in the apparent heats of ionization of the difference curves are obviously linked to the great differences obtained for denatured protein itself at the two temperatures. It will be shown in another paper that these differences are another manifestation of the differences in molecular identity between protein denatured at 0 and 25° for which kinetic evidence has already been reported.[6] In the present case the difference manifests itself in part as a difference in w for the two denatured forms. Thus the values for apparent ΔH obtained at high ionic strength, where w has, at most, a small effect, are more nearly the true heats of ionization. As already noted (discussion of difference curve in Fig. 7) the values found, although not constant over the entire curve, and on the average, somewhat high for histidine (suggesting a possible admixture with some amino groups) are closer to the values expected for histidine imidazole dissociations than for any other prototropic groups present in the protein.

Stoichiometry.—It may occur to the reader that there is some difficulty in identifying histidine residues with some of the unmasked groups, since Wyman's interpretation of his titration curve of oxyhemoglobin accounted for the combination with acid or base of all the histidine residues known to be present. This difficulty is only apparent, since Wyman did not take into account the dissociations of 4 terminal amino groups (the number of which was unknown when their experiments were done) nor of 8 ferrihemic acid groups,[13] all of which dissociate in or near the histidine range. The titration of these 12 groups within the expected range of histidine might well have been mistaken for the titration of histidine by Wyman. Such a confusion of identity might then account for about two-thirds of the 22 groups now shown to be masked in the native protein.

The present data for ferrihemoglobin at 25° are in good agreement with those of Wyman and Ingalls[18] between the most acid *p*H at which they worked (4.7) to at least *p*H 8.5, even though these workers used a slight lower ionic strength.

Rates of Denaturation Inferred from *p*H Drifts.—Earlier papers have shown that at 25° the rates of denaturation measured by spectrophotometry are close to those which may be inferred by the initial rate of acid-uptake calculated from *p*H drifts in titration measurements when they extend beyond 2–3 seconds. Such comparisons must be made with rate data obtained with buffers other than formate because of the specific protective effect of the latter. They are important because, if the rates are identical, there are no intermediate stages in which possibly only partial unmasking occurs.

In the course of the present measurements at 0°, with slower rates of denaturation than at 25°, new *p*H drift measurements have therefore been made to attempt a more critical comparison. These measurements are made by measuring *p*H as a function of time after flow is stopped. The rate constants calculated refer to disappearance of hydrogen ion in the first 15 seconds; they are shown as a function of *p*H in Fig. 10 for two batches of protein for 0.02 ionic strength. The results with the two batches of protein are consistently different, but the discrepancy is slight. The velocity constants decrease with decreasing H^+, with the same 2.5 power dependence found in the more accurate spectrophotometric kinetic measurements. However, the velocities are only about one-fifth as great as those found spectroscopically with HCl or acetic acid at this temperature.[19] This difference makes them correspond rather closely to the kinetic data obtained with formate (shown in the solid line), but this correspondence is probably a coincidence. The close agreement in the slope of *p*H dependence with the spectrophotometric data effectively eliminates electrode sluggishness as a possible explanation of the slower rate observed electrometrically. It must be concluded that denaturation (observed both spectroscopically and by changes in solubility) *precedes* unmasking. The over-all rate of increase in acid binding must be considered as proportional to

(17) J. Wyman, *J. Biol. Chem.*, **127**, 1 (1939).

(18) J. Wyman, Jr., and E. N. Ingalls, *ibid.*, **139**, 877 (1941).

(19) The velocities (measured spectophotometrically) found with formate occur at 0.36 higher *p*H at 0.5° than the velocities found with other acids. This displacement in *p*H dependence is very similar to the difference found at 25°, described in detail elsewhere.[2]

the velocity constant of denaturation which is *p*H dependent and to another "unmasking" constant which is independent of *p*H. This conclusion further confirms the reality of unmasking as a phenomenon distinct from changes in electrostatic interaction parameters incident to denaturation itself.

Equilibria Determined Titrimetrically.—If it is assumed, as hitherto, that all the protein exists in either fully masked (native) or fully unmasked (denatured) forms, it is possible to compare the equilibrium between native and denatured forms with similar equilibria determined spectrophotometrically.[2,6] Such a comparison is shown in Fig. 11 in which the equilibrium difference data at 0.02 ionic strength (Fig. 4) have been plotted as the logarithm of the ratio of denatured to native protein against *p*H. For comparison, spectrophotometric "equilibrium" data are shown for formate buffers and for HCl–KCl mixtures by two broken lines. The titration equilibrium results (which have been averaged for approach to equilibrium from both sides) lie closely to the spectrophotometric results at high *p*H (slight denaturation only) but appear to show less denaturation at *p*H values below about 4.4.[20] Denaturation is essentially complete in HCl–KCl at 0.5°, if judged spectrophotometrically, at *p*H 3.9; the averaged equilibrium difference curve still rises perceptibly at *p*H down to 3.6. If data obtained by approach to equilibrium from the regeneration side (in back-titration experiments) are used instead of averages, better agreement results and a single straight line without a break is obtained; however, its slope is lower than the slope obtained in the spectrophotometric experiments. No firm conclusions can be drawn since the spectrophotometric equilibrium data were corrected for irreversible loss, and similar corrections are not possible with the titration data. It is clear, however, that both equilibrium phenomena occur in the same *p*H range—if the differences in slope are real, they may be attributed in part to the difference in spectrophotometric and titrimetric rate constants described in the preceding section.

Recapitulation.—The principal kinds of evidence that indicate that there is a real increase in acid-binding groups when ferrihemoglobin is denatured in acid solutions and that the increase in acid binding cannot be attributed entirely to a change in molecular parameters that decreases the electrostatic interaction factors are summarized below.

1. A substantial difference between the acid bound by native and denatured protein persists in the presence of high enough salt concentrations to reduce greatly the effect of both w and Δw on the titration and difference curves. In such salt concentration, the maximum difference found shows a distinct plateau with respect to *p*H.

2. No differences in base binding between native and denatured proteins are found at *p*H values well above the isoelectric point; if the differences found on the acid side were due to electrostatic factors, similar differences in base binding should appear at *p*H values between 8 and 10. Furthermore, in one case (Fig. 5), the difference curve extends beyond the isoelectric point before vanishing.

3. At high salt concentrations at 0° and at both high and low salt concentrations, at 25°, the changes in *p*H at which the same amounts of acid are bound by both native and denatured protein are much larger than can be accounted for by any familiar electrostatic model without changes in the number of accessible groups.

4. Rate comparisons indicate that denaturation *precedes* the appearance of increased acid-binding capacity, suggesting that denaturation and the unmasking reaction are distinct rather than identical processes.

5. The large changes in the electrostatic interaction factor w inferred by Tanford from changes in viscosity due to denaturation depend on unconfirmed quantitative assumptions as to the causes of the viscosity change observed (expansion without important shape changes or aggregation, as well as increased permeability to solvent). It will be shown elsewhere that the viscosity changes themselves vary widely with a number of environmental factors present during the denaturation process. When measured under conditions comparable with those prevailing during the determination of "unmasking," the viscosity changes are substantially smaller than the changes which form the basis of Tanford's calculations.

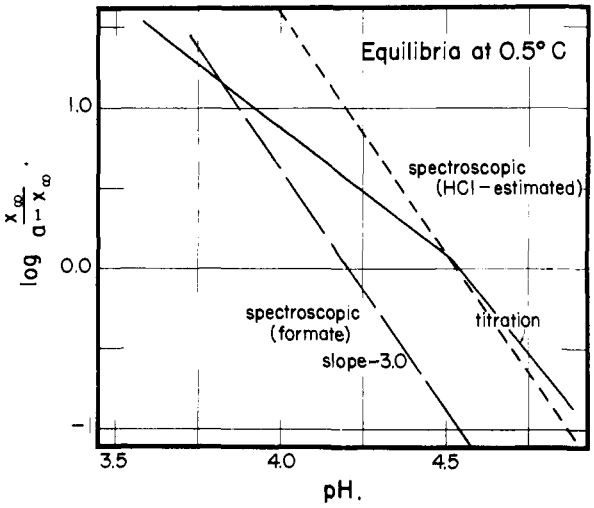

Fig. 11.—The equilibrium of native and denatured ferrihemoglobin determined from increments in acid binding (HCl) as a function of *p*H. Equilibrium curves determined spectrophotometrically are included for comparison.

Acknowledgments.—The authors are grateful to Professor George Scatchard for generous provision of facilities and for numerous opportunities to engage in fruitful discussions of this work.

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(20) This discrepancy is the reverse of what would be expected from incomplete binding of acid by the unmasked groups, *i.e.*, it would appear at the higher *p*H values.