[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE 39, MASS.]

Distinct Reversibly Denatured Ferrihemoglobins Produced at *p*H 3.5[1]

BY SHERMAN BEYCHOK AND JACINTO STEINHARDT

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The kinetics of the acid denaturation and subsequent regeneration of horse ferrihemoglobin indicate that different products are formed at 0 and 25° although the formation of both forms is accompanied by the unmasking of 22 imidazole groups. This conclusion is supported by: (a) analysis of the effects of ionic strength on their respective titration curves, which show that the 0° product has a lower electrostatic interaction factor w and hence is less compact, (b) analysis of the effects of temperature on the titration curves; striking anomalies in the apparent heats of ionization are shown to be attributable to the differences in w, (c) large differences in intrinsic viscosity, which confirm the greater compactness of the 25° product and (d) a preliminary estimate of a heat of transformation of one form into the other (in formate buffers, which unlike HCl, permit a readily attained labile equilibrium). The heat of transformation implies a dependence of heat of denaturation on temperature.

I. Introduction

When horse ferrihemoglobin is reversibly denatured by dilute acid at either 0 or 25° the products formed at both temperatures are spectroscopically indistinguishable and have been shown to possess twenty-two more acid-combining sites than the native protein.[2] Nevertheless anomalies in the temperature coefficients of the rates of denaturation and subsequent regeneration,[3] as well as anomalies in the effects of temperature on the titration curves,[2] have suggested that the product or products formed on exposure to *p*H 3–4.5 at 0° differs from those formed at 25°. The present paper confirms these differences more fully and directly and relates them to differences in molecular parameters which affect the electrostatic interaction term in the titration-equation and which also lead to wide (20%) differences in intrinsic viscosity. By exploiting these viscosity differences under conditions which permit equilibrium to be established between the two forms, it has been possible to demonstrate a substantial heat of transformation between them, and thus, a dependence of heat of denaturation on the temperature at which it occurs.

II. Experimental

All materials, methods and procedures were the same as previously described[2,4,5] except as stated below or in the followings ections.

Four different stock batches of ferrihemoglobin were used in obtaining the results presented here. No differences between the batches were observed.

In those experiments in which the temperature of measurement of a given property differed from the temperature at which the protein was denatured, the procedure for changing temperature was as follows:

1. **Regeneration Experiments.**—4 ml. of protein solution was denatured at *p*H 3.5 or 3.8 (0.02 *m* formate ion), depending on the temperature, from two to six half periods. This was then rapidly mixed with 71.0 ml. of buffer solution maintained at the temperature at which the measurement was to be made. The resulting solution (*p*H 5.15–5.17) was within about 1° of the desired temperature; less than one minute sufficed to bring the solution to within 0.1°. The rate constants given were calculated as previously described.[3]

2. **Back-titration Experiments.**—The denatured solution was transferred to a bath maintained at the temperature of the experiment and allowed to equilibrate for ten minutes. The completion of the measurement of acid bound required an additional 25–30 minutes.

3. **Viscosity Experiments.**—When denaturation was complete (over 10 half periods) the solution was transferred to the viscometer bath and equilibrated for ten minutes. It was then introduced into the viscometer and re-equilibrated for one minute after which measurements of flow times were begun.

Viscosity measurements were made in Cannon–Fenske capillary viscometers which had water flow times greater than 100 sec. at 25°. The correction for the kinetic energy of the effluent liquid was negligible in all cases recorded here. Temperature variations in the bath did not exceed 0.02°. Kinematic, rather than absolute, viscosities are reported here. Only in the calculation of the Huggins' constants (see below) were corrections applied for difference in density between solvent and solution. The absolute viscosities were then calculated in the manner suggested by Tanford,[6] for further computation of the Huggins' constant (see below).

When solutions of hemoglobin (at *p*H near 7) and formate buffers of known composition are mixed, the *p*H of the mixture differs slightly from that of the buffer alone. The binding of hydrogen ion by the protein results in a change of the buffer anion/acid ratio. The final anion concentration may be calculated from a knowledge of the initial buffer concentration, the dissociation constant of formic acid (at the resulting ionic strength) and the *p*H; or, alternatively, from knowledge of the hydrogen ion bound by the protein at the *p*H and ionic strength of the mixture. Both calculations give very nearly the same values for formate concentration. In the viscosity experiments described below, the stated concentration of formate ion was calculated by the first of the two methods, which involves fewer assumptions. Such calculations are not required for the unbuffered solution of HCl–KCl.

III. Results and Discussion

Effects of Temperature.—The first indication that the products formed at 0° and at higher temperatures may differ was given by the data shown in Fig. 1. It is evident that in the temperature interval 15.5–25° (although not above 25°) there is a constant separation of the logarithm of the first order rate constants for denaturation, *i.e.*, the ratio of the rate constants is independent of *p*H and corresponds to an apparent energy of activation for the rate-determining process of about 16,400 cal. A similar constant ratio prevails, at least up to *p*H 3.65, in the lower temperature interval 0.2–15.5°, but the ratio is much closer to unity, and corresponds to a much smaller energy of activation, about 4,300 cal. This value is much smaller than the range of values usually found in chemical reactions. Since the choice of

(1) A brief account of this work was reported at the meeting of the American Chemical Society in Boston on April 7, 1959.

(2) S. Beychok and J. Steinhardt, THIS JOURNAL, **81**, 5679 (1960). Earlier work is summarized in J. Steinhardt and E. M. Zaiser, *Advances in Protein Chemistry*, **10**, 151 (1955).

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

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

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

(6) C. Tanford, *J. Phys. Chem.*, **59**, 798 (1955).

the temperature separating the two intervals is quite arbitrary, it is wholly possible that the difference in apparent energies of activation in the upper and lower temperature ranges is even larger than in the calculations just given.

Two possible explanations for this anomaly have been offered.[3] The first, and least likely, is that there is an unexplained difference of about 12,000 cal. in the ΔH of ionization of the rate-controlling trigger groups previously hypothesized (at least 5000 cal. per group) in the two temperature intervals (this presupposes that the energy of activation of the unstable ionic species is not known in either interval). The second, and more plausible, is that the protein may be denatured in two or more different ways, characterized by different temperature coefficients. Thus, the products formed by the reactions having the higher temperature coefficients will predominate above some critical temperature (presumably near 15°) at which the rates are equal, and the products formed by the reactions having the smaller temperature coefficients will predominate below this temperature.[7]

This second hypothesis is strongly supported by differences in the effect of temperature on the regeneration of protein formed at 0 and 25°. These differences are shown in Table I, which represents the results of regeneration experiments at a *p*H value at which regeneration is essentially complete at both 0 and 25°. Protein denatured at *p*H 3.50 at both these temperatures was allowed to regenerate at the temperature at which it was denatured and at the second temperature as well; rate constants for all four regenerations were calculated.[3] At both temperatures of regeneration the protein denatured at 0° was regenerated over twice as fast as that denatured at 25°, although the rates of regeneration at the two temperatures differ by a factor of ten. The same result was found with material denatured at a slightly higher *p*H which has the effect of slowing down the rate of regeneration.[3]

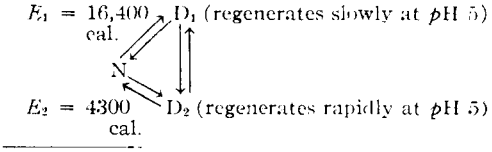
TABLE I

REGENERATION IN FORMATE AT *p*H 5.16 AFTER DENATURATION AT *p*H 3.50

Denatur., °C.	Regener., °C.	Corr. rate × 10^4	Velocity ratio
0.2	25	792	2.6
25	25	306	
0.2	0.2	75	2.3
25	0.2	36	
0.2	0.2	46[a]	(2.0)
25	1.7	25[b]	

[a] Denatured at *p*H 3.55. [b] Regenerated at *p*H 4.76.

We may recapitulate the information just presented in the following simple model (which omits all complications of the intermediate product in regeneration recently reported[3]).



(7) The differences in activation energy described may represent differences in activated state (different reaction paths) rather than different products, but the evidence for different products given immediately following makes it unnecessary to consider this alternative.

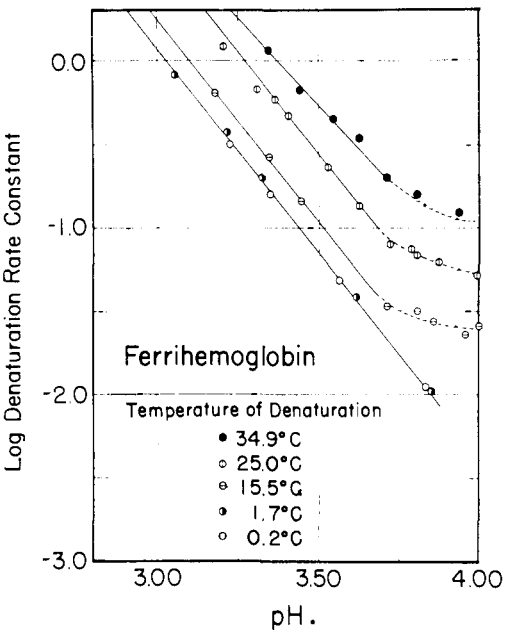


Fig. 1.—Rates of denaturation of horse ferrihemoglobin in 0.02 *m* formate buffers at five temperatures.

Here N represents native ferrihemoglobin, D_1 the denatured product or products formed at 25° and D_2 the denatured product or products formed at 0°. In the balance of this paper reference to D_1 and D_2 as the 25° form and the 0° form, respectively, does not necessarily imply that either is a homogeneous product or that there may not be more than two different products determined by the temperature of denaturation.

Effects of Ionic Strength on Titration Curves.—A further clear distinction between the two products may be demonstrated by analysis of the salt effect on the titration curves of native and denatured protein.[2] The analysis which follows also demonstrates a difference between the two forms in one specific physical molecular property.

The effect on the titration curves of increasing the ionic strength is to displace (raise) the *p*H at which any given amount of hydrogen-ion is bound. The displacements in *p*H as a function of acid bound are shown in Fig. 2. With native protein (steep line on right of figure) these displacements are the same at both 0 and 25°. This is entirely in accordance with expectation since the slope (when given in molecular weight units) of the straight line which roughly represents the points, should be 0.868 Δw; where Δw represents the change in the electrostatic interaction factor w in the familiar titration-equation due to changes in ionic strength.[2] Since w should be almost independent of temperature, the displacements at the two temperatures should be very nearly the same.

With the denatured protein, however, the slopes are not only smaller but also distinctly different at the two temperatures. Since the total number of titrating groups is the same at both temperatures and both ionic strengths[2] and pK_{int} is independent of ionic strength, the difference in slope can only mean that denatured protein formed at 0° not only has a smaller electrostatic interaction factor than

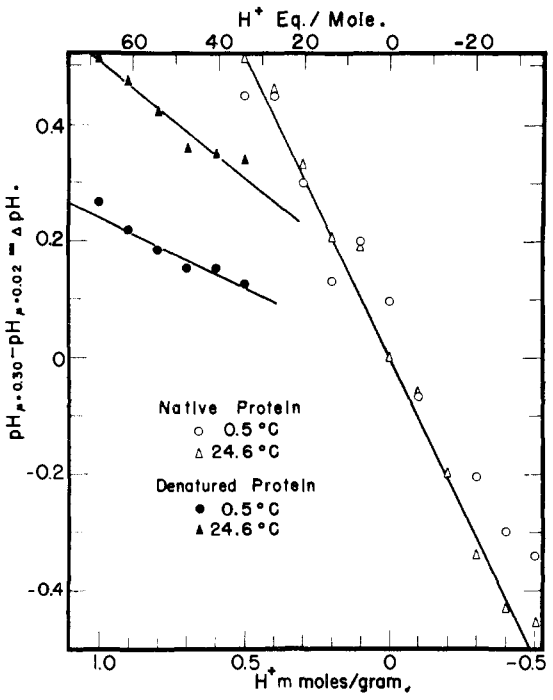


Fig. 2.—*p*H displacements in the titration curves of native and denatured protein caused by differences in ionic strength (0.02 and 0.3 *m*). The upper set of data for denatured protein is for denaturation at 24.6°, the lower is for protein denatured at 0.5°. Data for denatured protein is given only for the *p*H region in which no precipitation occurs.

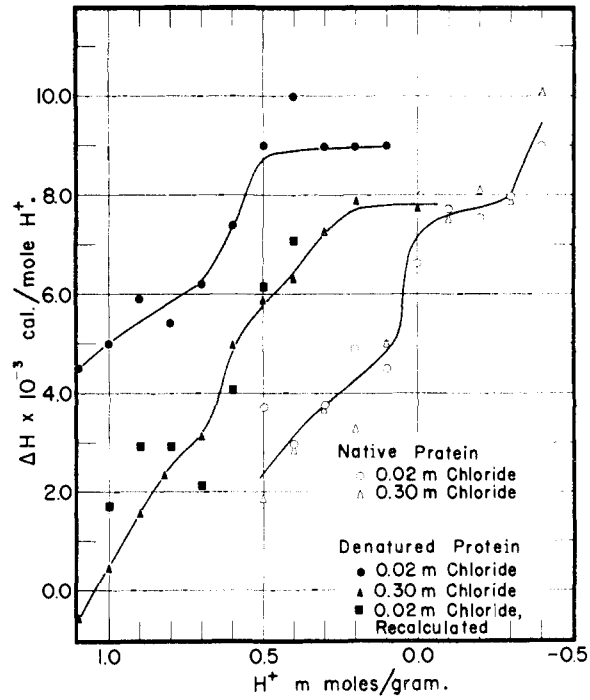


Fig. 3.—Apparent heats of hydrogen-ion dissociation of native and denatured ferrihemoglobin between 0.5 and 24.6° in 0.02 and 0.3 *m* chloride. The heats are calculated from the *p*H displacements in the titration curves at the two temperatures. The solid squares represent the data for denatured protein corrected for the *p*H displacement (due to the differences in *w*) shown in Fig. 4.

native protein, but also a smaller factor than denatured protein formed at 25° (the difference in *w* at the two salt concentrations is about 0.033[2] for native protein, in good agreement with theory[8]; it is about 0.018 for protein denatured at 25° and only 0.012 for protein denatured at 0°[2]). The two denatured forms must then differ in at least one of those molecular parameters which determine *w*, *i.e.*, radius, density, degree of asymmetry or permeability to solvent ions. When produced at 0° the product is either less dense or more asymmetrical or is more permeable to solvent ions. Paradoxically, it is the lower temperature which produces the greater "unfolding" or "expansion" of the molecule; the higher temperature produces the more "compact" forms.

Effects of Temperature on Titration Curves.—Demonstration of the existence of differences in the electrostatic properties of the denatured proteins when formed at two different temperatures permits the resolution of previously reported anomalies in the effects of temperature on the titration curves of the denatured protein.[2]

These anomalies are shown in Fig. 3 (for the time being ignore the "recalculated" points represented by squares). Here the average heat of acidic dissociation (calculated from the *p*H displacement of points on the titration curve brought about by the change in temperature) is shown as a function of the amount of acid bound, at two widely different ionic strengths. With native protein the results do not appear to depend on ionic strength and are in good agreement with Wyman's data for oxyhemoglobin.[9] At large amounts of acid bound (>0.4 mmole/g., corresponding to *p*H values below 4.8) low heats of dissociation, which are characteristic of carboxyl groups, are found. In the region just acid to the isoelectric point there is a rapid transition through values near 6000, which are characteristic of imidazole groups, to the higher values characteristic of amino groups, at higher *p*H. With denatured protein, however, the results differ with ionic-strength. At the lower ionic-strength the apparent heats are everywhere more than 1000 cal. higher than in the case of native protein—in fact, when the highest amounts of acid are bound, they appear to differ by as much as 4000 cal.[10] Only the values found at the higher ionic-strength are "normal" and in good agreement with those demonstrated for the native protein.

When it is recalled that the heats are obtained from a shift in *p*H between corresponding points on titration curves obtained at different temperatures, it becomes obvious that they include any effects which may be due to differences in the values of *w* which characterize the denatured proteins formed at these two temperatures. It has already been shown that the titration curves of the two forms should differ, except at high salt concentration, because of differences in the extents to which electrical

(8) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, pp. 512–522.

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

(10) Note that the values characteristic of histidine are found over a much wider region of H^+ bound than in the case of native protein. This difference corresponds to the unmasking of 22 groups (all or most of them histidine) in acid denaturation as previously described.[3]

interaction can occur between the charges that result when their acidic groups dissociate. This interaction is much smaller in denatured protein formed at 0°.

It should therefore be possible to determine true heats of acid-dissociation for denatured protein by determining the titration curves of denatured hemoglobin at two different temperatures *after denaturing at a single temperature.* The fact that the titration curves of denatured protein formed at 0 and 25° at low salt actually differ is shown in Fig. 4, in which both are measured at the lower temperature (the reason for this choice of the temperature for measurement will become apparent later). The curve for protein denatured at the lower temperature lies at slightly more acid values than the curve for protein denatured at 0°. The displacement is in the same direction as that brought about by increasing the temperature (when no such differences in molecular species exist) and thus adds a fictitious component to the apparent heat of ionization. This experiment provides a very direct demonstration that the products formed at these two temperatures are physicochemically distinct.

The titration curve at 0° and 0.02 *m* salt in Fig. 4 obtained with protein denatured at 25° may now be used in combination with the 25° titration curve of protein denatured at the same temperature[2] to obtain new heats of ionization which are free of complications due to the changes in molecular parameters which are present when denaturation does not occur at the same temperature. These "corrected" heats are shown in Fig. 3 as small squares. They fall very close to the same curve as the "normal" heats which were obtained at *high* salt concentration. The anomaly has disappeared.[11]

It should be noted as a corollary that the fact that the anomalously high ΔH does not appear in experiments at 0.3 *m* salt does not mean that there is no difference in the denatured products formed at 0 and 25° under these conditions. It merely serves to confirm the hypothesis that the two products have the same numbers and kinds of dissociating groups but differ markedly in parameters that affect the electrostatic interaction factor, w. The additional *p*H displacement due to changes in w is absent at high-ionic strength because w itself is very small in high salt.

Viscosity in HCl–KCl.—Since an obvious way for w to be affected is by differences in the asymmetry or size of the denatured protein, recourse may be had to measurements of viscosity to determine whether such differences can be demonstrated. Information about size or shape can be obtained from both the intrinsic viscosity η_i or the way in which the solution viscosity, η, varies with concentration of dissolved protein. With most macromolecules in moderately dilute solution the dependence of η on concentration is given by[12]

$$\frac{\eta/\eta_0 - 1}{c} = \eta_i + k'\eta_i^2 c \qquad (1)$$

in which η_0 is the viscosity of the pure solvent and c is the concentration. The Huggins' constant, k', often gives important information about the molecular asymmetry or rigidity of the dissolved particles.[13]

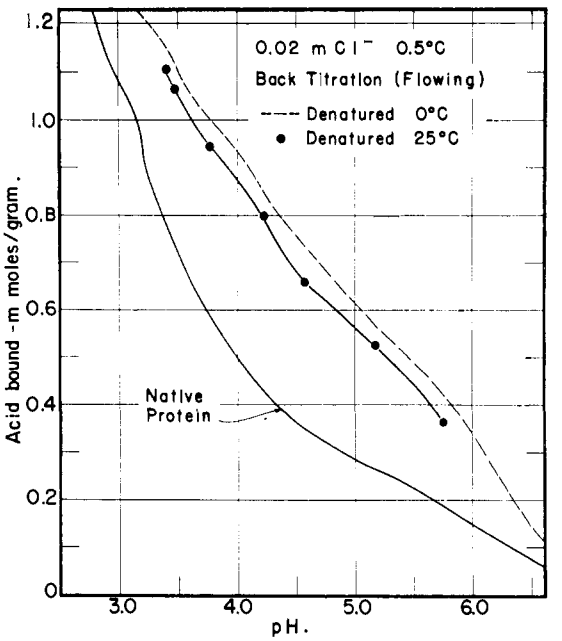


Fig. 4.—The effect of temperature of denaturation on the titration curves (back-titrations with base) of denatured protein. The back-titrations were carried out at 0.5° after denaturation at both 0° and 25°.

Figure 5 shows that eq. 1 may be applied to data obtained with ferrihemoglobin denatured at *p*H 3.5 in HCl–KCl mixtures at both temperatures and measured at 0° (the ordinate is proportional to the reduced viscosity, with a proportionality constant very near unity). Unlike native hemoglobin, measured at *p*H 7 (bottom of Fig.), denatured protein has a high η_i (intercept) and an *anomalously high* concentration dependence. Tanford has previously reported the great increase in η_i accompanying acid-denaturation, but his data are not characterized by the great concentration dependence shown in Fig. 5[14] (the difference in η_i, compared with native protein, is so large that the kinetics of denaturation have been successfully followed using a viscometer instead of a spectrophotometer). The most striking feature of the data in Fig. 5, however, is that protein denatured at 0° at either ionic strength has an intrinsic viscosity which is about 20% higher than protein denatured at 25°—*i.e.*, it is, as was concluded earlier, less symmetrical or less dense or more permeable to the solvent.

The anomalously high dependence of viscosity on concentration which is found with both kinds of denatured protein but not with the native protein, is reflected in values of the Huggins' constants between about 6 and 8.5 as indicated in Fig. 5. Most linear polymers (flexible coils) in good solvents give

(11) The residual scatter is due to failure to smooth the data of Fig. 4.

(12) M. L. Huggins, THIS JOURNAL, **64**, 2716 (1942).

(13) H. L. Frisch and R. Simha, in F. Eirich, editor, "Rheology, Theory and Applications," Vol. I, Academic Press, Inc., New York, N. Y., 1956.

(14) C. Tanford, THIS JOURNAL, **77**, 6421 (1955). Tanford's η_i for 25° on which he based his calculations of w is higher than any of the values in Fig. 6 by about 25%.

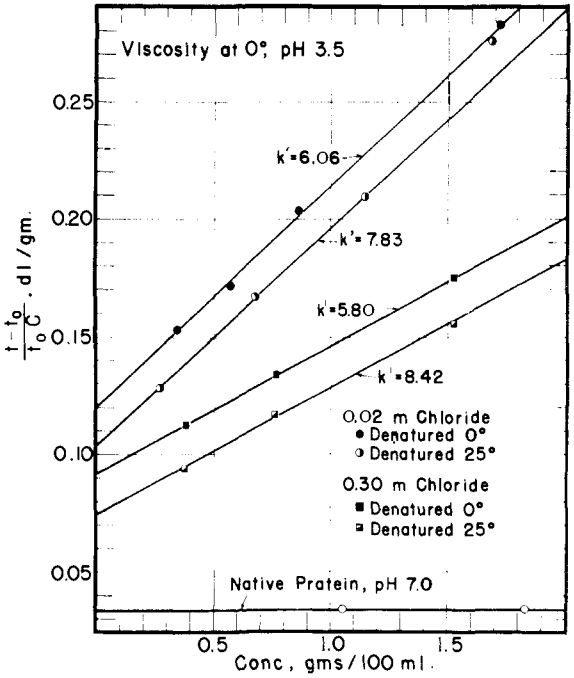


Fig. 5.—The viscosity (in HCl–KCl) at *p*H 3.5 of two forms of denatured ferrihemoglobin and of native ferrihemoglobin at 0° as a function of concentration. Results at two ionic strengths are shown. The ordinate (deciliters per g.) represents the fractional increase in flow-time over the flow-time of solvent per unit concentration, and is proportional and nearly exactly equal to the reduced viscosity ($\eta_{specific}/C$). k' (the Huggins' constant) is equal to the slope divided by the square of the intrinsic viscosity.

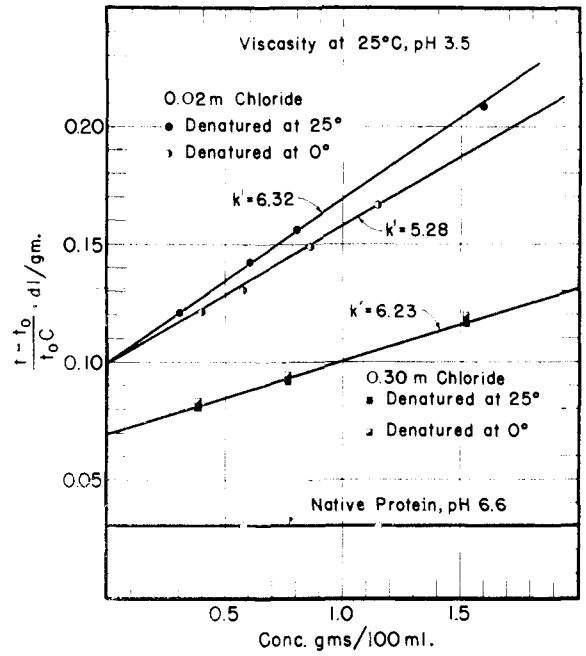


Fig. 6.—The viscosity of two forms of denatured ferrihemoglobin and of native hemoglobin, at 25° and *p*H 3.5 (HCl–KCl). Results at two ionic strengths are shown. The ordinate and k' are explained in Fig. 5. The broken line intercepting the ordinate shows the value of η_i reported by Tanford at this temperature at 0.04 ionic strength.[18]

values of about 0.3 or slightly higher. The theoretical value for highly asymmetric rigid rods is about 0.7, about one-tenth of the value observed. Compact spheres commonly give values under 2.5. There is a strong presumption that values as high as those obtained with denatured ferrihemoglobin indicate the effects of concentration-dependent aggregation, *i.e.*, at each concentration the point obtained lies on a different (inaccessible) concentration function which would (if accessible) extrapolate to a different η_i characteristic of the particular molecular size characteristic of the state of aggregation at the particular concentration. Thus, it is not possible to conclude whether the denatured forms are aggregates of rigid rods or of isotropically swollen spheres.

Changes in ionic strength do not appear to affect the concentration-dependence strongly (the Huggins' constants are not strongly affected: thus the aggregating forces do not appear to be electrostatic in nature), but the intrinsic viscosities (and therefore some of the molecular parameters) are strongly affected by ionic strength. Both molecules at infinite dilution appear to be more compact at the higher ionic strength.

We have chosen to present the measurements made at 0° first because at this temperature there is no detectable conversion of one denatured product into the other. The results are consequently clear-cut. At 25°, however, this is not the case. Protein denatured at 0° is fairly rapidly converted at 25° to the form produced by denaturation at 25°, within the time required to make the first viscosity determination. This difference in behavior is shown in Fig. 6. At 0.3 *m* chloride (lowest line) the data for protein denatured at both temperatures are practically identical. At 0.02 *m* chloride they are almost so—in fact, the intrinsic viscosities are identical. The denatured protein formed at 0° can be transformed in a short time at 25° to essentially the product which is formed initially in denaturation at 25°. Note, however, that a change of 25° in the temperature at which the measurements are made has very little effect on the intrinsic viscosity (<3% more viscous at the lower temperature) of protein denatured at 25° and has only effected a moderate reduction in the Huggins' constant. The smallness of these effects will be found useful later.

It may be objected that the kinetic evidence for the existence of different denaturation products shown in Table I shows no sign of the reversion of one form of denatured protein to the other which has just been demonstrated. It must be recalled, however, that regeneration is rapid and starts instantly on raising the *p*H above about 5. Thus, *even if transformation occurs at pH 5* (not demonstrated) the regeneration process is well on its way to completion in the time interval required for a viscosity determination. In titration experiments, however, which take as long as viscosity measurements, but which are conducted at *all* *p*H values, the same transformation shown in Fig. 6 can be demonstrated. Thus, in Fig. 4, to show a difference in the products formed by denaturation at 0 and 25°, the titrations were carried out at 0°.

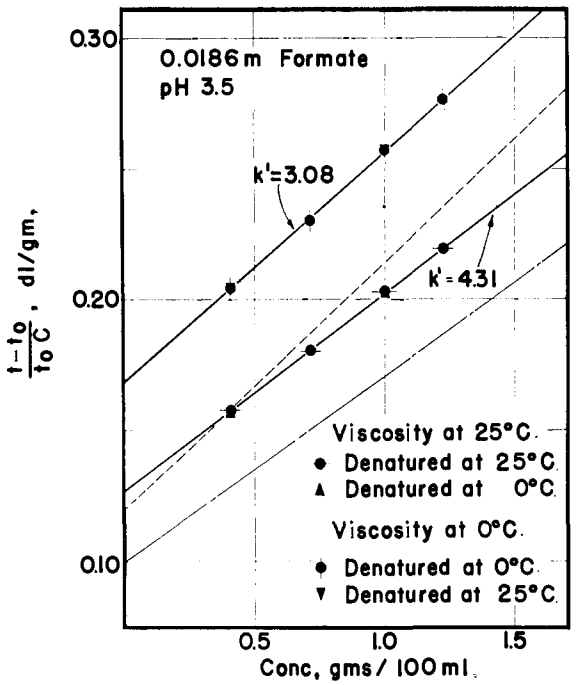


Fig. 7.—The viscosities of denatured ferrihemoglobin in formate buffers containing 0.0186 *m* formate ion at *p*H 3.5. The broken lines represent the data of Figs. 5 and 6 for protein denatured and measured in HCl–KCl at 0° (upper line) and denatured and measured at 25° (lower line) at 0.02 ionic strength.

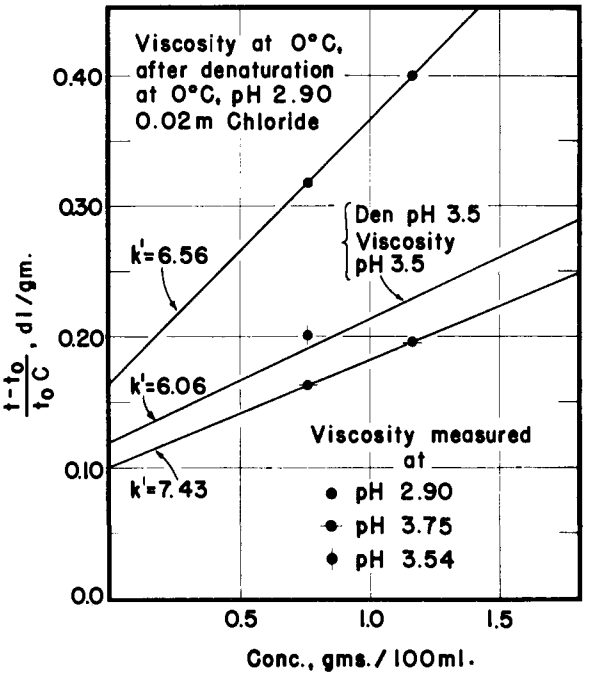


Fig. 8.—The effect of *p*H on viscosity of denatured ferrihemoglobin in HCl–KCl (0.02 *m*) at 0°. The *p*H of denaturation was, in every case, 2.90. The line labeled "Den. *p*H 3.5, Viscosity *p*H 3.5" represents the data in Fig. 5 for protein denatured at 0° in 0.02 *m* chloride.

When the measurements are made at 25°, no difference in acid-binding behavior can be demonstrated.

Viscosity in Formate Buffers.—It has been known for some years that ferrihemoglobin is more stable to acid denaturation in formate buffers than in other carboxylic acid buffers or in HCl.[15] This increase in stability causes a shift of over 0.3 unit in the log *k vs. p*H relationship which characterizes denaturation with other acids. The Soret band is also slightly less intense at 406 mμ in formate. Since the kinetic work (Fig. 1 and Table I), which first raised the presumption that different products were produced by denaturation at 0 and 25°, was carried out with formate buffers, it is to be expected that the viscosity differences found with HCl-denatured protein would also be found in formic acid denaturation.

Figure 7 shows that this expectation is realized but there are important differences which appear as new manifestations of the specific effect of formate ion. The intrinsic viscosities are characterized by even larger differences than when denatured in HCl (a difference of over 30% instead of 20%). Furthermore the lowest η_i is 27% higher than it is when the protein is denatured and measured in HCl–KCl at the same *p*H and at only slightly higher ionic strength. Protein denatured in formate is thus considerably less compact than protein denatured in chloride. There is a lower dependence in formate of viscosity on concentration, indicating that there is a lower concentration-dependent tendency to form aggregates—however, the Huggins' constants are not as low as would be required for unaggregated compact spheres (2.5). There is either a lower tendency to aggregate or else, as may be indicated by the higher intrinsic viscosity, aggregation may be more nearly complete at the highest dilutions.

Figure 7 also shows another significant difference in formate buffers. Regardless of the temperature at which denaturation occurs, the viscosities appear to depend only on the temperature at which the measurements are made. The results *at both temperature* thus resemble the results with HCl–KCl at 25° only. The transformation of D_1 into D_2 and its reverse reaction thus occurs quite rapidly at either 0 or 25° when formate is present—although apparently not rapidly enough (at higher *p*H) to obscure the kinetic differences shown in Table I.

Since this is the case it is cogent to inquire whether the results in formate show any differences between D_1 and D_2 at all, or whether, indeed, there is any need to evoke two distinct products to explain the viscosities in this buffer. The evidence on these points is clear. Attention has already been drawn, in discussing the HCl–KCl data of Figs. 5 and 6, to the fact, that the intrinsic viscosities (and to a lesser extent the Huggins' constants) of protein denatured at 25° were almost completely unaffected by the temperature of measurement. The substantial differences in intrinsic viscosity in formate at 0° and 25°—equal to the differences previously demonstrated as distinguishing D_1 from D_2 in HCl–KCl—can only mean that in formate also D_1 is a larger fraction of the total denatured protein at 25° than it is at 0°.

Equilibrium between D_1 and D_2 in Formate.—If there are only two denatured forms (D_1 and D_2)

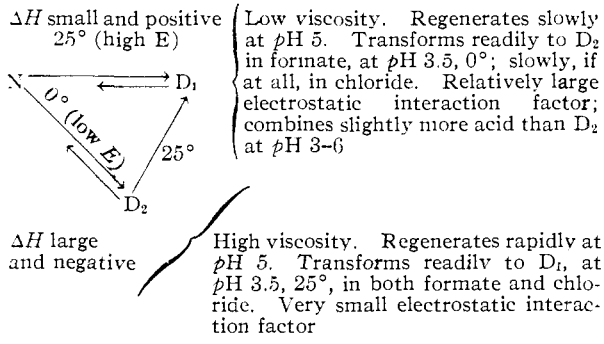
(15) E. M. Zaiser and J. Steinhardt, THIS JOURNAL, **75**, 1788 (1954).

present in this temperature range, then the reversibility of the reaction $D_1 \rightleftarrows D_2$ in formate should permit determination of the equilibrium between these forms and, from the temperature dependence of the equilibrium constant, the heat of transformation may be calculated. For example, if the transformation is 90% complete in each direction at each of the temperatures in Fig. 7, a value of ΔH of about 30,000 cal. would be deduced. The heat absorbed in denaturation itself for at least one of these temperatures cannot be less than half of ΔH for the $D_1 \rightarrow D_2$ transformation and may be greater by an indeterminate amount. However, the protonation of 22 unmasked imidazole groups, which is incident to denaturation by acid, will appear (by its attendant heat evolution) to reduce the true ΔH of denaturation by about 140,000 cal. when ΔH is measured in other ways. The values estimated earlier[16] from the *p*H displacement of the equilibrium by changes in temperature have been shown to be of doubtful significance because of the complex regeneration reaction[3] which involves the transient formation of an intermediate.

Effect of *p*H on Viscosity on Denatured Protein.—Since substituting formate ion for other buffer anions in kinetic experiments on denaturation has the effect of lowering by a constant amount, the *p*H, at which the same rates are obtained, it appeared possible that the peculiar effects of formate ion on viscosity might be manifested in other acids at a high *p*H, *i.e.* about 3.8–3.9. The effect of *p*H on viscosity of denatured protein at 25° at a single ionic strength was therefore investigated.

The results obtained at three *p*H values with protein denatured at a single *p*H are shown in Fig. 8. It is clear that the *p*H has a marked effect on intrinsic viscosity. The Huggins' constant is not affected. However, in HCl ηi at *p*H 3.75 is not the same as in formate at the lower *p*H—and its relation to concentration bears no resemblance to that prevailing in formate. The effect of formate on viscosity is thus highly characteristic and specific. The *p*H of denaturation is without effect on the viscosity, although the viscosity depends on the *p*H at which it is determined.

Recapitulation.—All of the evidence presented in this paper which indicates that ferrihemoglobin denatured by acid at 0° differs from the denatured protein formed at 25°—and which thus constitutes distinguishing properties of the two products—may be summarized in an expanded form of the diagram used earlier in this paper.



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(16) E. M. Zaiser and J. Steinhardt, THIS JOURNAL, **76**, 2866 (1954).

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, SCHOOL OF MEDICINE, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT]

Pantethine Analogs. The Condensation of Pantothenic Acid with Selenocystamine, with Bis-(β-aminoethyl) Sulfide and with 1,2-Dithia-5-azepane (a New Ring System)[1,2]

BY WOLFGANG H. H. GÜNTHER[3] AND HENRY G. MAUTNER

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As part of a project concerned with the development of potential coenzyme A antagonists, the following compounds were synthesized: selenopantethine, bis-(β-pantothenoylaminoethyl) sulfide and N-pantothenoyl-1,2-dithia-5-azepane. These compounds were prepared by the condensation of pantothenic acid with selenocystamine, bis (β-aminoethyl) sulfide and 1,2-dithia-5-azepane, respectively. The last compound represents a new heterocyclic system.

In the design of antimetabolites with potential carcinostatic activity, little emphasis has been placed on possible antagonists of coenzyme A or compounds related to it. Yet the literature contains several reports which suggest that tumor tissue might be more sensitive to the action of antagonists of coenzyme A than normal tissue. Thus, the capacity of tumors to utilize acetate and to oxidize fatty acids appears to be lower than that of non-neoplastic tissue,[4] rat tumors are reported to be deficient in coenzyme A and in pantothenic acid-content, as compared to normal tissues,[5] and in at least some neoplasms the coenzyme A content parallels certain synthetic capacities of the tumor.[6] Reports that the utilization of acetate is the first cell function, in a bacterial system, to

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(3) James Hudson Brown Memorial Post-doctoral Fellow, 1958–1959.

(4) J. P. Greenstein, "Biochemistry of Cancer," Academic Press, Inc., New York, N. Y., 1954, p. 374.

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(6) P. Emmelot and L. Bosch, *Brit. J. Cancer*, **9**, 327 (1955).