C. **Trifluoroacetic Acid.**—One-half gram of L-PBG (MW_w 680,000) was dissolved in 25 ml. of trifluoroacetic acid. Hydrogen bromide was bubbled into the solution for 15 minutes, and the reaction was allowed to stand for 2 hours. It was then poured into several volumes of ether, filtered and extracted with ether in a Soxhlet extractor, giving 0.25 g. of product, $[\eta]^{0.2\ M\ Na_2SO_4}_{pH\ 7.3}$ 1.4.

In later preparations, *e.g.*, those reported in the table, the trifluoroacetic acid was removed by freeze-drying (KOH trap to protect pump) after eliminating most of the hydrogen bromide on the aspirator. The L-PGA then was extracted with ether or acetone to remove benzyl bromide.

D. **Benzene.**—This is the most convenient solvent for preparing L-PGA. γ-Benzyl-L-glutamate NCA was polymerized in benzene at 1% concentration as described.[3] For MW_w determination a sample of the viscous polymer solution was then removed and the L-PBG isolated by precipitation into ethanol. A gas inlet tube bearing a calcium chloride drying tube on the vent was attached to the flask containing the remainder of the polymer solution. Hydrogen bromide was then bubbled into the solution for about an hour, after which the solution became a thick gel. It was allowed to stand overnight, and the supernatant benzene was drawn from the reaction flask by means of an aspirator the next day. The L-PGA usually is a small, hard lump at this time. If it is not, the reaction should be allowed to stand until the L-PGA has become a hard lump; if the reaction is stopped before this time, the product will be incompletely debenzylated. After removing the bulk of the benzene, the flask was placed first on aspirator vacuum to eliminate most of the hydrogen bromide, and then on a vacuum pump (use KOH trap) to evaporate the remaining benzene. The L-PGA was extracted with acetone in a continuous extractor until all the colored by-products and benzyl bromide were removed, and finally dried. The yield was the theoretical, and the results of several experiments are given in Table I.

E. **Sulfur Dioxide.**—A 125-ml. pressure bottle containing 2 g. of L-PBG (MW_w 680,000) was dried at 110°. Anhydrous sulfur dioxide (50 ml.) was condensed into it in a Dry Ice-bath (protected by a calcium chloride drying tube); the bottle was closed and allowed to warm to room temperature until the polymer dissolved. It was then cooled again in Dry Ice, opened, and hydrogen bromide bubbled in for 15 minutes (drying tube). The bottle was closed again and allowed to stand two hours at room temperature. After cooling again in Dry Ice, it was opened and the sulfur dioxide poured out, leaving the L-PGA behind. The rest of the SO_2 evaporated and the product was washed thoroughly with ether, leaving 1.2 g. of L-PGA containing no benzyl groups and having $[\eta]^{2\ M\ Na_2SO_4}_{pH\ 7.3}$ 1.64.

Anal. Calcd. for $C_5H_7NO_3$: C, 46.5; H, 5.4; N, 10.8. Found: C, 46.8; H, 5.9; N, 10.2.

Optical Rotation Measurements.—Optical rotations were measured with a Rudolph high precision photoelectric polarimeter, model 200, using a General Electric H-100-A4 mercury lamp as the light source. The 546 line was isolated with a Baird interference filter in conjunction with Corning Glass filters #9780 and #3484. Measurements were made at 22°; concentrations were 0.2%.

The sample of L-PGA used for the optical rotation measurements was #186-C prepared by method D. It was dialyzed for 24 hours at *p*H 7.3 then lyophilized, $[\eta]^{0.2\ M\ NaCl}_{pH\ 7.3}$ 0.93, DP_w 630, MW_w 82,000.

Anal. Calcd. for 80% sodium salt and 20% free L-PGA: C, 40.9; H, 4.3; Na, 12.2. Found: C, 40.4; H, 4.3; Na, 12.0.

Acknowledgment.—We are pleased to acknowledge the support of this work by the Office of the Surgeon General, Department of the Army, and the valuable technical assistance of Misses Evelyn DesRoches and Ann Gounaris in the preparative work, and Mr. Kenneth Norland in the optical rotatory measurements.

BOSTON 15, MASS.

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

Kinetic and Equilibrium Measurements of the Regeneration of Acid-denatured Horse Ferrihemoglobin[1]

BY JACINTO STEINHARDT, ETHEL M. ZAISER AND SHERMAN BEYCHOK[2]

RECEIVED APRIL 3, 1958

Measurements are presented of the rates of denaturation and regeneration of horse ferrihemoglobin over more than one *p*H unit in formate buffers at 0.2°. The kinetics of denaturation (which is accompanied by a large increase in acid-binding groups) are first-order and uncomplicated, as previously reported for higher temperatures. Evidence is presented that the product formed varies continuously as a function of the *p*H and temperature of denaturation. Rate measurements on regeneration, now made in a direct manner for the first time, show that regeneration, although first-order also, yields first an intermediate product, spectroscopically similar to native protein but characterized by its effect of inhibiting the reaction by which it is formed. The inhibitor reverts, on standing, to apparently normal native protein which can be denatured again at the original rate. The highly anomalous kinetics of regeneration are uniquely explained by the existence of this inhibitor, which is also demonstrated directly by experiments devised for that purpose. The rate of the regeneration reaction, contrary to earlier reports, is independent of *p*H. Equilibrium data are given, including values of ΔH, as are temperature coefficients for two of the underlying reactions. The difficulties of interpreting the equilibrium, or of relating ΔH to the activation energies measured, are discussed in detail.

I. Introduction

Previous papers[3-5] have reported that when horse ferrihemoglobin is denatured by acid, in the *p*H range 3.0–4.2, the reaction is accompanied by an increase in its acid-binding capacity which corresponds to the addition (in one step) of about 36 basic groups per molecule. The identity of the new basic groups has been discussed at length elsewhere[6]; the evidence strongly favors liberation of an equal number of carboxylate (or carboxylate plus a few imidazole) and lysine ε-amino or guanido groups. At the *p*H of denaturation the lysine or guanido groups immediately combine with protons from carboxyl groups, so that the over-all effect is an apparent increase in carboxylate only. This increase, like the denaturation itself,[7] is almost

(1) A brief account of this work was reported at the meeting of the American Chemical Society in New York on September 12, 1957. Some of the data were presented at the meeting of the American Society of Biological Chemists at Atlantic City, N. J., in April, 1954.

(2) Certain of the measurements in this paper were made by Robert J. Gibbs, whose assistance is here gratefully acknowledged.

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

(4) E. M. Zaiser and J. Steinhardt, *ibid.*, **76**, 1788 (1954).

(5) E. M. Zaiser and J. Steinhardt, *ibid.*, **76**, 2866 (1954).

(6) J. Steinhardt and E. M. Zaiser, *Advances in Protein Chem.*, **10**, 151 (1955).

(7) Denaturation was followed quantitatively by both changes in absorption at 406 mμ and by changes in solubility at the isoelectric point.

fully reversible on raising the pH. Evidence for the existence of this type of reversible "unmasking" of acidic and basic groups during protein denaturation exists for several other proteins and fits in very consistently with other facts about the reactivity of native and denatured proteins.

By combining kinetic and equilibrium data, at each of several pH values, rate constants have been measured for the denaturation reaction and calculated or inferred for the regeneration reaction over the pH range 3.2 to 4.2 and at several temperatures between 15.5 and 34.9° [similar effects have also been observed with carbonylhemoglobin[8,9] but with this protein only incomplete reversal can be obtained, and thus only denaturation data have been reported].

Since, in the preceding work, the rate constant for regeneration was obtained only indirectly by combining the sum of rate constants for denaturation and regeneration ($k_1 + k_2$) which gave the observed velocity of denaturation, with the ratio (k_1/k_2) assumed to have been determined in equilibrium experiments, it is desirable to determine it also by direct measurement of the rate of the regeneration process in order to confirm the correctness of the model used in the calculations. Because both reactions were presumed to be first order and therefore governed by the sum of the rate constants ($k_1 + k_2$), k_1 could be determined unambiguously by studying denaturation at an acid pH in which k_2 is demonstrably negligible (reaction proceeds practically to completion). Similarly regeneration should be studied under conditions in which k_1 makes a negligible contribution. In the range in which k_1 and k_2 are both important equilibrium occurs, and the half-periods of denaturation and regeneration reactions should be both observable and exactly equal.

The present research therefore started with measurements at 25 and 15.5° of the rates of regeneration as a function of pH of protein initially denatured at pH near 3.5 to various extents up to 95%. The same formate buffers previously employed were used again. A number of unexpected phenomena appeared at once, all of which were inconsistent with the model of a simple first-order reversible system previously presented.

Because the critical initial stages of the unexpectedly rapid regeneration reaction could not be definitively established at the higher temperatures previously reported, the regeneration reaction was slowed by working at 0.2°. In order to study and interpret equilibria at this temperature, it therefore became necessary to extend the earlier denaturation experiments to 0.2° also. This paper thus permits determinations of temperature coefficients, activation energies, and heats of reaction over a wider range of temperature than was covered earlier and has required extensive revision of the concepts presented earlier. Because of the anomalies noted above, the scope of the experiments was enlarged to include the effects of denaturation time and pH of denaturation on the kinetics and equilibria involved in regeneration. The results have provided clues to essential modifications of the simple model presented previously[3,5,6] and have led to the discovery of substantial, although transient, differences between the regenerated and the original native protein.

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

(9) E. M. Zaiser and J. Steinhardt, THIS JOURNAL, **73**, 5568 (1951).

II. Experimental

Except as described below, all preparations, procedures and solutions were the same as those previously described.[3-5] pH measurements were made at 23–27°, and the values obtained were adjusted to the temperature of the specified experiment, using the values of Harned and Owen[10] for temperature corrections of the pK of analogous buffer systems without protein present (protein concentrations never exceeded 0.18% and were most often 0.06% or lower). All experiments were carried out at total ionic strength 0.02 (potassium formate).

Spectrophotometric runs were followed by observing the change in absorbancy of ferrihemoglobin (MHb) at 406 mμ, using a 1 mm. light path for 0.06% or 0.18% MHb solutions. With more dilute solutions a 10 mm. light path was used.

For regeneration experiments, acid-denatured ferrihemoglobin usually was prepared by the treatment of 0.765% protein solution at either the temperature of regeneration, or at 15° or at 25°, at stated conditions of pH and time. At the end of the denaturation period a small volume of denatured protein solution (usually containing residual native MHb since the times of denaturation were selected to cover from about 50% to virtually complete denaturation) was diluted by rapid addition of a large volume of another formate buffer (at the temperature of regeneration) of the same formate concentration but at higher pH, such that the final solution contained the desired protein concentration at various pH values between 4.08 and 5.39. Regeneration was then followed as a function of time after mixing, at 0.2, 1.7, 15.5 and 25.0°.

It was observed that even under conditions where there should be no measurable denatured protein at equilibrium (pH $\geq$ 5.16) the final optical density, D_M, after long periods of regeneration, falls short of the value for completely native protein, D_N, by a small amount which increases with the time of exposure to low pH during the preliminary denaturation process. Evidently a small amount of protein is denatured in a competing reaction which yields material which does not regenerate at all at the lowest temperature studied (some of it appears to regenerate exceedingly slowly at 15 and 25°). The effect of the presence of this material (referred to hereafter as "lost protein") has been allowed for by using the appropriate value of D_M in place of D_N in all calculations. It can be shown that lost protein is produced practically entirely from native protein rather than from already denatured protein (it is formed most readily under conditions which produce denaturation, and when native protein is still present). It is formed only during the initial denaturation rather than during regeneration (unless the latter is carried out at pH values at which partial denaturation can occur).

There is implicit in all calculations based on the spectrophotometric data the assumption that reversibly denatured ferrihemoglobin and "lost protein" have approximately the same relatively low extinction coefficients at 406 mμ. Observations made on solutions which were prepared to contain considerable amounts of lost protein indicate that this assumption is valid. Further support for this assumption comes from the close comparison, on kinetic analysis, with data obtained from precipitation studies, in which no denatured protein or "lost protein" is present in the assayed solution.

Experiments at 0.2° (±0.1°) were made in a 40-liter ice-water bath maintained at 0.2°, and samples were removed as needed. When a large number of experimental values were desired over a short time interval, the reaction was followed by taking readings on a solution kept in one cuvette in the Beckman DU Spectrophotometer for up to 1 to 2 hr. The temperature was kept at 0.2° by continuous circulation around the cell compartment of water from the ice-water bath.

(10) Harned and Owen, "Physical Chemistry of Electrolytic Solution," Reinhold Publ. Corp., New York, N. Y., 1950.

The data obtained by precipitating denatured protein[3,5] were confined to one temperature, 15.5°, since it proved impossible to dissolve enough sulfate at 0.2° to effect complete precipitation, and no other completely satisfactory precipitating agent was found for the experimental conditions at the lower temperature.

Results and Discussion

Denaturation at 0°.—Preliminary experiments established the desirability of slowing the regeneration reaction by working at temperatures near 0° in order to define its critical initial stages more closely. It was therefore also necessary to extend the earlier work on denaturation to such temperatures, in order to be able to test the relationship of rates to equilibria. A summary of the results of denaturation experiments (log velocity constant against *p*H) at 0.2 and 1.7° is shown in Fig. 1,

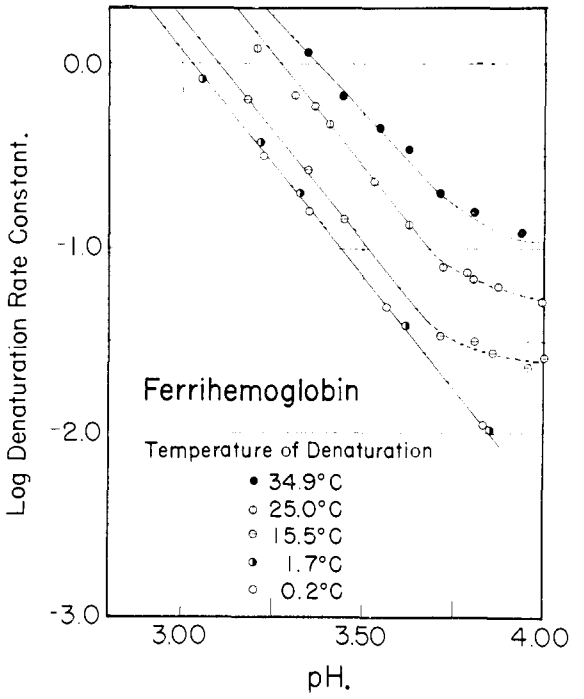


Fig. 1.—Rates of denaturation of horse ferrihemoglobin as a function of *p*H in 0.02 *M* formate buffers at 0.2 and 1.7°, compared with data at three higher temperatures reported earlier.

which also displays the previously reported data at higher temperatures. The kinetics of individual denaturation experiments at 0.2° are not shown since they do not differ in any other respect from the straight-forward first-order kinetics found at the higher temperatures.

It is evident that the effect on denaturation velocity of reducing the temperature from 15.5 to 0.2° (or 1.7°) is very small; the reduction in velocity is very much smaller than was found earlier in reducing the temperature by a smaller amount, from 25 to 15.5°. The change in velocity between the latter two temperatures, independent of *p*H, corresponded to an apparent energy of activation of 16,400 cal.[5] The velocity difference in the lower temperature interval corresponds to an average value of only 4,300 cal. and may be even lower at the lower end of the temperature range (there is hardly a discernible difference between data obtained at 0.2 and 1.7°). Such a remarkably small effect of temperature is rare for any chemical reaction and is particularly unexpected for denaturation of a protein.[11]

The existence of a lower temperature effect at low temperatures than at high suggests that the character of the denaturation brought about at 0° is qualitatively different than at 15°, or at least that the reaction follows a different reaction path at the lower temperature. Thus, the velocity of the reaction characterized by the higher temperature coefficient would be retarded much more by a drop in temperature than would that of the reaction with the low coefficient, so that the former might predominate at temperatures over 15° and the latter near 0°. It may also be shown that protein denatured at 0° regenerates more rapidly than protein denatured at 25° when both are regenerated at the lower temperature; and it also will be shown here that protein denatured at different *p*H values regenerates at different rates. These facts tend to support the view that the denatured reaction product at 0° is different from the denatured protein formed at higher temperatures,—that, in fact, the denatured product varies, perhaps continuously, as shown by its rate of regeneration, with the conditions of denaturation. Most, but not all, of the regeneration data reported in the present paper refer to the material denatured at 0.2°. Whatever difference may exist between it and protein denatured at higher temperatures, the denaturation appears to be initiated in both cases by formation of the same unstable ionic species, by reversal of dissociation of certain critical acidic groups,[6] since the slope (2.5) of the *p*H–log velocity relationship is the same at both 0.2, 15.5 and 25°.

Regeneration Kinetics.—If regeneration of denatured protein is first order, as earlier hypothesized,[6] a plot of log ($D_M - D$) against time should be linear (D_M is defined before). Representative results of regeneration experiments, in which varying initial extents of denaturation preceded regeneration, are shown in Fig. 2. There is a consistent departure from linearity, well outside any experimental error. The same data are tested for conformity with the requirements of a second-order process in Fig. 3, in which the reciprocal of the amount of denatured protein remaining is plotted against time. Here there is an approximation to linearity after completion of the initial 10 to 20% of the reaction which is more rapid than the rest, but other even more obvious anomalies appear. Thus the slopes of the straight lines shown differ widely and appear to depend on the amount of denatured protein present initially (or on the length of exposure to denaturing conditions). In a second-order process, these slopes, which represent the regeneration velocity constant, should all be the same. A third unexpected departure from the requirements of a second-order process, an anomalous strong dependence of slope (velocity constant) on

(11) The 12,100 cal. difference between the two apparent energies of activation is too great to be ascribed to any plausible difference in the effect of temperature on the dissociation of the trigger groups previously hypothesized.[3,6] Such a contribution could hardly be expected to exceed 3000 cal.

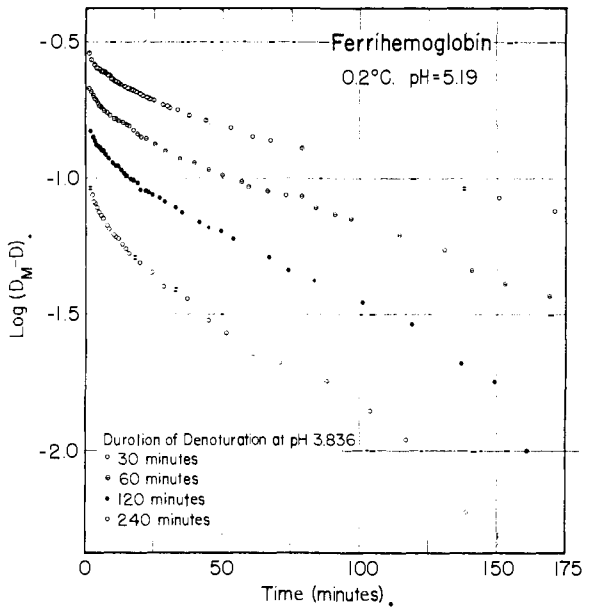


Fig. 2.—Regeneration of horse ferrihemoglobin denatured at *p*H 3.84 for various lengths of time. Denaturation and regeneration both occurred in 0.02 *M* formate buffers at 0.2°. The data do not conform to first-order kinetics.

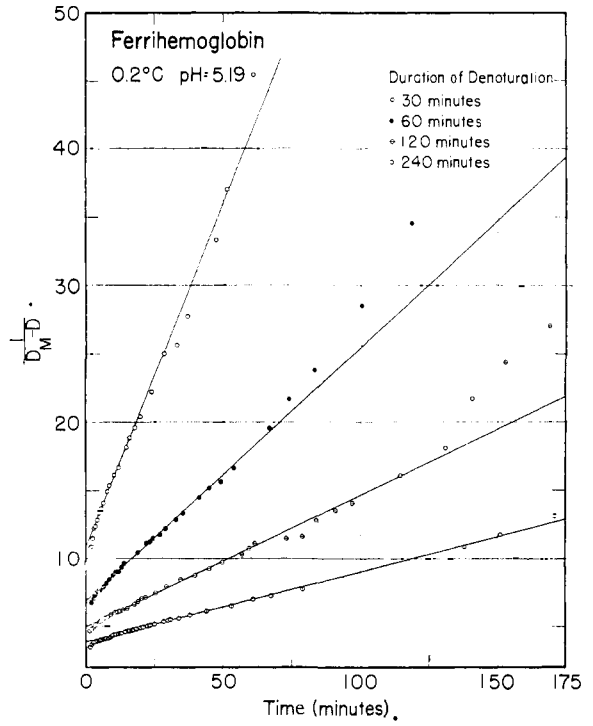


Fig. 3.—The data shown in Fig. 2 replotted to test conformance to second-order kinetics. Note presence of initial spurt and anomalous dependence of subsequent velocity on denaturation time.

concentration, is shown in Fig. 4, in which the total protein concentration has been varied thirty-fold, with various identical lengths of exposure to acid for each concentration. Thus, initial concentration of denatured protein rather than duration of exposure to acid appears to determine the slope. Explana-

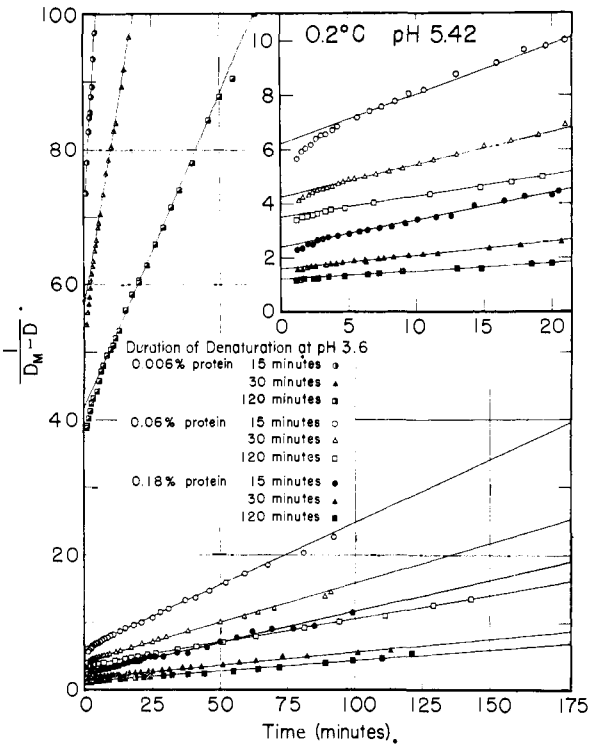


Fig. 4.—Anomalous dependence of apparent second-order velocity constant for "second stage" of regeneration reaction (after spurt) on protein concentration and on denaturation time.

tion of such dependence of velocity on *initial* as well as instantaneous concentration must be a feature of any theoretical model proposed.

A fourth anomaly is illustrated in Table I which shows that, in common with equilibria involving only first-order processes, but unlike any involving processes of higher order, there is no effect of protein concentration on the proportions of native and denatured protein present at equilibrium. The bracketed quantities are somewhat less reliable than the others; in such extreme dilution (0.006%) the loss of protein onto the reaction-vessel walls affects the accuracy of the measurements. However, within their limits of precision no effect of concentration can be discerned.

TABLE I

REGENERATED AT *p*H 4.35 AFTER DENATURATION FOR TIME INDICATED AT *p*H 3.83, 0°

Duration of denaturation (min.)	0.06% x_0/a	0.06% % denatured	0.006% x_0/a	0.006% % denatured
60	(43.2)	(17.2)	(44.2)	(14.3)
127	64.6	27.0	61.7	26.4
193	80.2	29.7	81.9	29.8

In summary, if attempts are made to account for the kinetics by uncomplicated first- or second-order isotherms, the following contradictions present themselves: (1) the reaction is not first-order, but the equilibrium reached is independent of concentration, a property which is characteristic only of reversible first-order reactions; (2) the reaction departs substantially from second-order kinetics during its initial stages, which appear to be more

rapid than the later stages (the opposite of an induction period); (3) if, nevertheless, the reaction is interpreted as predominantly second order, the velocity constants obtained appear to depend on the extent and conditions of the preceding denaturation.

It can only be concluded that the reaction is characterized by features which have not been encountered previously, and which preclude any possibility of interpretation by simple first- or second-order processes.

Model of the Regeneration Reaction.—Over a period of some years, numerous efforts have been made to explain the data shown, and a very large additional body of essentially similar data, on the basis of models which have embodied various *ad hoc* combinations of successive and parallel reaction-steps of either first or second order, with widely varied assumptions as to the heterogeneity of both native and denatured material. All of these efforts (some of which led to substantial mathematical or computational difficulties) have been unequivocally unsuccessful.

The uniquely successful model presented in this paper was developed as the result of emphasis on attempting to explain velocity constants which appear to vary with amount of denaturation, or with denaturation time. Velocity constants may appear to vary when they are proportional to concentrations of inhibitors. If either of these were to be formed or destroyed during denaturation, the velocity of regeneration would vary in a predictable way as the denaturation time was changed. This simple idea proved untenable when tested but led by extension to a related concept which not only accounted for all of the data, but also successfully predicted phenomena that had not yet been observed. The successful model simply assumes that the *precursor* of an inhibitor of regeneration is formed during denaturation; and that this precursor forms the inhibitor under the conditions that bring about regeneration. All the evidence presented here will show that the inhibitor is the *freshly regenerated protein* itself, and the precursor therefore simply all the denatured protein.

This view of denaturation is expressed in eq. 1; the equation assumes that all the processes involved are first order

$$\frac{dx}{dt} = \frac{-k_2 a}{(x_0 - x) + k_I} x \qquad (1)$$

in which k_2 is the first-order regeneration velocity constant, a is the total protein (native + denatured + regenerated), x is the denatured protein remaining at time t and x_0 is the initial concentration of denatured protein. The constant k_I has been included to provide that the initial rate (when $x = x_0$) should not be infinite. $k_2 a x/k_I$ is thus the velocity at time zero. The occurrence of the quantity a in the numerator is justified empirically, but it should be noted that without it the absolute rate would be independent of concentration (it is not), *i.e.*, the reaction would be zero order, instead of first order, although the rate would be time-dependent, as in reactions of first or higher order. Under the conditions of our experiments the constant k_I is evidently very small (and thus of no importance for other than time periods much shorter than could be observed). Consequently its existence has been disregarded, and eq. 1 has been used as if k_I were not included.

The integrated form of (1), neglecting k_I, is

$$\log \frac{x_0}{x} + \frac{x}{x_0} - 1 = \frac{k_2 a}{x_0} t \qquad (2)$$

It is obvious that if the quantity on the left side of the equation is plotted against time a straight line should result, the slope of which will be inversely proportional to x_0, the initial amount of denatured protein, *i.e.*, an apparent dependence of "velocity constant" on extent of denaturation, similar to the dependence found, is predicted.[12]

$$\log \frac{D_M - D_0}{D_M - D} + \frac{D_M - D}{D_M - D_0} - 1 = \frac{k_2 [D_M - 0.167]}{D_M - D_0} t \qquad (2')$$

Figure 5 shows the data previously presented replotted in terms of equation 2. It will be observed that the fit is excellent, even at the shortest times (no "spurt" is found). The data scatter only at over 75% completion where very small uncertainties in the end-value of the optical density (D_M) can have a large effect. The same satisfactory fit is observed (Fig. 6) when the data obtained over a thirty-fold range of protein concentration are similarly tested. The data of Figs. 5 and 6 also show the inverse dependence of slope on x_0 (and direct dependence on a) which was predicted; they would, in fact, all fall very close to a single straight line (k_2 truly constant) if the ordinate of each individual point were multiplied by the appropriate value of x_0/a. That this is true for the data of Fig. 5 is shown in Fig. 7 in which a/x_0 for each experiment has been plotted (large open circles) against the slopes of the lines through the data drawn in Fig. 5. If the straight line through the large open circles actually passed through the origin, all of the products of the slopes multiplied by the appropriate a/x_0 values would have been shown to be identical, and k_2 would be a true velocity constant. It is apparent that this is almost exactly the case; however, the small deviation is not certainly within the experimental error and may be real.

The smaller points which cluster about the same line in Fig. 7 represent experiments in which the preliminary denaturation occurred at *p*H values slightly above 3.8, and with regeneration at either *p*H 5.2 or 5.4. Obviously the k_2 values (corrected slope) for all these experiments are the same within the narrow limits, even though the preliminary denaturation time was varied between 30 and over 1800 minutes and regeneration occurred at two different *p*H values. It will be shown later that the regeneration *p*H is indeed without effect over much wider limits. With large variations in *p*H of *denaturation*, however, k_2 is affected.[13] Thus, samples of data obtained after preliminary denaturation with roughly four times more acid, and two times less acid, are also shown in Fig. 7; the fact that they do not superimpose on the other data (central straight line) indicates that the regeneration ve-

(12) In terms of observable optical densities at 0.06% ferrihemoglobin, eq. 2 becomes

(13) Changes in temperature of denaturation have been shown to have the same effect—the *p*H effect and the temperature effect are undoubtedly inter-related.

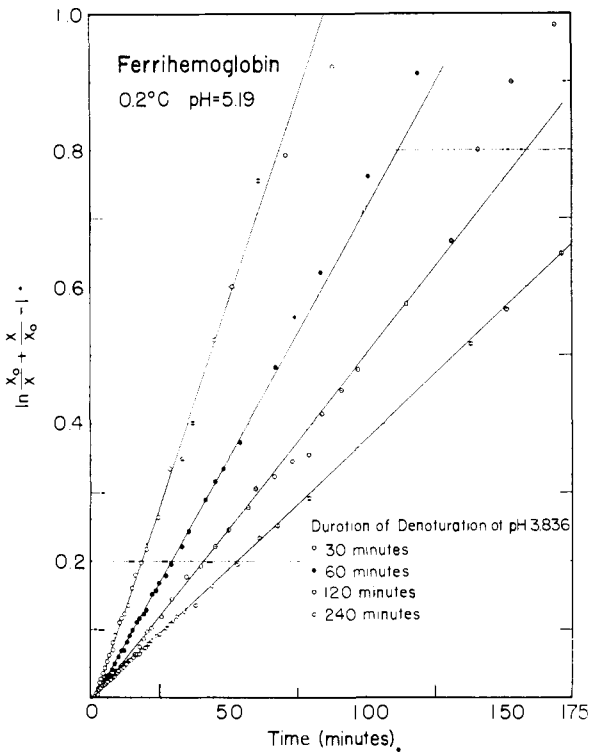


Fig. 5.—The data shown in Figs. 2 and 3 replotted to show conformance with eq. 2 (first-order regeneration reaction inhibited by product).

locity (k_2) depends on the pH of denaturation when it varies this widely. k_2 is larger when the protein is denatured in more acid solutions, *i.e.*, more rapidly.[14] It therefore appears as stated earlier that the "denatured" protein is not uniquely defined even at a single denaturation temperature.

All of these data, plus others obtained under other conditions (but excluding pH values at which regeneration stops short of completion because of approach to equilibrium), are summarized in Table II, in which the last two columns represent individual and average values of k_2. These values, for all regenerations carried out after denaturation at pH between 3.8 and 4.0 (including those at higher concentrations of protein), are in excellent agreement with one another. It is evident, however, that k_2 rises when the initial denaturation occurs at more acid pH (doubling by pH 3.15) and falls when the protein is denatured more slowly (at less acid pH). There is also a reduction in rate when the protein is denatured at 25° instead of at 0.2°. This is not surprising in view of the possibility previously noted in this paper of differences in reaction product when protein is denatured at widely different temperatures.

Since the regeneration experiments summarized in Table II were carried out at several different pH values (4.76 to 5.39), it should be noted that the regeneration pH is without effect on the rate. It will be seen that protein denatured at pH 3.92 was regenerated at both 5.16 and 5.39, with no significant

(14) The lines drawn through the data obtained with denaturation at pH 3.15 and 4.15 do not go through the origin, but there are too few points to justify attaching significance to this discrepancy.

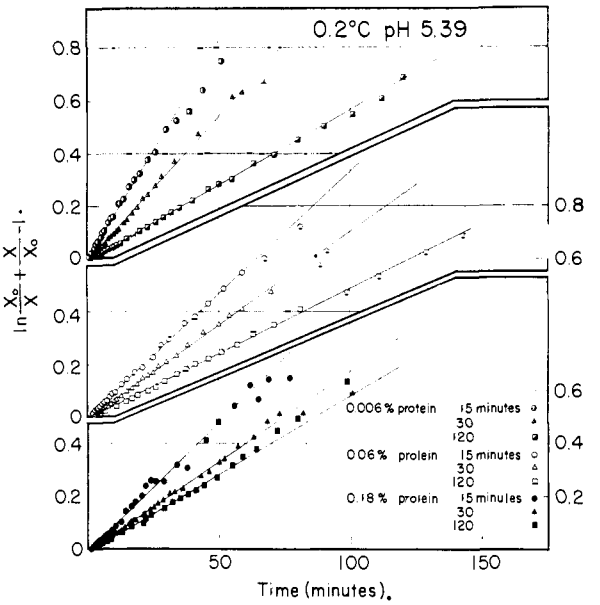


Fig. 6.—The data shown in Fig. 4 replotted to show conformance with eq. 2. The anomalous dependence on concentration has disappeared.

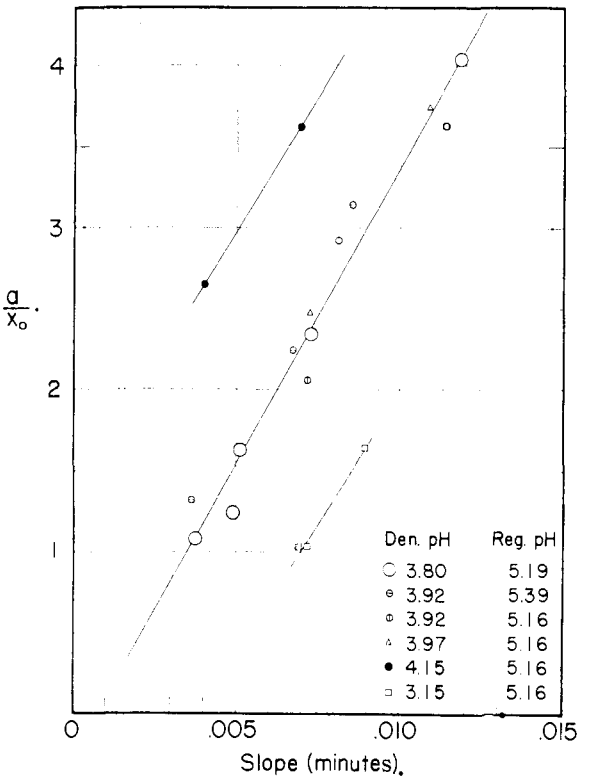


Fig. 7.—The relation between initial state (concentration of protein and fraction initially denatured) and slopes of lines shown in Figs. 5 and 6.

resulting difference in k_2. Figure 8 shows that much wider differences in regeneration pH had equally negligible effects (less than a twofold difference over a range of more than one pH unit, an interval which corresponds to more than a thirty-fold difference in rate of *denaturation*). Even the

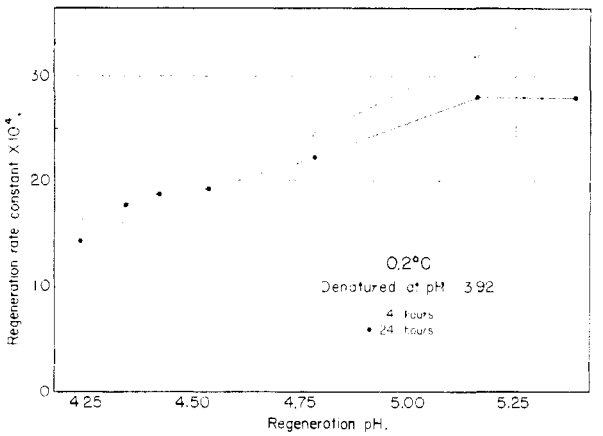


Fig. 8.—Absence of important dependence of regeneration rate constant on *p*H. Data shown at *p*H 4.75 and below are based on approximate application of eq. 2 to reaction systems that do not go to completion.

twofold difference indicated is doubtful, since the points in the figure obtained at *p*H values below 4.76 represent experiments in which regeneration was not complete. The rates shown were calculated without taking this equilibrium into account, since the kinetic law for the reversal region is exceedingly complex—thus the values obtained for k_2 were certainly too low.

Up to this point, it has been shown that eq. 1 describes all the regeneration data obtained at 0.2°. Before proceeding further to apply the equation to experiments specifically devised to test it critically, it is appropriate to inquire whether the model it describes is inherently plausible. In what ways is it reasonable to assume that *freshly regenerated protein* can act as an inhibitor of the regeneration of the denatured protein which remains? At least five different possibilities which could lead to eq. 1, have been considered.

(1) Freshly-regenerated protein may break reaction chains in which activation energy is passed on from molecules which already have reacted to those which have not. Since there is no obvious reason why freshly regenerated protein should differ from other forms of protein in accomplishing chain-breaking, this possibility has not been considered further.

(2) Freshly-regenerated protein may pre-empt reaction sites on catalytically active surfaces. There is no other evidence for the existence of, or necessity for, such surfaces.

(3) Freshly-regenerated protein may combine reversibly with D to form a complex (DI) which does not regenerate. This leads to equation 1, but only when D + (DI) equals x. Thus, (DI) would have to have the same absorption as D, which seems inherently unlikely.

(4) Freshly-regenerated protein may combine with, and thus make unavailable for reaction with denatured protein, excess amounts of an essential reactant (such as heme).

(5) The inhibitor may not be freshly-regenerated protein itself, but a substance whose formation runs parallel to that of regenerated protein, at least under the conditions of the present experiments. This possibility becomes less likely the more widely the experimental conditions may be varied without altering the validity of eq. 1.

TABLE II

0.2°, 0.02 ionic strength, 0.06% protein, formate buffers.

Denaturation *p*H	Denaturation time, min.	% Reversibly denatured[c]	Regeneration *p*H	Cor. rate $\times 10^4$	Av. $\times 10^4$
3.80	30	24.8	5.19	29	
	60	42.9		31	
	120	61.6		32	
	180	80.6		39	
	240	92.6		35	33
3.92	150	32.0	5.39	26	
	300	50.0		31	
	1200	75.8		28	29
3.92	110	27.6	5.16	31	
	150	34.3		28	
	300	48.4		35	31
3.97	180	26.8	5.16	29	
	360	40.5		29	29
4.15	660	27.6	5.16	19	
	1830	37.8[d]		20	19.5
ca. 3.78	50	46.3	5.39	26	
	100	63.9		33	
	400	77.6		21	27
ca. 3.78(0.18%)	50	42.5	5.39	26	
	100	57.5		35	
	400	79.9		32	31
ca. 3.55	15	47.2	5.39	47	
	30	66.6		47	
	120	89.5		43	46
ca. 3.55(0.18%)	15	40.4	5.39	39	
	30	60.5		42	
	120	86.3		52	44
ca. 3.15	*ca.* 4	61.4	5.16	55	
	16	97.4		70	
	32	96.9		67	64
ca. 2.9[a]	*ca.* 3	87.1	5.16	101	101
3.50[b]	5	66.3	4.76	25	
	10	86.5	(at 1.7°)	24	
	20	91.6		24	
	40	91.8		18	23

[a] Rate has initial spurt. [b] Denatured at 25°. [c] Lost protein excluded. [d] Reaches equilibrium.

Experimental Tests of the Model.—Although nothing further can be ventured now as to the likelihood of the alternative mechanisms listed above, it is easy to demonstrate by *direct test* that *freshly*-regenerated protein, and only freshly-regenerated protein, acts as an inhibitor of the regeneration reaction. Merely adding to the reaction mixture native protein which has never been denatured has no effect whatever on the kinetics or on k_2. Furthermore, it is possible to compare results when the same initial proportion of denatured protein is arrived at in two different ways, *i.e.*, in one case by denaturing until a given amount is present, and second, by mixing quantities of more completely denatured protein with enough native protein to reduce the proportion of denatured protein to the same figure. Since x_0/a is the same in both

cases, the slopes obtained should be the same if eq. 1 is valid. That this is indeed the case is shown in Fig. 9; the small difference in slope is accounted for

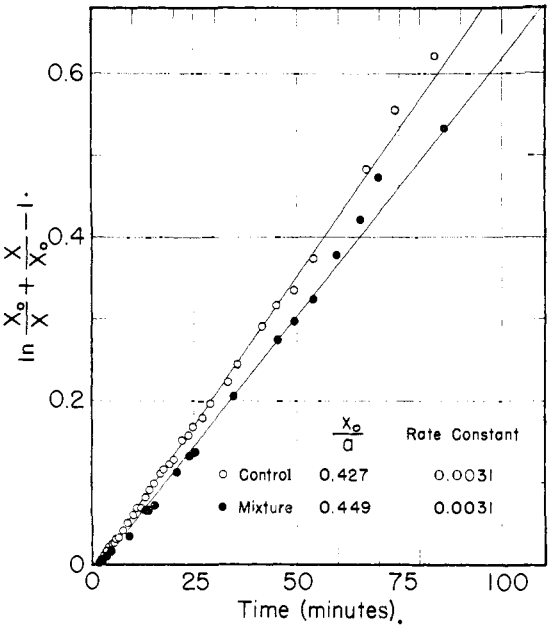


Fig. 9.—Test of dependence of regeneration rate on fraction of protein denatured initially.

exactly by the small differences in x_0/a. Finally, the direct addition of *freshly-regenerated protein* to a reaction mixture not only reduces the initial rate very substantially but causes the kinetics to depart radically from eq. 2 in a predictable way. Thus when freshly-regenerated protein is present *at the beginning* of the reaction, eq. 1 should be written (again omitting the very small term k_1) as

$$\frac{dx}{dt} = \frac{k_2 a x}{(x_0 - x) + I} \qquad (3)$$

where I is the initial concentration of freshly-regenerated protein added. On integration, this becomes

$$\left(1 + \frac{I}{x_0}\right) \ln \frac{x_0}{x} + \frac{x}{x_0} - 1 = \frac{k_2 a}{x_0} t \qquad (4)$$

which differs from eq. 2 by more heavily weighting the logarithmic term on the left-hand side by an amount proportional to I. Thus when data obtained with inhibitor added are plotted by means of the simpler (unweighted) ordinate used in Fig. 9, a straight line will no longer result; eq. 4 shows that, however, toward the end of the reaction (when the logarithmic term is no longer important) the curve should approach linearity, and possess the same slope as in the absence of inhibitor. Figure 10 shows that both these expectations are realized. In the experiment shown, freshly-regenerated protein (to the extent of 27% of the protein present) was added to the reaction mixture at time zero. The initial rate was greatly slowed, but eventually the slope becomes parallel to that of the control. A further test has been made by determining the time at which the regenerating control has reached a concentration of freshly-regenerated protein equal to about the same percentage (27) of the total pro-

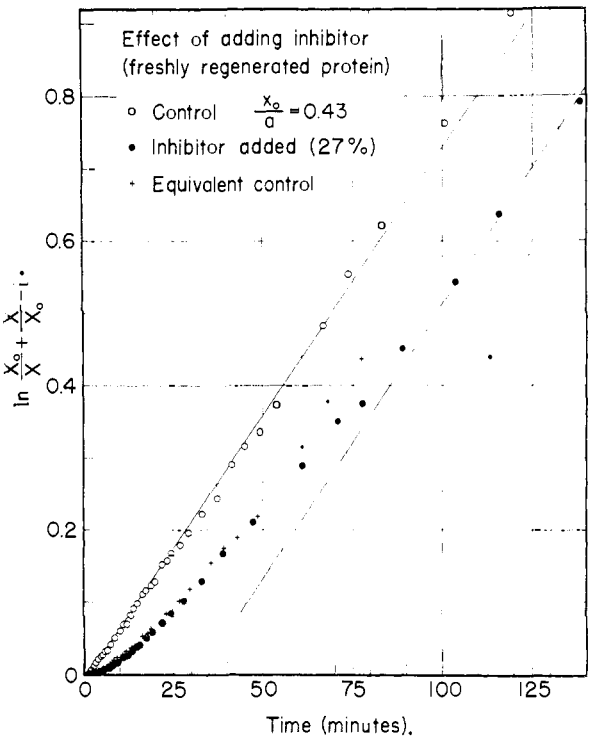


Fig. 10.—Test of effect of adding inhibitor (fully regenerated protein). The experiments shown are described in the text.

tein. This time was then called time zero, as if it were the beginning of the experiment, and all later points plotted at corresponding times. The results of this calculation, labelled "equivalent control," are shown by crosses in Fig. 10. The agreement with the experiment in which 27% of freshly-regenerated protein was added at the beginning is very good indeed. Very significantly *these results are not obtained* if the regenerated protein added is allowed to stand for a few hours before it is added.

The conclusion seems inescapable that the inhibitor is actually freshly-regenerated protein, or another substance which is formed exactly parallel to it. In addition, regenerated protein loses its power to inhibit the regeneration reaction within a few hours after it is formed.

Regeneration at Higher Temperatures.—The possibility of a fortuitously parallel formation of inhibitor other than newly regenerated protein may be examined further in the light of regeneration data obtained at 15.5 and 25°. Such data are shown in Fig. 11. In these experiments in which the regeneration temperatures were changed the protein was initially denatured at 25° instead of at 0°. Nevertheless, eq. 2 is obeyed.[15] Thus these experiments do not support the possibility that an inhibitor other than newly regenerated protein is involved.

In one important respect, not immediately evident in the figure, the data at 15.5 and 25° differ from those obtained at 0.2°. The constancy of k_2 does not ex-

(15) The only serious discrepancies occur in the case of protein denatured for 5 minutes (75%) and then only after regeneration has proceeded to well over 60% (in the 25° case to about 75%). These discrepancies are apparently fortuitous since they do not appear in other sets of data at these temperatures at slightly lower regeneration *p*H.

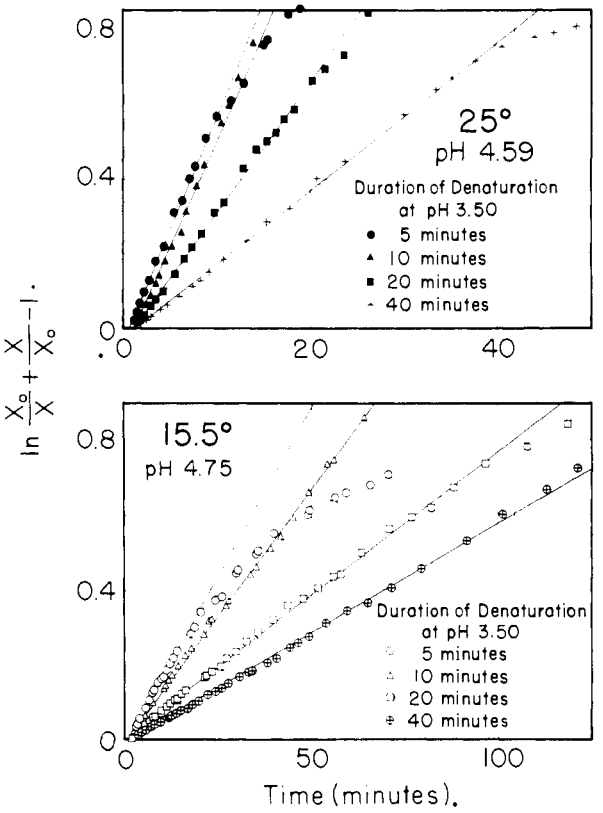


Fig. 11.—Test of compliance with eq. 2 of regeneration data obtained at 15.5 and 25° after initial denaturation at 25°. The extent of denaturation has been allowed to go higher than in the experiments at 0.2°.

tend to the longer denaturation times. This is shown in Table III. The values of k_2 in Table III show satisfactory consistency between values at the same temperature and denaturation time but different pH, but there is a well-marked tendency of k_2 to fall with denaturation times of eight half-periods and longer. This effect may not be peculiar to denaturation at 25° or to regeneration at temperatures above 0°, since it manifests itself only for experiments in which the initial denaturation was much more severe than in any carried out at 0.2°. Thus the longest denaturation time included in the data of Table II was approximately eight half-periods and all but two of the entries represent four half-periods or less. With such durations the k_2 values at the higher temperatures are also consistent. There is thus a suggestion that fully denatured protein exposed for long periods to a denaturing pH undergoes very slowly secondary changes which reduce the velocity with which it can regenerate.

TABLE III

REGENERATION AT 15.5 AND 25° AFTER DENATURATION AT pH 3.5 AT 25°

T, °C.	Regeneration pH	Denaturation time (min.) at 25°	$k_2 \times 10^4$
15.5	4.75	5	104
		10	104
		20	65
		40	49
	4.44	5	108
		10	108
		20	69
		40	48
25	4.59	5	350
		10	405
		20	263
		40	142
	4.44	5	344
		10	420
		20	282
		40	197

No effort has been made to calculate temperature coefficients for regeneration except within the narrow 15–25° temperature interval of Table III since such calculations are only valid when the initial material, denatured protein, was demonstrably the same at every temperature of regeneration, *i.e.*, the same temperature of denaturation was used in all the experiments. Within this interval the energy of activation for regeneration is approximately 22,000 cal., as compared with the value for denaturation in the same interval, 16,200 cal., previously reported.[5]

Equivalence of Spectrophotometric and Solubility Criteria of Regeneration.—It has been shown in earlier papers that the same results are obtained if denaturation is followed spectrophotometrically or by means of the drastic changes in solubility that classically defined denaturation. Figure 12 shows that this identity of results also is obtained in following the course of regeneration. Data are shown for 15.5° rather than for 0°, because at the lower temperature it is not possible to dissolve the amount of sodium sulfate required to prevent wholly regeneration of remaining denatured protein when the pH is raised to the isoelectric point.[3]

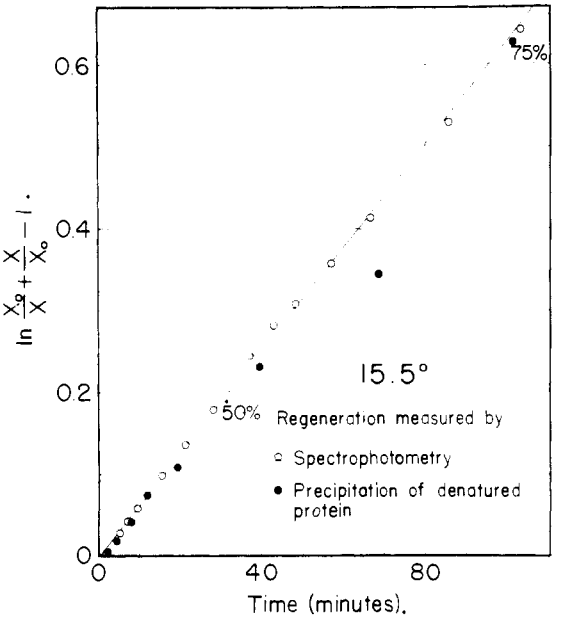
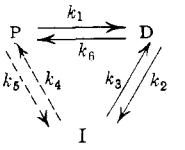


Fig. 12.—Demonstration that regeneration kinetics based on light-absorption measurements at 406 mμ give the same results as direct measurements of denatured protein present by precipitation at the isoelectric point.

Equilibrium between Native and Regenerated Protein at 0°.—It has been reported earlier that equilibrium occurs between native and denatured ferrihemoglobin at *p*H values between about 3.8 and 4.7. The model of the regeneration reaction proposed in this paper implies an interpretation of this "equilibrium" phenomenon which is different from the one described previously. Thus, it becomes necessary to consider the equilibria previously observed to have been predominantly between denatured and regenerated protein (inhibitor) rather than between native and denatured protein, even although regenerated protein reverts eventually to the true native form, when the *p*H is high enough. We may make this conclusion plausible by first representing the system as



The dotted arrows are used as a convenient indication that the reactions to which they refer are too slow to contribute importantly to the results observed. Even if these reactions are omitted entirely the integrated form of the kinetic equations for this model is unwieldy and contains numerous combinations of constants which cannot be evaluated directly.

The closed reaction cycle shown above may be justified in detail as follows

(a) Since in denaturation at acid *p*H simple first-order kinetics prevail and no latent periods are ever observed, either k_1 is much larger than k_3k_5 or k_3 is much larger than k_5—*i.e.*, the amount of I present at these *p*H values is always very small.

(b) D does not revert to P directly, at an appreciable rate, since the kinetics described in this paper show that the principal product formed in the "regeneration" reaction is an inhibitor, I (I and P are spectroscopically indistinguishable).

(c) I reverts very slowly to P, since the inhibitory property gradually disappears. Furthermore, the rate of re-denaturation of "regenerated protein," at least after it has been stored for some hours, is the same as that originally observed.

(d) I is in equilibrium with D at *p*H values between 3.8 and 4.7 since in this region "regeneration" stops short of completion, although "regenerable" protein is still present.

It is apparent that a number of pseudo-equilibria may occur and that these may be different according to initial conditions, and the way in which the experiments are carried out. There is a possibility that a true equilibrium will not be directly observed since the step I → P (and its reverse) is so slow that the irreversible loss of protein previously alluded to competes with it very strongly.

These conclusions, based on observations already reported, are strongly supported by the actual course of regeneration over very extended periods, in the equilibrium region of *p*H. Figure 13 shows representative data obtained at *p*H 4.16 for one, two and eight half-periods,[16] at 0.2°, after an initial denaturation at *p*H 3.5 at 15°. The figure includes for comparison denaturation data at the same *p*H. The main difference between data obtained with the different denaturation times is ascribable to the fact that a good deal of P is initially present after one and two half-periods, but very little after eight. At such acid regeneration *p*H, the density reaches a well-marked maximum in the two half-period case, but not in the eight half-period case. This maximum is in part due to the slow reaction P → D continuing after the "regeneration" reaction D ⇆ I has reached equilibrium. Unfortunately part of the decline in density is also due to irreversible loss (measured by determining the final density at *p*H 5.16), so a simple analysis is not possible. That the decline in optical density is not due solely to the irreversible effect is shown by: (a) close agreement in the 72 hour values, which are separated by an amount accounted for by the loss during the initial denaturation; and (b) the different times at which the decline is observed in the different experiments, as well as its absence in the case of the longest denaturation time. Most of the denatured protein present at equilibrium can be "regenerated."

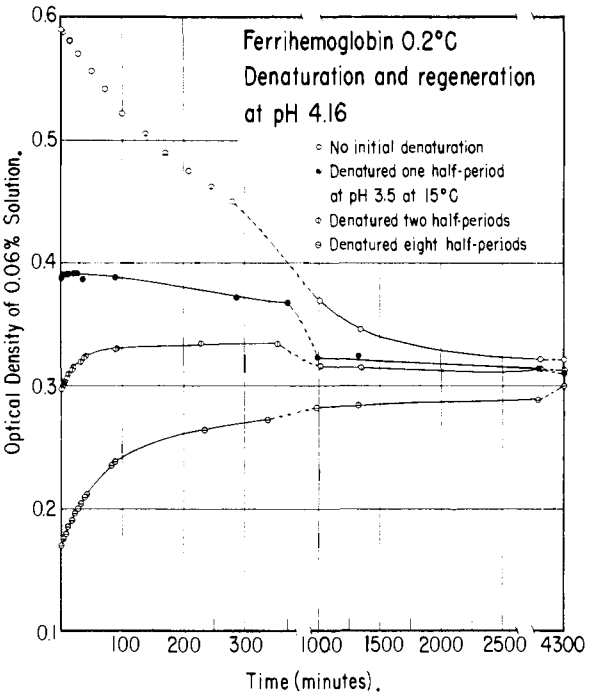


Fig. 13.—Course of regeneration at 0.2° at *p*H 4.16 after denaturation at *p*H 3.5 for one, two and eight half-periods at 15°. Denaturation data at the same *p*H value are also shown.

The long-time values reached after denaturation for eight half-periods (after correction for irreversible loss) are more nearly attributable to the equilibrium D ⇄ I, but even here the very slow reaction I ⇄ P undoubtedly plays some part in determining the true point of equilibrium between I + P and D.

The "equilibrium" data shown in Fig. 14 represent the values obtained after 24 hr., which are essentially stationary thereafter when corrected for

(16) Regeneration measurements were continued over a sufficiently long time to reveal the maximum clearly only in the experiments with denaturation at 15°, but similar maxima exist in the experiments with denaturation at 0.2.

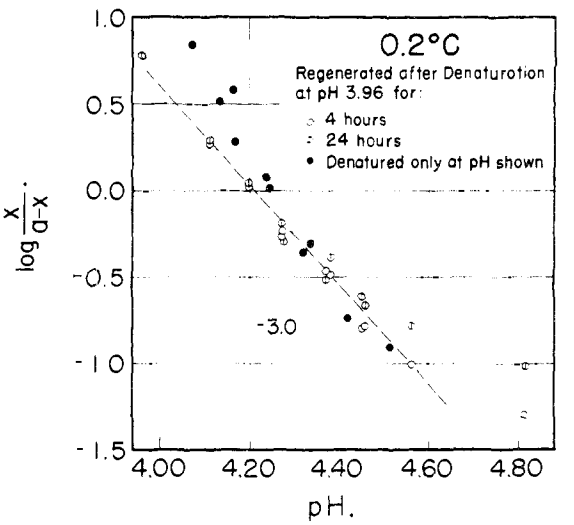


Fig. 14.—Equilibrium data obtained in regeneration experiments at 0.2° as a function of *p*H compared with data obtained in denaturation experiments. The choice of ordinate is explained in the text.

progressive irreversible loss—the latter correction is very appreciable in the more acid range, since it must take into account losses during regeneration as well as the loss during the initial denaturation, which suffices when regeneration occurs in the less acid range. The ordinate used has been selected for convenience only. The true equilibrium equation is expected to be

$$\frac{(I + P)(I + P + C)}{D} = K'_{eq} \qquad (5)$$

where K'_{eq} contains combinations of four velocity constants, and C is equal to K_I, as defined for eq. 1, multiplied by $(1 + k_4/k_5)$. If I + P is small compared with C, as it is at sufficiently low *p*H, the above expression may be written, with good approximation, as

$$\frac{I + P}{D} = \frac{a - x}{x} = \frac{K'_{eq}}{C} \qquad (6)$$

Equation 6 would lead to the linear relation found with the regeneration data at all except the highest *p*H values. The failure of denaturation data to fall on the same straight line at the more acid *p*H values indicates that final equilibrium was not attained, presumably because at such acid *p*H values adjustment of the equilibrium always lags its displacement by irreversible loss.

No further analysis is attempted here since it is clear that the equilibrium constant to be derived at 0° from such pseudo-equilibria (even though carefully corrected for irreversible loss) cannot be simply related to the ratio k_1/k_2 for the velocity constants of denaturation and regeneration reactions as previously believed, but rather to the ratio of the velocity constants for the formation of I from D to the product of the two velocity constants governing the disappearance of I. It is likewise unprofitable at this time to attempt to determine whether or not the dissociations of the unmasked groups, as well as the trigger-groups, enter into the *p*H-dependence of the apparent equilibrium—they will only have a strong effect if they combine with hydrogen ion almost quantitatively in this range of *p*H.

Significant calculations of the effect of temperature on the "equilibria" shown can only be made by assuming that: (a) the use of a different denaturation temperature in the regeneration experiments at higher temperatures does not vitiate the comparison; (b) irreversible losses, which were less accurately estimated at the higher temperatures, were not large; (c) equilibrium was actually attained in the older denaturation experiments.

None of these assumptions appear safe, and in fact the data at the higher temperatures when plotted as in Fig. 14 have a slightly different slope. The apparent heat of reaction ΔH will therefore depend on *p*H. Its value between 0.2 and 15.5° at *p*H 4.2 is very approximately 32,000 cal., and it increases at higher *p*H (a value near zero was found between 15.5 and 25°). Approximate values for the apparent values of E_1 and E_2, the activation energies for the denaturation and regeneration reactions, are already available for the temperature interval 15.5 and 25° ($E_1 \cong 16{,}400$ cal., independent of *p*H,[6] and $E_2 \cong 22{,}000$ cal.); and for E_1 only, in the temperature interval 0.2–15.5° ($E_1 \cong 4{,}300$ cal.). However, it has been shown above that the usual simple relation, $\Delta H = E_1 - E_2$, cannot be expected to apply in the present system.

Acknowledgment.—Thanks are due to Prof. G. Scatchard of The Department of Chemistry, Massachusetts Institute of Technology, for generous provision of necessary facilities and for providing opportunities for helpful discussions.

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