Anal. Calcd. for $C_{22}H_{17}NO_2S$: C, 73.51; H, 4.76. Found: C, 73.84; H, 4.65.

Ethyl *o*-1-(Naphthyl)-benzenesulfonate was obtained in excellent yield by refluxing the sulfonyl bromide in ethyl alcohol for 15 minutes. After crystallization from alcohol the product melted at 108–109°.

Anal. Calcd. for $C_{18}H_{16}O_3$: C, 69.21; H, 5.16. Found: C, 69.41; H, 5.29.

Conversion of Va to 1-Phenylnaphthalene.—A solution of 1.4 g. of *o*-(1-naphthyl)-benzenesulfinic acid (Va) and 6 g. of mercuric chloride in 40 ml. of alcohol was refluxed for 1.5 hours. The solid precipitating on cooling was extracted three times with 25-ml. portions of acetone, and the residue from evaporation of the acetone was crystallized from alcohol. The crude *o*-(1-naphthyl)-benzenemercuric chloride, m.p. 169–175°, thus obtained was refluxed for eight hours with hydrochloric acid and the product steam distilled. Nitration of the resulting oil gave a crude nitro compound, which after purification was shown by a mixed melting point determination to be identical with the product prepared by nitrating 1-phenylnaphthalene.

Addition of Bromine to III.—One gram (0.0037 mole) of III was suspended in 40 ml. of carbon tetrachloride and treated with 0.59 g. (0.0037 mole) of bromine in 10 ml. of carbon tetrachloride. After standing two days at room temperature the mixture was warmed to effect solution and the product allowed to crystallize. After an additional crystallization from 25 ml. of carbon tetrachloride, 0.70 g. (44%) of material melting at 170–172° was obtained. Two further crystallizations raised the melting point to 175–176°.

Anal. Calcd. for $C_{16}H_{12}Br_2O_2S$: C, 44.88; H, 2.82. Found: C, 44.43; H, 2.63.

Later attempts to repeat this experiment were unsuccessful. Under apparently identical conditions evolution of hydrogen bromide was observed and a mixture was obtained the melting point of which could not be raised above 165° by crystallization. The aromatized sulfone (X) was identified as one of the products of the bromination. By pulverizing III and shaking with bromine in carbon tetrachloride, the time for complete up-take of the bromine was reduced to two hours; no hydrogen bromide was apparent during the reaction and a 33% yield of dibromide VIII, m.p. 175–176°, was obtained.[14]

Benzo[d]naphtho[2,1-b]thiophene 7-Dioxide (X).—The dibromide (VIII) (1.2 g., 0.0028 mole) dissolved rapidly in 35 ml. of hot 2% alcoholic potassium hydroxide, and an insoluble product soon formed. After refluxing the mixture for two hours, the solid was filtered and washed free of potassium bromide. The yield of X, m.p. 228–229°, was 0.68 g. (90%). The melting point remained unchanged after two further crystallizations from alcohol.

Anal. Calcd. for $C_{16}H_{10}O_2S$: C, 72.16; H, 3.78. Found: C, 72.37; H, 3.70.

Dehydrobromination of VIII to X was also accomplished with triethylamine in benzene solution.

Benzo[d]naphtho[2,1-b]thiophene (XI).—Reduction of 1.1 g. of VIII with excess lithium aluminum hydride in ethyl ether[8] gave material melting, after crystallization from alcohol, at 101–102.5°; yield 65%. Two more crystallizations from alcohol raised the melting point to 102–102.5°.[15]

Anal. Calcd. for $C_{16}H_{10}S$: C, 82.01; H, 4.30. Found: C, 81.91; H, 4.51.

Oxidation of XI in acetic acid solution with a ten-fold quantity of 30% hydrogen peroxide gave a practically quantitative yield of X after a 20-minute reflux period.

5,6,6a,11b-Tetrahydronaphtho[2,1-b]benzo[d]thiophene 7-Dioxide (VI).—Five grams of III in alcohol solution was reduced in a Parr apparatus using 0.1 g. of 5% palladium-on-charcoal catalyst. Addition of water to the hot alcohol solution from which the catalyst had been removed gave 4.5 g. (89%) of crystalline material melting at 179–181°. Two recrystallizations from acetone–alcohol and two from benzene–pentane raised the melting point to 180–181°. Mixed m.p. with III, m.p. 183–184°, was approximately 161–171°.

Anal. Calcd. for $C_{16}H_{14}O_2S$: C, 71.08; H, 5.22. Found: C, 71.47; H, 5.02.

***o*-(3,4-Dihydro-1-naphthyl)-benzenesulfinic Acid (VII).**—A solution of 0.93 g. of VI dissolved in 50 ml. of 2% alcoholic potassium hydroxide was refluxed for one hour. The solution was cooled and acidified with 6 *N* hydrochloric acid. Slow addition of 180 ml. of ice water yielded 0.85 g. (92%) of the sulfinic acid, m.p. 121–122°. Three recrystallizations from 50% aqueous alcohol did not raise the melting point.

Anal. Calcd. for $C_{16}H_{14}O_2S$: C, 71.08; H, 5.22. Found: C, 71.28; H, 4.77.

(14) We wish to thank Dr. Glenn D. Cooper for carrying out these later experiments.

(15) In an attempted synthesis of this compound from phenyl 1-amino-2-naphthyl sulfide by a Pschorr ring closure, Neumoyer and Amstutz[9] obtained a 0.4% yield of a product melting at 174.2–175.7° (sulfone, m.p. 258.4–259.7°); the sulfur analysis of neither this compound nor that of the sulfone agreed with the calculated values for XI and X.

EVANSTON, ILLINOIS RECEIVED MAY 21, 1951

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

Kinetic Evidence on the Mechanism of the Acid Denaturation of Horse CO Hemoglobin[1]

BY ETHEL M. ZAISER AND JACINTO STEINHARDT

Equilibrium measurements have shown that exposure of horse carbonylhemoglobin to dilute acid initiates a time-dependent reaction which liberates 36 acid-binding groups. The relation of this process to denaturation has now been explored by kinetic measurements. The rates of denaturation of COHb by acid have been determined as a function of *p*H, ionic strength and temperature, by three different methods: loss of characteristic absorption at 5380 Å., loss of solubility at the isoelectric point, and increase in available acid-combining groups. The reaction is at least partially reversible. The initial formation of ferrihemoglobin plays an essential part in the process as usually observed. The velocity constants measured by all three methods are identical. It is concluded that the liberation of 36 basic groups incident to denaturation occurs essentially as an all-or-nothing phenomenon, *i.e.*, no delayed acid combination occurs in undenatured molecules, or in denatured molecules in amounts smaller than the full complement.

Over a wide range of *p*H values, the acid-binding capacity of horse carbonylhemoglobin when determined by a technique permitting measurement at 3 seconds after mixing with acid[2] is less than the amount predicted from its amino acid composition, and less than one-half of the amount found by the usual methods of titration which require long periods for attainment of equilibrium.[3] The amount of acid bound increases with time at rates which increase with the acid concentration. After brief exposure to acid at *p*H 2.8 or below, a back-titration curve may be obtained which, on comparison with the 3-second curve (Fig. 1) reveals that the acid bound has increased by as much as 0.52 mmole/gram (Fig. 1, inset curve 2). This

(1) A preliminary account of this work was presented at the meeting of the American Chemical Society at Chicago, Ill., in September, 1950.

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

(3) E. J. Cohn, A. A. Green and M. Blanchard, THIS JOURNAL, **59**, 509 (1937).

corresponds to the amount that would be bound if 36 basic groups, not originally accessible, have been unmasked by exposure to acid in a time dependent reaction. On merely waiting a sufficient time (12 to 24 hours) at higher *p*H values (2.8–3.5) the number of basic groups liberated is almost as great as when a preliminary exposure of the protein to *p*H (<2.8) is followed by back-titration after a few minutes (Fig. 1, inset curve 1).[4] At low *p*H's the *apparent* number of groups liberated *appears* to diminish because the reaction is so rapid that the 3-second curve is no longer an adequate representation of the acid bound at zero time, *i.e.*, in the instantaneous acid–base equilibrium. The basic groups released have been tentatively identified as carboxylate (COO^-) groups, or as equal numbers of carboxylate and imidazole.[2]

Changes in the absorption spectrum[2] and in the solubility of the protein at the isoelectric point (denaturation), appeared to occur in the same range of *p*H and at the same times as the increase in acid-binding capacity. Such parallelism, if confirmed in detail, would indicate the possibility of establishing a definite connection between the liberation of basic groups and structural changes in the molecule which manifest themselves in loss of specific properties and solubility at the isoelectric point. More detailed studies of the rates of these changes promised to yield a clearer demonstration of the interdependence of denaturation and liberation of basic groups than could be obtained by observations on equilibrium states. They might also be expected to yield further information as to the smaller number of groups involved in triggering the reaction.[2]

The kinetics studies to be presented here are of three kinds: (a) spectrophotometric determination of the rate of disappearance of native protein, as manifested by fading of the COHb band at λ 5380 Å.; (b) measurement of the amount of protein rendered insoluble in salt solutions at the isoelectric *p*H, as a function of time of exposure to acid; and (c) measurement of the delayed acid uptake as a function of time as well as *p*H—this involved determination of whole titration curves as a function of time.

The last method is laborious and is applicable only to solutions unbuffered except for the protein; consequently measurements were made only for 3 seconds, 10 minutes and 24 hours, as previously reported.[2] The first two methods were employed in the presence of formate buffers of sufficient capacity to suppress the *p*H drifts attendant on the liberation of basic groups by acid. It is shown here that the results with buffers may be freely compared with those made with HCl alone, when allowance is made for drifts in *p*H in the latter.

Experimental

Kinetic experiments were conducted at 3 and 25° at an ionic strength of 0.02 (except where the effect of ionic strength alone was to be tested) for the purpose of comparison with titration data already reported.[2] The preparation and characterization of the crystalline horse carbonylhemoglobin and the methods of determining *p*H and obtaining titration and back-titration curves at different times after

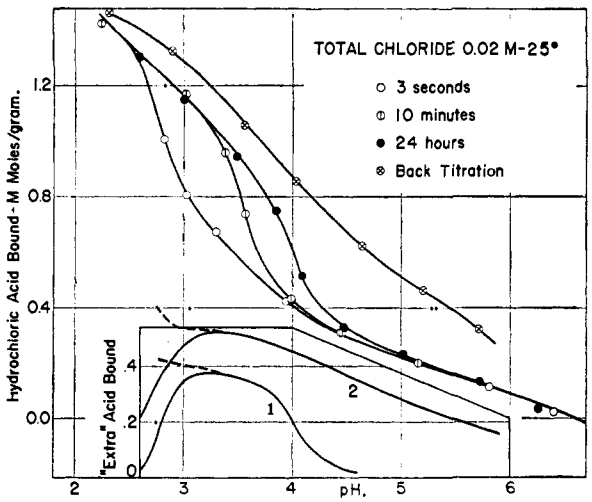

Fig. 1.—The amounts of hydrochloric acid combined with COHb in solutions of constant total chloride concentration as a function of *p*H and time.[2] The two inset curves at the bottom are obtained by difference: #1 by subtracting the 3 second data from the 24 hour data; and #2 by subtracting the 3 second data from the back-titration data. The dashed segments of these curves are corrected as explained in the text.

mixing have been described previously.[2] The preparations contained very small amounts of ferrihemoglobin (never over 2%).

Two series of formate buffers were used: one in which the total formate ($HCOOH + HCOO^-$) was kept constant at 0.02 *M* and varying amounts of KCl added to produce an ionic strength of 0.02; and one in which the ionic strength of 0.02 was due solely to $HCOO^-$, the amounts of undissociated HCOOH being varied to change the *p*H.

Spectrophotometric Determination of Rate of Disappearance of COHb.—The disappearance of the COHb band at 5380 Å. was measured in a Beckman model DU spectrophotometer as a function of time of exposure of 0.05% protein to formate buffers of ionic strength 0.02 in the *p*H range 3.1 to 4.2, and as a function of ionic strength over a limited range. For each measurement a fresh sample was removed from the reaction mixture which was kept always at 25°. As in the titration experiments,[2] the solutions were saturated with CO, and practically all the protein was present as COHb. However, since air in traces, or in deliberately introduced larger quantities, was always present (see Discussion) COHb may be initially transformed in part to ferrihemoglobin (this reaction is acid catalyzed) which is subsequently denatured, as well as directly to denatured COHb (or its subsequent oxidation product). Thus the reaction mixture may contain COHb, ferrihemoglobin, and the denatured forms of either or both. At 5380 Å. native COHb has a substantially higher absorption than the other possible products, the absorptions of which are closely similar at this wave length, and independent of *p*H in the range of interest. If the very small differences are disregarded, the ratio of the change in optical density to the final change is a direct measure of the proportion of COHb which has altered, although it does not distinguish between the possible products, or indicate their proportions. Ferrihemoglobin is, however, denatured more rapidly than is COHb at *p*H values above 3.3, and it is denatured much more rapidly than it is formed at *p*H values below 4.0.[5] Thus in the range 3.3–4.0 the reaction mixture contains only negligible amounts of this protein. Consequently, the rate of change in density, which closely parallels the rate of disappearance of native COHb, is equivalent to the rate of formation of denatured protein, and has been calculated accordingly. At *p*H values above about 4.0 the reaction rate calculated in this way refers predominantly to another reaction, the formation of ferrihemoglobin which is not destroyed.

Velocity constants were calculated from the slope of a

(4) When salt is absent, the same maximum increase (0.52 mmole/g.) is found.

(5) J. Steinhardt and E. M. Zaiser, in preparation.

plot of the logarithm of $(D - D_\infty)$ against time where D is the optical density, and D_∞ its final value. The total density change (0.191 for a 0.05% protein solution) was constant to better than 3% over the pH range investigated.

Precipitation Method for Following Rate of Disappearance of COHb.—Denatured protein was precipitated from solutions of COHb which had been exposed to formate buffers for varying lengths of time by adjusting the pH to the isoelectric value 6.6.

Preliminary experiments indicated that certain of the time-dependent changes ("denaturation") brought about by exposure to acid buffers were reversed on bringing the pH to neutrality in order to precipitate denatured protein. These results are consistent with observations of Holden[6] among others. They are also consistent with results previously published by the authors[2] who showed that the reaction liberating base stops short of completion at pH values above 3.8. Thus the "equilibrium" titration curve obtained in about 24 hours is different from the back-titration curve obtained after exposure to strong acid (pH 1.9).

The experimental effects of this complication were completely eliminated by adding sodium sulfate (final concentration 12.5%) immediately before neutralizing. Control experiments on solutions of completely denatured COHb and ferrihemoglobin showed that this procedure prevented "renaturation" on the addition of base, probably because it precipitated most of the denatured protein before the base was added. If salt is not added first some of the denatured protein always reverts to the native form (as distinguished by spectroscopic or solubility criteria) during the process of neutralization; indeed, very slow or stepwise neutralization will convert large proportions of initially denatured COHb or ferrihemoglobin into a substance which is soluble at pH 6.6 and, as Holden[6] also observed, indistinguishable spectrophotometrically from native ferrihemoglobin.

Spectrophotometric measurements on the filtrates at 5000, 5380 and 6300 Å. permit determination of the amounts of native protein present as COHb and as ferrihemoglobin.

The agreement of analyses based on optical densities at any pair of these wave lengths is confirmation that the filtrates contain only COHb and ferrihemoglobin. Because the filtrates were very dilute most analyses were made by using data at 5000 and 5380 Å., at which the absorption coefficients are much greater than at 6300 Å. The following relations between protein concentration in g./liter and optical density/cm. (D) were employed

$$[\text{COHb}] = 1.592D_{5380} - 1.084D_{5000}$$
$$[\text{FerriHb}] = 1.733D_{5000} - 0.603[\text{COHb}]$$

Ferrihemoglobin is always present, but it is readily shown that when sodium sulfate (12.5%) is present and the pH of the reaction mixture was initially at 4.0 or below, all the ferrihemoglobin found *is formed in the filtrate from* the native COHb which did not precipitate with the denatured protein. Thus, this ferrihemoglobin represents undenatured COHb in the reaction mixture before precipitation.[7] Consequently, under the conditions used here (salt in filtrate, pH of reaction below 4.0), the rate of disappearance of total undenatured protein from the filtrates (COHb plus ferrihemoglobin) is the rate which corresponds to the rate of disappearance of COHb alone in the measurements at 5380 Å. previously described which are based on the total reaction mixture.

Results and Discussion

Spectrophotometric Determination of Rate of Denaturation.—It became immediately evident that excellent first order rate constants for the disappearance of native COHb could be obtained, and that the rate had an extremely high order of dependence on pH. However, the reproducibility of the first experiments left much to be desired. This lack of reproducibility was traced to extreme sensitivity of the reaction rate to traces of oxygen, which were never wholly excluded. Acid catalyzes the formation of ferrihemoglobin when oxygen is present, and the ferrihemoglobin formed is denatured more rapidly than COHb itself (measurements of the rate of denaturation of ferrihemoglobin are to be reported elsewhere). The susceptibility to oxygen is also shown by a paradoxical inverse dependence of denaturation rate on protein concentration (due to the fact that a greater proportion of the protein in dilute solutions is transformed to ferrihemoglobin by the oxygen present). If, instead of attempting to exclude oxygen, small amounts of air are deliberately introduced (not enough to reduce significantly the proportion of the pigment present as COHb) reproducible rate constants are always obtained. At the same time, the anomalous dependence of rate on protein concentration disappears. This procedure was therefore followed, rather than attempting the much more difficult procedure of working under sufficiently anaerobic conditions; the latter would have excluded any possibility of comparison with the equilibrium measurements presented earlier,[2] since in those experiments traces of air were not excluded.

Figure 2 illustrates the first order kinetics, the effect on the rate of excluding or of adding air, and the reproducibility of the rates which are obtained when air is added. It also indicates the great difference in rate which is brought about by a small difference in pH.

The effect of pH is illustrated more completely in Fig. 3, which includes data for two temperatures. Between pH 3.2 and 3.8 at 25° the reaction velocity changes about one-hundred fold, and exhibits a consistent dependence on the 3.2 power of the hydrogen-ion concentration. At 2–3°, the dependence on pH is slightly smaller. The effect of temperature is small as compared with the very large values often found for protein denaturation; if the rates are compared at the same pH (which may not necessarily correspond to the same concentration of the reacting species at each temperature[8]) the apparent energy of activation varies from about 8000 calories at pH 3.72 to about 12,000 calories at pH 3.20. The difference in pH dependence of the velocity at the two temperatures on which these differences in apparent activation energy depend, and a fractional exponent itself, is not unexpected in a reaction which proceeds by more than one path. The small activation energies found are consistent with other work with hemoglobin[9] and with the view previously expressed that the reacting species is formed by the addition of hydrogen-ion to a small number (two were suggested) of carboxylate groups,[2] themselves characterized by extremely small heats of dissociation.

At pH values much above 3.8 the dependence of log velocity on pH would not remain the same when the rate is measured by density changes in the reaction mixture. The rate tends to become nearly independent of pH. This is because the rate of denaturation becomes much slower than another reaction, the formation of ferrihemoglobin. The latter, which varies very much more slowly with

(6) H. F. Holden, *Australian J. Exp. Biol. Med. Sci.*, **14**, 291 (1936).

(7) When sodium sulfate is not used, all the ferrihemoglobin found in the filtrate represents reversed denatured protein.

(8) J. Steinhardt, *Kgl. Danske Videnskab. Selskab, Mat.-fys. Medd.*, **14**, No. 11, 1937.

(9) H. K. Cubin, *Biochem. J.*, **23**, 25 (1929).

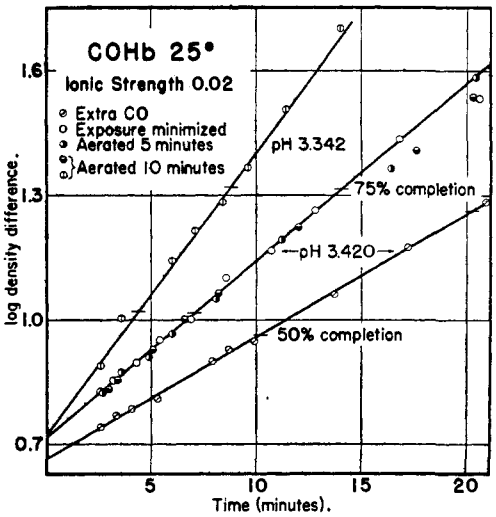

Fig. 2.—Representative kinetic data on the denaturation of COHb, illustrating the effects of *p*H, and of excluding or adding air. The points labeled "extra CO" represent experiments under the closest approach to anaerobic conditions.

*p*H, becomes chiefly responsible for the density changes observed. This complication does not arise when the measurements are made by the precipitation method (described below).

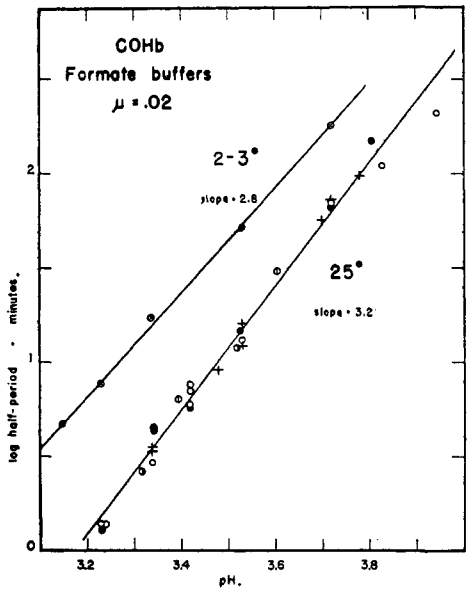

Fig. 3.—The dependence of the first-order rate constants on *p*H and temperature: circles, spectrophotometric method; crosses, precipitation method; 25° O, total formate 0.02 *M*; ⊙, formate ion 0.02, no KCl; ●, total formate 0.02 *M*, air added; ◑, formate ion 0.02, no KCl, air added; 2-3° ⊗, total formate 0.02 *M*.

The reaction rate is strongly dependent on ionic strength. Since it does not distinguish between formate and chloride ion, the change in rate with *p*H at a constant ionic strength (Fig. 3) is not due to changes in the ratio of these ions.[10] Figure 4 shows that a small increase in ionic strength, from

(10) There are indications, however, that certain other ions are not equivalent. Ferrihemoglobin is denatured more rapidly in acetate buffers than in formate buffers of the same ionic strength.

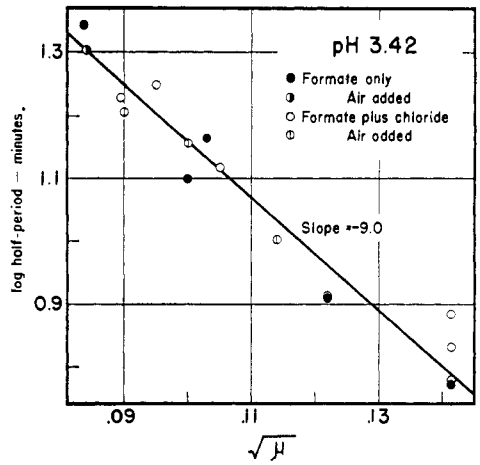

Fig. 4.—The effect of the ionic strength on the rate of denaturation of COHb in formate or formate-KCl buffers at *p*H 3.42.

0.007, the smallest concentration of buffer permissible, to 0.02, brings about a more than threefold increase in rate. A linear relation of log velocity to the square root of ionic strength appears to hold over the limited ranges explored. The slope 9 is very large but no larger than has previously been encountered for protein denaturation, *e.g.*, a slope of 13 for the denaturation of crystalline pepsin.[8]

Determination of Rate of Denaturation by Loss of Solubility.—Rate measurements based on the rate of loss of protein soluble at the isoelectric point are also included in Fig. 3. It is clear that they are the same both absolutely, and in relation to *p*H, as the measurements made by following the change in spectroscopic properties in the reaction mixture. Since these changes are parallel they both measure the rate at which molecules are transformed to denatured proteins.

Determination of the Rate of Denaturation by Increase in Acid-binding Capacity.—If it is assumed that the first-order denaturation reaction just described also involves the liberation of 36 acid-binding groups per molecule (0.52 mmole per gram), then the amount x liberated at any given time as a function of hydrogen-ion activity is given by

$$x = 0.52\left(1 - e^{-0.693\left(\frac{H}{H_{1/2}}\right)^{3.2}}\right) \qquad (1)$$

if the possibility of reversal at high *p*H is disregarded. Here $H_{1/2}$ represents the hydrogen-ion activity at which one-half the groups (0.26 mmole per gram) are liberated at the time in question, the exponent 3.2 is taken from the data in Fig. 3; and −0.693 is the natural logarithm of 1/2.

Data previously reported[2] (see also Fig. 1 of this paper) permit the calculation of the amounts liberated at a fixed time, 10 minutes, at 0.02 ionic strength. Under these conditions *the titration data* show that 0.26 mmole/g. are liberated at *p*H 3.48 ($H_{1/2}$ = −antilog 3.48). The kinetic data (Fig. 3), on the other hand, show that the half-period at *p*H 3.48 is exactly 10 minutes. Further verification of the basic assumption is given in Fig. 5 which shows that eq. 1 reproduces

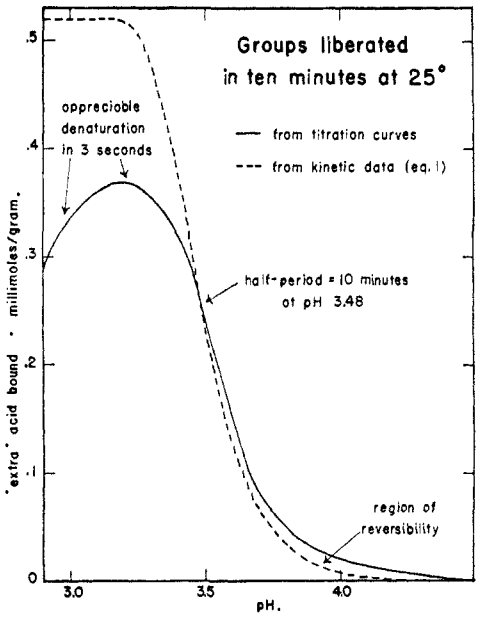

Fig. 5.—The variation with pH of the extent of liberation of acid-binding groups at ten minutes: a comparison of an experimental difference curve from titration data (at 3 seconds and 10 minutes) with a curve calculated from independent measurements of the dependence of rate of denaturation on pH (eq. 1).

the experimental results at 10 minutes very well—except for amounts well in excess of half-liberation. The discrepancy at higher values is inevitable, since the largest amounts liberated when salt was present did not appear to exceed 0.36 mmole/g., due, in part, to the fact that appreciable amounts reacted in less than 3 seconds; thus the 3-second curve did not approximate to a zero-time base line at high concentrations of acid.[11]

(11) When salt is absent the maximum amount liberated in 24 hours is the same as the maximum difference between the 3-second curve and back-titration curve.

A further check of consistency can be made by calculating the amounts liberated in 3 seconds or less at the lowest pH values used in the titration experiments. If 3.0 seconds is taken as the average time at which protein solution bathed the glass-electrode, an extrapolation of Fig. 3 shows that half the acid-binding groups should be liberated in this time at pH 2.77. Using this value for $p\mathrm{H}_{1/2}$, the amounts liberated in the same time have been calculated, and are applied as corrections to the data for the apparent amounts liberated, given as the inset curves in Fig. 1. The effect is to show the amounts liberated as a flat plateau independent of pH at values down to 2.9.

The excellent agreement in the correspondence of the absolute velocity constants at pH 3.48 and the general agreement in other respects appears to warrant the conclusion that although other reactions may intervene in more strongly acid solutions[2] the same velocity constant applies for the liberation of acid groups as for loss of characteristic spectroscopic absorption and for loss of solubility at the isoelectric point.

Significance of the Agreement in Velocity Constants.—Two conclusions appear warranted. One is that in this protein the liberation of a large number (36) of acid-binding groups is an inseparable part of denaturation as usually defined. The second, perhaps most surprising, is that all of these groups are liberated or made available essentially as a single or unit process, *i.e.*, no undenatured molecules exist in which any measurable liberation of groups has occurred; and no denatured molecules exist with measurably fewer than the full complement. No other conclusion appears to be compatible with the demonstrated identity of the kinetics.

Acknowledgment.—We wish to express our appreciation to Prof. G. Scatchard for his critical interest in this work.

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