moles-SH per 36,000 even though pooled β-lactoglobulin, containing both genetic species in unknown ratio, was used.[35-37] Secondly, Green and Aschaffenburg[27] have demonstrated a dyad axis of symmetry in both molecules, indicating that the two halves have very nearly, if not identical configurations. All these data suggest that each protein is made up of two identical chains of *ca.* 18,000 mol. wt., and strengthen the assumption that a tagged amino acid would be distributed equally between both subunits.

Model I would be more elegant than II or III with regard to the genetic control of the synthesis of the β-lactoglobulins. As pointed out earlier, it is known that the difference between β-A and β-B is controlled by a single pair of genes. In I, each half of the molecule could be synthesized on the same RNA template, controlled by one specific DNA locus. The two halves would then combine spontaneously to the AA and BB molecules (either on the template or in solution) at the intracellular *p*H. Although not excluded by this argument, the synthesis of models II and III is less straightforward than the first one.

On the basis of the above, it seems more likely that the correct structure is represented by I. This viewpoint is strongly supported by experiments involving tryptic digestion of β-A and β-B, followed by 1-dimensional ionophoresis, which will be reported elsewhere.[38] The acid dissociation phenomenon can be best depicted then by the mechanism of Fig. 5. Here, each species is composed of identical half molecules, there being two differences between β-A and β-B, namely a difference in chain folding at the surface of subunit contact and the presence of one extra titratable carboxyl on each subunit of β-A.

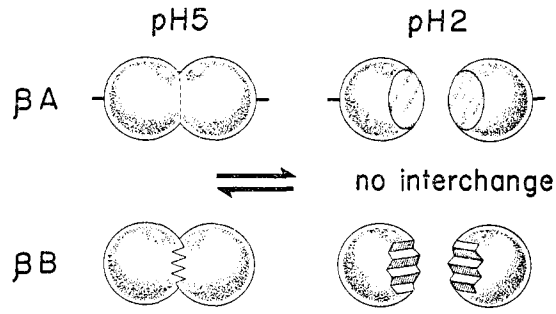

Fig. 5.—Schematic representation of the low *p*H dissociation of β-A and β-B.

Due to the difference in tertiary structure no cross combination can take place. Although the model advanced in Fig. 5 seems to represent the simplest explanations of the experimental facts, the possibility of models II and III has not been completely eliminated by these experiments. To settle this question, more detailed structural information on the two lactoglobulins is necessary. Such a study has been presently undertaken in our Laboratory.

Acknowledgments.—We would like to thank Professor S. J. Singer for suggesting to us the radioactive tagging technique for the solution of the hybridization problem as well as for making available to us prior to publication his manuscripts with Dr. H. A. Itano. We would also like to thank Drs. Murray Strassmann and Bernice Friedman of Cancer Research Institute, Philadelphia, who made their counting equipment available to us.

(35) L. W. Cunningham and B. J. Nuenke, *J. Biol. Chem.*, **234**, 1447 (1959).
(36) M. G. Horowitz and I. M. Klotz, *Arch. Biochem. Biophys.*, **63**, 77 (1956).
(37) A. F. S. A. Habeeb, *Canad. Jour. Biochem. & Physiol.*, **38**, 269 (1960).
(38) R. Townend and V. M. Ingram, unpublished results.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, CLINICAL ENDOCRINOLOGY BRANCH, NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MARYLAND]

The Properties of Thyroglobulin. IV. Denaturation Kinetics

BY HENRY METZGER AND HAROLD EDELHOCH

RECEIVED JULY 28, 1960

The rate of thyroglobulin denaturation has been measured as a function of *p*H and temperature. Typically large values of the thermodynamic activation parameters were found at neutral *p*H values. In alkali a significant increase in rate occurred while ΔH^* and ΔS^* were considerably reduced from their value at lower *p*H's. Salts served to increase denaturation rates in neutral as well as in alkaline solution. Certain heavy metal cations were effective in enhancing denaturation rates at rather low concentrations.

Protein denaturation has traditionally been defined in terms of a modified solubility near the isoelectric point of the protein. In this paper a study of the influence of temperature and *p*H on the rate of thyroglobulin denaturation is presented as measured by solubility criteria. As Kauzmann[1] and others have pointed out, however, such an approach to denaturation has the disadvantage that it draws attention away from "the most significant aspect of the phenomenon, namely its intimate relationship to protein structure." To provide this sort of correlation between the modified solubility and molecular configurational properties, the studies presented in the companion papers V and VI were undertaken. In addition, the properties of thyroglobulin in other solvents, where major unfolding occurs, are compared with those described as denaturation in aqueous media.

(1) W. Kauzmann, *Adv. Protein Chem.*, **XIV**, 1 (1959).

Methods and Materials

In this paper "denatured thyroglobulin" will refer to thyroglobulin which had been subjected to conditions which resulted in a complete loss of its solubility in acetate buffer at *p*H 5.0 and ionic strength 0.5. Since thyroglobulin is

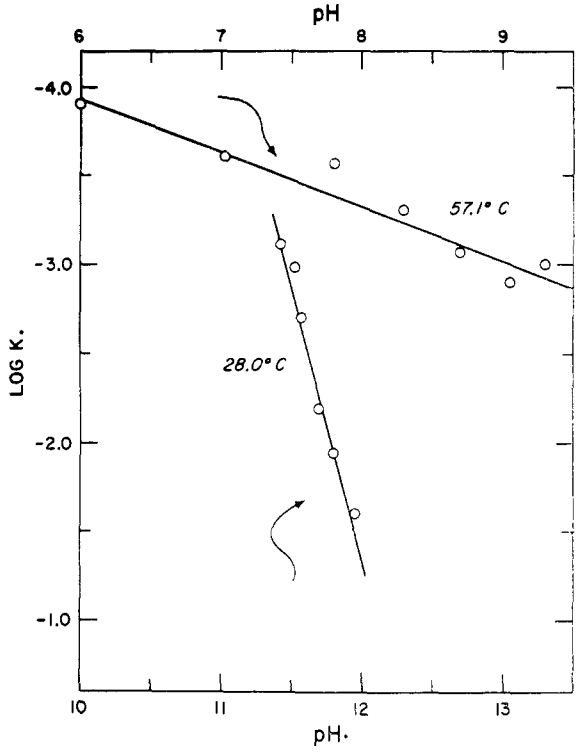

Fig. 1.—pH dependence of the first order rate constants of thyroglobulin denaturation at neutral (57.1°) and alkaline (28.0°) pH values in 0.10 M KNO_3. k is the velocity constant in reciprocal seconds.

rapidly denatured at its isoelectric point, *i.e.*, pH ~4.5,[2] the pH of the precipitation buffer must be controlled rigorously.[3] Moreover denatured thyroglobulin which is precipitated in this acetate buffer requires a rather high centrifugal field for complete sedimentation. Centrifugation at 14,000 r.p.m. for ten minutes at room temperature in a Servall angle-head rotor was necessary to effect complete separation. To determine the concentration of the soluble and hence native protein, aliquots of the supernatant solutions were diluted in a pH 7.5 phosphate buffer of 0.5 ionic strength and the optical density measured at 280 mμ in a Beckman DU spectrophotometer. The optical density of a 1% thyroglobulin solution previously had been determined to be 10.5 at 280 mμ.[4]

Linear relationships were observed when the logarithm of the optical density of the supernatant solution was plotted against time thus indicating first order kinetics. Rate constants were determined from the slope. The activation energy was calculated from the Arrhenius expression and equated with the heat of activation, ΔH^*. Free energies of activation (ΔF^*) were obtained from the relation $k = k'T/h\, e^{-\Delta F^*/RT}$ and entropies of activation (ΔS^*) from $\Delta F^* = \Delta H^* - T\Delta S^*$. The undefined symbols have their standard significance.[5]

In the experiments performed at elevated temperatures, protein solutions were adjusted to the desired pH at room temperature. They were then warmed to within 5° of the experimental temperature and were then placed in a constant temperature bath and stirred to facilitate temperature equilibration.

In the experiments performed near room temperature the protein solutions were equilibrated at the experimental temperature and then brought to the appropriate pH with base. These runs were performed in the alkaline pH range and were monitored throughout with a Radiometer pH meter. A rapid flow of water saturated nitrogen was blown over the solution.

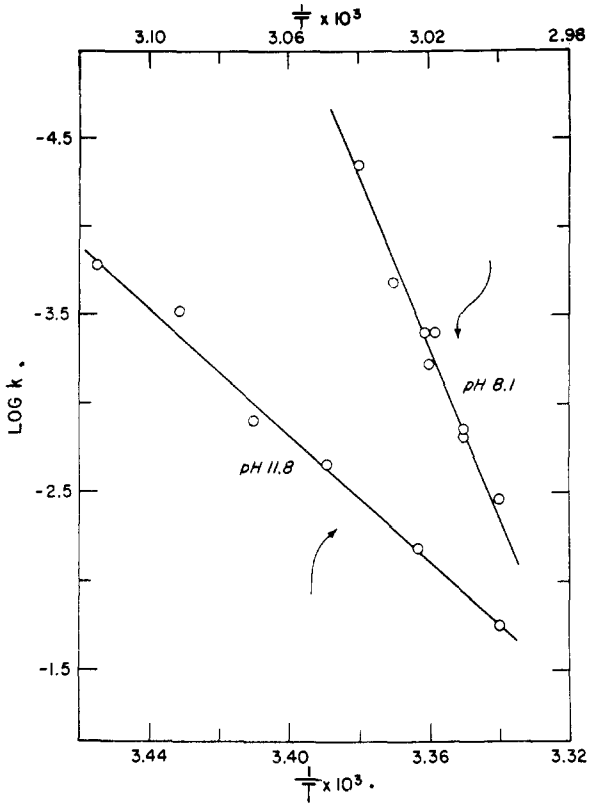

Fig. 2.—The temperature coefficient of the first order rate constants of the thermal (pH 8.1) and alkaline (pH 11.8) denaturation of thyroglobulin in 0.10 M KNO_3. k in reciprocal seconds.

The Radiometer pH meter was standardized with Beckman buffers. No pH changes associated with the denaturation process were observed. The temperature of all reactions was controlled to within 0.05°. The thyroglobulin concentration in the reaction mixture was usually close to 1%.

Thyroglobulin solutions were prepared by differential centrifugation of saline extracts of thyroid tissue as previously described.[4] These solutions contained about 90–95% of S-19 thyroglobulin and 5–10% of a faster moving component which should be identical to the S-30 reported by Schulman, *et al.*, in thyroid extracts.[6] All solutions were prepared in 0.10 M KNO_3 and were stored at 3° until used. Fresh glass distilled water was used and all salts were reagent grade.

Results

Rates of Denaturation at Neutral pH Values.—In the neutral pH range elevated temperatures (*i.e.*, 55 to 60°) were required to produce measurable rates of denaturation. Between pH 6 and 9 the first order velocity constant of denaturation increased gradually with pH. A linear dependence of the logarithm of the rate constant on pH was observed in this range at 57.1°. The data are illustrated in Fig. 1. The line through the points has a slope of 0.30.

The temperature coefficient was investigated at pH 8.1 and the data appear in Fig. 2 as an Arrhenius

(2) M. Heidelberger and K. O. Pedersen, *J. Gen. Physiol.*, **19**, 95 (1935).

(3) After introduction of treated thyroglobulin into the acetate buffer, the solution should be allowed to stand for at least 30 minutes at room temperature in order to facilitate complete precipitation.

(4) H. Edelhoch, *J. Biol. Chem.*, **235**, 1326 (1960).

(5) See, for instance, S. Glasstone, J. K. Laidler and H. Eyring "The Theory of Rate Processes." McGraw-Hill Book Co., Inc., New York, N. Y., 1941.

(6) S. Shulman, N. R. Rose and E. Witebsky, *J. Immunol.*, **75**, 291 (1955).

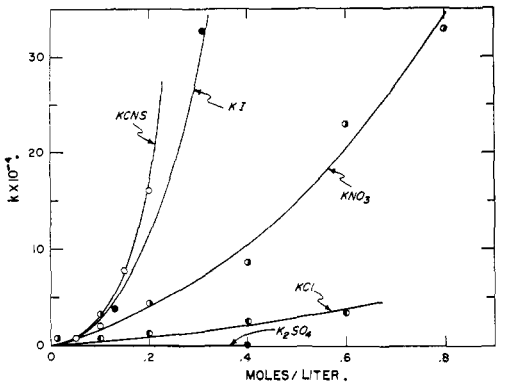

Fig. 3.—The influence of various anions on the rate of thyroglobulin denaturation at *p*H 8.0 and 55.0°.

plot. It can be seen that the rate increased very rapidly with temperature. The activation energy obtained from the slope was 160 kcal.

Rates of Denaturation at Alkaline *p*H Values.—Above *p*H ~9.5 the rate of denaturation no longer followed the *p*H dependence reported in the preceding section. The rate increased sufficiently faster than at *p*H values slightly above 11 measurable rates were observed at room temperature. Furthermore the rate now became markedly sensitive to *p*H and showed an inverse dependence on the 3.5th power of the hydrogen ion activity as shown in Fig. 1. The temperature coefficient, measured at *p*H 11.8 was close to half of that found at *p*H 8.1. The thermodynamic activation parameters for thyroglobulin denaturation at *p*H 8.1 and 11.8 are collected in Table I.

TABLE I

EFFECT OF *p*H ON THE THERMODYNAMIC ACTIVATION PARAMETERS OF THYROGLOBULIN DENATURATION[a]

*p*H	Temp. range, °C.	ΔF^*	ΔH^*	ΔS^*
8.1	56–60	−25.1	160	420
11.8	16–26	−21.5	80	198

[a] ΔF^* and ΔH^* in kcal./mole; ΔS^* in cal./mole/degree; 0.10 *M* KNO_3 present in all solutions. ΔF^* decreased with increasing temperatures; values in Table I are average values over the temperature range indicated.

Effect of KNO_3 on Rates of Denaturation.—The effect of salt concentration on the rate of denaturation was studied at *p*H 8.0 where thyroglobulin should have a relatively small negative charge and at *p*H 11.4 where electrostatic effects may play a more significant role. The isoelectric point of thyroglobulin is ~*p*H 4.5.[2] As shown in Figs. 3 and 4 the acceleration of the rate of denaturation due to KNO_3 was very similar at both *p*H values and consequently tends to exclude nonspecific electrostatic factors as a major determinant in the mechanism of denaturation.

Effect of Different Anions on Rates of Denaturation.—Fig. 3 shows the influence of various anions on the rate of thyroglobulin denaturation at *p*H 8.0. As noted by others[7] major differences in rate were observed with different anions and the relative effectiveness conformed to the classical Hofmeister series, *i.e.*, $CNS^- > I^- > NO_3^- > Cl^- > SO_4^=$. KCNS

(7) R. B. Simpson and W. Kauzmann, *J. Am. Chem. Soc.*, **75**, 5139 (1953).

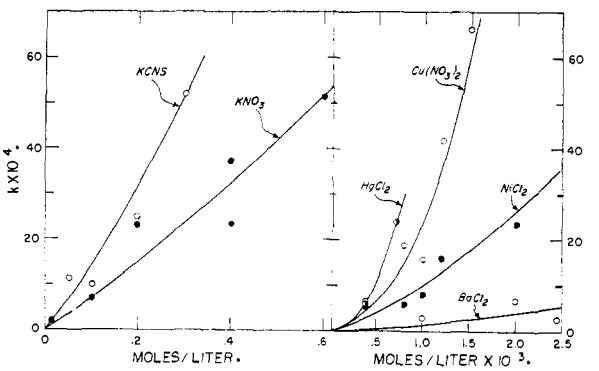

Fig. 4.—Left, effect of KNO_3 and KCNS on the rate of thyroglobulin denaturation at *p*H 11.4 and 28.0°; right, effect of several divalent cations on the rate of thyroglobulin denaturation at *p*H 11.4 and 28.0°.

had a more profound effect than KNO_3 at *p*H 11.4 as well as at *p*H 8.0 (see Fig. 4). K_2SO_4 had little or no effect on the rate at either *p*H 8.0 or 11.4. The rate of denaturation at *p*H 8.0 in 0.2 *M* NaCl was the same as in 0.2 *M* KCl. However, in 0.2 *M* LiCl the rate was several fold faster.

Effects of Divalent Cations on Rates of Denaturation.—Since certain heavy metal ions have a high affinity for particular ionizable amino acid residues in proteins,[8] the influence of several cations were also investigated. In Fig. 4 it is seen that Hg^{++}, Cu^{++} and Ni^{++} appreciably enhanced the rate of denaturation at *p*H 11.4, while Ba^{++}, at comparable concentrations, had very little effect. Since the amount of metal ion bound was not evaluated, we have plotted its total concentration on the abscissae.

Effect of KCNS on Thyroglobulin Composition at 25°.—The profound effect that KCNS had on the rate of denaturation led us to investigate its effect on the molecular properties of thyroglobulin. In strong KCNS solutions S-19 thyroglobulin fragments into two slower sedimenting components, *i.e.*, S-17 and S-12.[9] In Table II are recorded the

TABLE II

COMPOSITION OF THYROGLOBULIN COMPONENTS IN KCNS AT *p*H ~7[a]

KCNS, *M*	Protein concn. ≅ 1.57 S-19	S-17	S-12
0.5	58	21	21
1.0	22	52	26
1.5	15	59	26

[a] Composition of components was determined from areas in schlieren boundaries; no corrections have been applied to these areas.

ultracentrifugal analyses of thyroglobulin solutions in concentrated KCNS. Experiments performed on the same thyroglobulin preparation where KNO_3 replaced KCNS showed only the S-19 thyroglobulin. It is of interest to note that in 1.0 and 1.5 *M* KCNS more S-17 is formed than S-12. Other condi-

(8) For a review of this field, see F. R. N. Gurd and P. Wilcox, *Adv. Protein Chem.*, **11**, 311 (1956).

(9) For significance of these symbols, see papers I (ref. 4) and V (ref. 10) in this series.

tions leading to these two components have resulted in greater amounts of S-12.[4,10]

When the *p*H of 1.5 *M* KCNS was reduced from neutrality to 5.0 with acetate buffer, the solution became increasingly turbid over a period of several hours. After centrifugation at 14,000 x *g*. it was found that 60% of the thyroglobulin had become insoluble. On the other hand 1.5 *M* KNO_3 had no effect on the solubility of thyroglobulin in *p*H 5.0 acetate buffer.

Reversibility of Alkaline Denaturation.—Thyroglobulin solutions which had been exposed to alkaline conditions were denatured by a first order process when the extent of denaturation was determined by pipetting aliquots directly into a strong acetate buffer at *p*H 5.0. If such alkaline treated solutions were, however, carefully titrated with dilute HCl to lower *p*H values and subsequently quenched in *p*H 5.0 acetate buffer, the modification in solubility was no longer observed. The rate of recovery of solubility was complex and seemed to be dependent on both the *p*H and the length of time that the solution was exposed to the high *p*H. For example, when a thyroglobulin solution was brought to *p*H 12.3, the loss in solubility at *p*H 5.0 was complete almost immediately. If this solution was first adjusted to more neutral *p*H values (~7) after only a few minutes at *p*H 12.3, the recovery of solubility was rapid and complete. If it was kept at *p*H 12.3 for longer periods of time (> 15 minutes) and then was back-titrated, it regained its solubility much more slowly and incompletely. These findings suggest that a slow secondary reaction occurs after the initial loss in solubility which leads to a more permanent impairment in molecular structure and to irreversible loss in solubility.

Reversibility of Thermal Denaturation.—Lundgren[11] reported that the *rate* of precipitation in a borate–succinate buffer at *p*H 5.2 of a thermally denatured thyroglobulin (100°–10 min.) depended on the time the solution was incubated at room temperature after heating. From this data Lundgren concluded that the thermal denaturation of thyroglobulin was reversible by cooling at neutral *p*H. We observed similar effects; however, the final *amount* of insoluble thyroglobulin formed was not affected. Reducing the *p*H to 6.5 of a thyroglobulin solution denatured at *p*H 9.6 and 57° and incubating between 25–57° also did not have any influence on the final amount precipitated in *p*H 5.0 acetate buffer. As reported elsewhere[10] the optical rotation and molecular composition of 65° heated thyroglobulin solutions also did not change on cooling.

Discussion

The large activation energies observed for thyroglobulin denaturation are characteristic of protein denaturation reactions. It has been demonstrated in a number of proteins, *e.g.*, hemoglobin,[12] ovalbumin[13] and pepsin,[14] which show a marked dependence of ΔH^* on *p*H, that ionizable residues engaged in internal bonding are somehow involved in the activation process and appear in a free form in the denatured molecule. Scheraga[15] has suggested that the *p*H region in which ΔH^* changes occur can be associated with the pK's of the hydrogen bonded ionizable groups that participate in the activation process.

At 28° thyroglobulin is denatured at observable rates between *p*H ~11.4 and 12.0. At *p*H 11.8 the heat of activation is about half of the value found at *p*H 8.1. In this *p*H range both the ε-amino and tyrosyl groups ionize.

Since certain heavy metal cations bind preferentially to specific residues, their influence on denaturation rates may provide a means of distinguishing between different groups possessing similar pK values. The relative efficacy of the divalent cations shown in Fig. 4 is in the same direction as their affinities to amino groups in low molecular weight compounds.[8] However, the data bear only a qualitative relationship to their binding constants.

As is demonstrated in a companion paper[10] most if not all of the phenolic hydroxyl groups in thyroglobulin behave abnormally when studied by spectrophotometric titration procedures. Since the binding constants of the phenolic groups to the heavy metal cations used are not available, we cannot exclude this group as one of the functional groups stabilizing the thyroglobulin molecule.

About 80 kcal. are required to activate these ionizable groups in thyroglobulin. If these groups are hydrogen bonded to certain acceptors with heats of formation of 2 to 4 kcal. it is evident that only relatively few groups are involved in the activation process since the heat of ionization of phenolic groups is about 6 kcal., while that of amino groups is about twice this value. Since the rupture of intramolecular hydrophobic bonds may liberate heat,[1] the number of hydrogen bonds activated may be somewhat larger if hydrophobic bonds are also involved in the activation process.

Macromolecular Changes Associated with Denaturation.—It is reported in paper V[10] that moderate heating of thyroglobulin solutions (40–54°) in 0.01 *M* KNO_3 at *p*H 9.6 results in the breakdown of native thyroglobulin (S-19) into two slower sedimenting components, *i.e.*, S-12 and S-17. Although the rate of decomposition increases rapidly with temperature, the products cannot be classified as denatured since they are soluble at *p*H 5.0 in acetate buffer, have almost the same specific rotation as native thyroglobulin and show only minimal changes in viscosity[10] and polarization of fluorescence.[16]

At temperatures where denaturation occurs (> 54°) the rate of disappearance of S-19 thyroglobulin becomes very rapid while the rate of denaturation may still be quite slow. It is apparent therefore that under these conditions the

(10) H. Edelhoch and H. Metzger, *J. Am. Chem. Soc.*, **83**, 1428 (1961).

(11) H. P. Lundgren, *J. Biol. Chem.*, **138**, 293 (1941).

(12) E. M. Zaiser and J. Steinhardt, *J. Am. Chem. Soc.*, **76**, 1788, 2866 (1954).

(13) W. F. Harrington, *Biochim. Biophys. Acta*, **18**, 450 (1955). For activation energy data see tables in H. Eyring and A. E. Stearn, *Chem. Revs.*, **24**, 253 (1939).

(14) H. Edelhoch, *Biochim. Biophys. Acta*, **38**, 113 (1960).

(15) H. A. Scheraga, *Ann. Rev. Phys. Chem.*, **x**, 191 (1959). M. Laskowski, Jr., and H. A. Scheraga, *Abstr. Am. Chem. Soc.*, 126th Meeting, 60C, New York, N. Y., Sept. 1954.

(16) R. F. Steiner and H. Edelhoch, *J. Am. Chem. Soc.*, **83**, 1435 (1961).

thermal dissociation reaction precedes the denaturation reaction. It is noteworthy that the latter process produces no further change in the sedimentation characteristics of the S-12 and S-17 components. Only a small (5°) irreversible increase in specific rotation and normalization of only a small fraction of the anomalous tyrosyl groups was found.[10] When thyroglobulin was heated at temperatures below ~54° in 0.10 *M* KNO_3, the dissociation reaction was largely inhibited. Increase in temperature resulted in a loss in solubility without much change in sedimentation pattern. Consequently the loss in solubility appears to be associated with only marginal changes in the secondary and tertiary structure[17] of thyroglobulin.

Below *p*H ~11.2 in 0.10 *M* KNO_3 at room temperatures S-19 thyroglobulin disappears completely and two new *p*H 5 soluble components with essentially the same sedimentation constants (*i.e.*, $S^0_{20,w}$ = 12 and 17)[18] are formed. These molecules appear to be very similar to those formed by heating at *p*H 9.6 in 0.01 *M* KNO_3. Increasing the *p*H (>11.2) results in the formation of a new molecular species with a slower sedimentation constant ($S^0_{20,w}$ = 8.9). Significantly greater destruction of the fine structure of thyroglobulin accompanies the formation of this component as deduced from viscosity and polarization measurements. The ionization of a few specific groups by alkali thus not only reduces the activation energy but also leads to the formation of a smaller and a somewhat more disorganized molecule.

KCNS is known to show pronounced effects on both the kinetics and equilibrium properties of protein solutions.[7,19] In thyroglobulin it appears to function by reducing the temperature necessary to accomplish its splitting into slower sedimenting components. By stimulating this process it is also apparently facilitating the conversion of the newly formed components from a soluble to a denatured formed. In KNO_3 slightly higher temperatures (or alkalinity) are needed to accomplish this latter transition. In KCNS, the process leading to the loss of solubility is stimulated by higher concentrations of salt.

The Properties of Renatured Thyroglobulin.—Despite the fact that under appropriate conditions the solubility of denatured thyroglobulin is recovered, evidence has been accumulated to show that "renatured" thyroglobulin does not possess identical properties with the native (untreated) protein. As demonstrated in paper V various studies indicate that reconstituted thyroglobulin still showed hydrodynamic, optical rotatory and spectral properties that were distinguishable from the native form.[10] In addition, if "renatured" molecules were denatured again they were now considerably less stable to alkali. For instance when the denaturation rate was measured (*p*H 11.6, 0.10 *M* KNO_3, 22°) the rate constant for the renatured solution was 27 times larger than for the native solution.

In addition to the enhancement in the rate of denaturation of renatured thyroglobulin, the *p*H–dissociation curve of the breakdown of S-19 thyroglobulin into S-12 and S-17 (*cf.* paper I) was also modified. The salient feature of this curve was that it was shifted by about 0.4 to 0.5 *p*H units in an acid direction when compared with the same preparation of untreated native thyroglobulin. We may surmise therefore that the reformed S-19 molecule has not recovered all of the bonds which constitute its structure in the native state.

It is interesting to note that an increase in denaturation in alkali of 27-fold corresponds to a lower *p*H of 0.4 *p*H units since the *p*H dependence (*vide supra*) was 3.5 in this region.

(17) For a recent discussion of these terms, see article by K. V. Linderstrøm-Lang and J. A. Schellman, in "The Enzymes," Vol. I, 2nd Ed., ed. by P. D. Boyer, H. Lardy and K. Myrbäck, Academic Press, Inc., New York, N. Y., 1959.

(18) In paper I (ref. 4) we deduced that the component sedimenting between S-19 and S-12 had a sedimentation constant ($S^0_{20,w}$) value of 15. Actually the experimental points were moderately scattered and an $S^0_{20,w}$ of about 17 could fit the data equally well. We have therefore assumed that the component sedimenting between S-19 and S-12 in alkaline solutions is identical to that formed at lower *p*H values on heating in 0.01 *M* KNO_3.

(19) D. C. Carpenter and J. J. Kucera, *J. Phys. Chem.*, **35**, 2619 (1931).