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The Properties of Thyroglobulin. V. The Properties of Denatured Thyroglobulin

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RECEIVED JULY 28, 1960

The properties of thermally (60°, *p*H 9.6) and alkali (25°, *p*H 12) denatured thyroglobulin have been examined by sedimentation, viscosity, light scattering and optical rotation. Fragmentation into two slower sedimenting particles, which were not denatured, preceded both the thermal and alkaline denaturation. At slightly higher temperatures or *p*H's denaturation occurred. The dissociation processes were accompanied by only marginal changes in configurational properties. Denaturation by heat (*p*H 9.6) produced no further change in the sedimentation coefficients of the fragments and only a small change in optical rotation (~5°). Denaturation by alkali produced a new sedimentable species and was accompanied by moderate changes in optical rotation and viscosity. Alkali denaturation was largely reversible on slow reduction in *p*H. The loss in solubility that occurred on denaturation was therefore associated with only limited molecular configurational changes. Considerably larger modifications in viscosity and optical rotation were observed in either 8 *M* urea or 5 *M* guanidine. Evidence is also presented which purports to show that ultracentrifugally homogeneous thyroglobulin possesses a compositional heterodispersity which is reflected in its behavior on thermal and alkaline fragmentation.

In the companion paper (IV)[1] it has been shown that thyroglobulin may be denatured, as indicated by a loss in solubility near its isoelectric point, either (1) by heating at neutral *p*H values or (2) by raising the *p*H at room temperatures. In addition it has been observed that S-19 thyroglobulin disappears and forms two slower sedimenting components (S-12 and S-17) which are soluble at *p*H 5.0 at either (1) temperatures slightly below that required for thermal denaturation (<55°) or (2) *p*H values slightly lower than the values required to produce alkaline denaturation (<11.2).

The properties of thermally dissociated and denatured as well as alkali dissociated and denatured thyroglobulin have been investigated and are contrasted with the molecular configurational states that obtain in detergent and concentrated urea and guanidine solutions. The effects of more dilute urea and guanidine solutions will be reported elsewhere.

Methods and Materials

The procedures used for measurements of sedimentation coefficient, viscosity and reduced intensity, $R = I_{90}r^2/I_0$, of light scatter were the same as described in paper I.[2] Corrections of sedimentation data for radial dilution or Johnston–Ogston effects[3] were not performed unless otherwise stated. Sedimentation constant ($S^0_{20,w}$) refers to the value of the sedimentation coefficient ($S_{20,w}$) which was obtained by extrapolation to zero protein concentration.

Optical rotatory measurements were performed with a Rudolph Model 80 photoelectric polarimeter. Measurements were performed at room temperatures (22 to 25°) and are recorded in this way. All optical rotational data conformed to a single term Drude equation. The critical wave length (λ_c) was determined from the slope of a plot of $[\alpha]\lambda^2 = [\alpha]\lambda_c^2 + k$. The specific rotations have been corrected for the refraction of the solvent to that of water by the Lorentz factor $n_w^2 + 2/n_s^2 + 2$.

A Beckman Model GS *p*H meter was used to obtain the *p*H of thyroglobulin solutions. It was calibrated with Beckman *p*H 7.0 and 10.0 standard buffers. The optical densities (O.D.) recorded for the spectrophototitration procedure were measured on a Beckman Model DU spectrophotometer. Difference spectra were obtained on a Cary Model 14 recording spectrophotometer.

Thyroglobulin preparations were identical with those described in paper IV. Urea was recrystallized before use and showed little absorption near 280 mμ. Guanidine was decolorized with Norite and recrystallized. Other reagents were reagent grade and were used without further purification.

Results

Velocity Sedimentation. 1. The Effect of Alkali.—It has been reported in paper I that thyroglobulin is rapidly denatured above *p*H ~11.4 and that concomitantly a new sedimenting boundary is formed with a sedimentation coefficient of about 8 in a 1.0% protein solution.[2] This boundary spreads rather rapidly and shows considerable asymmetry by the time it is near the bottom of the cell. The schlieren pattern of this boundary therefore represents a composite of two (or more) components with quite similar sedimentation coefficients. The concentration dependence of this composite boundary has been determined from its peak before it becomes significantly asymmetric. Its sedimentation coefficient fits the relation $S = 8.9\ (1-0.17c) \times 10^{-13}$ between 0 and 1% protein concentration.

2. The Effect of Temperature. A. <55°.—At *p*H 9.6 in *0.01 M* KNO_3 thyroglobulin was fragmented into two slower sedimenting components by heating at temperatures above ~40°. In Fig. 1 is shown the concentration dependence of the components formed by heating *p*H 9.6 solutions at 53° for 45 minutes. The fastest moving boundary has a sedimentation constant of 19.2 which is characteristic of native thyroglobulin. The slowest boundary has a $S^0_{20,w}$ value of 12.0, which is identical to the predominant component formed in *dilute* alkaline solutions at 25°. However, the intermediate boundary has an $S^0_{20,w}$ value of about 17.1.[4] For convenience the above mentioned components will be referred to as S-19, S-12 and S-17, respectively.

In Fig. 2 is reported the ultracentrifuge data showing the rate of disappearance of native thyroglobulin and the rate of formation of the S-12 and S-17 components at 46 and 51°. At neither temperature did the reaction lead to the complete loss of S-19. After an initial rapid reaction a plateau was reached in which the composition changed only

(1) H. Metzger and H. Edelhoch, *J. Am. Chem. Soc.*, **83**, 1423 (1961).

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

(3) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

(4) This component with $S^0_{20,w}$ = 17.1 is probably identical to the component which is formed on alkaline breakdown of S-19 and referred to as S-15 in paper I. Actually the data on which the $S^0_{20,w}$ value of 15 was based showed considerable scatter (*cf.* Fig. 5, ref. 2) since it was determined in solutions in which it was the minor component. An extrapolated value of $S^0_{20,w}$ of 16 to 17 could represent the data equally well.

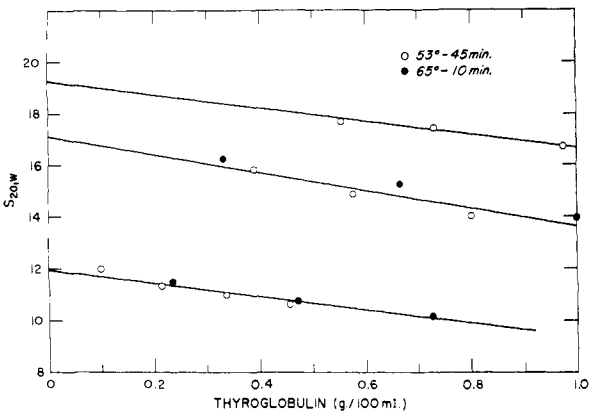

Fig. 1.—The concentration dependence of sedimentation of thyroglobulin components obtained by heating solutions at *p*H 9.6, in 0.01 *M* KNO_3, 0.02 *M* glycine. The concentrations of components are expressed in terms of the total concentration of protein in each moving boundary.

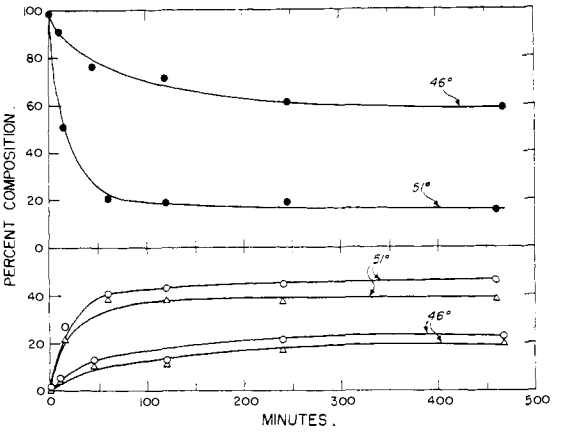

Fig. 2.—Effect of prolonged heating on the composition of thyroglobulin components at *p*H 9.6 in 0.01 *M* KNO_3, 0.02 *M* glycine: ●, S-19; ○, S-12; △, S-17.

very slowly with time. The height of the plateau was governed by the temperature. Data at other temperatures showed similar behavior. The initial rates of reaction increased considerably with temperature. Very little dissociation occurred below 40° whereas S-19 disappeared completely at 53° in several hours.

To all appearances the sedimentation data for the thermal decomposition of native thyroglobulin resembles an equilibrium process. However, the following experiments, undertaken to demonstrate a reversible reaction, were uniformly unsuccessful. All the following experiments were performed at *p*H 9.6 (0.02 *M* glycine buffer) in 0.01 *M* KNO_3. (1) No change in the ultracentrifuge pattern was found when a solution which had been heated at 51° for 120 minutes was cooled to 46° and allowed to incubate for 8 hr. (2) The S-12 component was isolated by a separation cell from a solution heated for 30 minutes at 50°. After standing at room temperature for 5 hr., no faster sedimenting components were observed. After reheating this solution for 30 minutes at 50°, again no change in sedimentation pattern was evident. (3) A solution which had been heated for 260 minutes at 46° was centrifuged in a rotor prewarmed to 46°. The schlieren boundary pattern was identical to that seen when the same sample was cooled and centrifuged at 22°. (4) 0.5 and 2.5% thyroglobulin solutions were heated for identical periods of time. The 2.5% solution was then adjusted to the exact concentration of the dilute solution in order to equalize Johnston–Ogston effects. Both solutions were centrifuged simultaneously in the same rotor by using a wedge disc cell in addition to the regular cell. The reaction occurring in the more dilute solution showed a slightly greater rate. It seems plausible to assume that the inequality in rate resulted from a small difference in the activity of thyroglobulin at the two concentrations studied. The low salt concentrations used in these experiments would tend to result in variations in thyroglobulin activity at different concentrations.[5] (5)

(5) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, *J. Am. Chem. Soc.*, **72**, 4641 (1950).

Varying the total concentration of protein in solutions containing a mixture of components did not produce any change in composition, as determined either by light scattering or sedimentation.[2]

Since the foregoing experiments failed to display any evidence of a reversible reaction with either temperature or concentration, it must be concluded that none exists under constant conditions of *p*H and salt concentration.

2. **B. >55°.**—At *p*H 9.6 in 0.01 *M* KNO_3 the rate of thyroglobulin denaturation was too slow to measure conveniently below ~55° whereas above 60° it proceeded very rapidly. Denatured thyroglobulin solutions obtained by raising the temperature to 65° (or 85°) showed two components whose sedimentation coefficients agreed, within experimental errors, with those found with heated but undenatured solutions. The data appear in Fig. 1 and Table I. It is of interest to note that at the highest temperatures explored at *p*H 9.6, *i.e.*, 85°, no evidence was found of components sedimenting slower than the S-12.

3. **The Effect of Ionic Strength.**—When native thyroglobulin was heated at either 53° or 65° in 0.10 *M* KNO_3 at *p*H 9.6 only about $^1/_3$ of the S-19 boundary disappeared to form slower sedimenting components (*cf.* No. 1 and 5 in Table I). Furthermore, when a solution of thyroglobulin which had been heated at 53° for ten minutes in 0.01 *M* KNO_3 was adjusted to 0.10 *M* KNO_3 the composition approached that observed by heating at 53° in 0.10 *M* KNO_3 for 10 minutes (*cf.* Table I for analysis of ultracentrifuge boundaries). When KNO_3 was added at 53° the extent of reversal was somewhat greater than what occurred at 25°.

It has been demonstrated earlier that the composition of thyroglobulin solutions, at neutral or slightly alkaline *p*H values[2] or in detergent solutions,[6] is influenced considerably by the ionic strength of the medium. It appears that the degree of association of thermally derived subunits of thyroglobulin is also subject to similar control.

When the *p*H of a 53° heated thyroglobulin solution was reduced from 9.6 to 6.5 at 25°, very little

(6) H. Edelhoch and R. E. Lippoldt, *J. Biol. Chem.*, **235**, 1335 (1960).

TABLE I

ULTRACENTRIFUGAL COMPOSITION OF HEATED THYROGLOBULIN SOLUTIONS[a]

No.	Heat treatment	pH	KNO_3, M	>S-19	S-19	S-17	S-12
1	53°, 10 min.	9.6	0.10		70	15	15
2	53°, 10 min.	9.6	.01		20	30	50
3a	53°, 10 min.	9.6 $\xrightarrow{b}$ 6.5	.01		15	35	50
b	53°, 10 min.	9.6 $\xrightarrow{c}$ 6.5	.01	5	60	5	30
c	53°, 10 min.	9.6	0.01 $\xrightarrow{b}$ 0.10		40	25	35
d	53°, 10 min.	9.6	0.01 $\xrightarrow{c}$ 0.10		50	30	20
4	53°, 10 min.	6.5	0.01		60	20	20
5	65°, 10 min.	9.6	.10	20	55	5	20
6	65°, 10 min.	9.6	.01			25	75

[a] All solutions contained 0.02 *M* glycine and were about 1% in protein concentration. Composition determined from the relative areas of schlieren boundaries; no corrections were made for the Johnston-Ogston effect. All centrifuge experiments were performed at room temperature. [b] Solution adjusted at 25°. [c] Solution adjusted at 53°.

change in components occurred. However, when the same procedure was carried out at 53°, almost complete reversal occurred (*cf.* Table I).

As indicated in Table I the composition of thyroglobulin solutions denatured at 65° depended on the ionic strength of the solution. Thermally denatured thyroglobulin therefore cannot be representative of a particular molecular entity since its composition varies with the concentration of KNO_3.

Optical Rotation.—In Table II are collected the optical rotatory constants, in terms of $[\alpha]_{550}$ and λ_c for thyroglobulin in a variety of solvents. All the dispersion data fit the simple Drude equation and typical plots appear in Fig. 3.

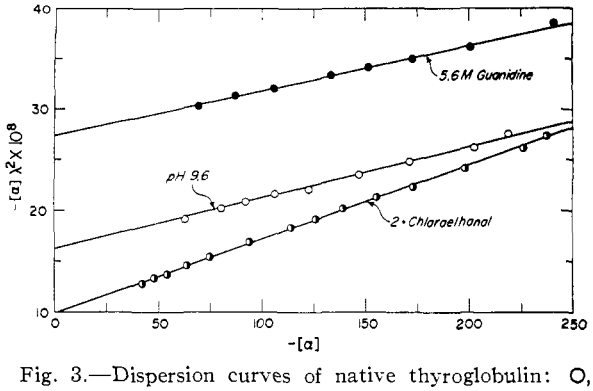

Fig. 3.—Dispersion curves of native thyroglobulin: O, *p*H 9.6, 0.01 *M* KNO_3, 0.02 *M* glycine; ●, in 5.6 *M* guanidine; ◑, in 90–10% (v./v.) 2-chloroethanol–water solution.

From Table II it can be seen that little change occurred in either $[\alpha]_{550}$ or λ_c when native thyroglobulin was largely transformed to slower sedimenting molecules by (1) heating at 53°, (2) raising the *p*H to values less than ~11.0 or (3) in *very* dilute detergent solutions. Denaturation of a *p*H 9.6 solution at 65° produced only a 5° change in $[\alpha]_{550}$ whereas denaturation by alkali at *p*H 12.3 resulted in about a 15° increase in levorotation. The change in specific rotation that occurred with heating did not reverse on cooling. On the other hand the increase observed at *p*H 12.3 was found to be almost completely reversible when the *p*H was reduced to ~7.

At higher detergent concentrations, where dissociated thyroglobulin is extensively unfolded,

TABLE II

THE SPECIFIC OPTICAL ROTATION AND DRUDE CONSTANT FOR THYROGLOBULIN SOLUTIONS IN A VARIETY OF SOLVENTS[a]

*p*H	KNO_3, *M*	Reagent	Heat treatment	$-[\alpha]_{550}$	λ_c
6.0	0.10	..	.....	62–64	225–235
9.6	0.01	0.02 *M* Glycine		64	225
	.01	.02	53°, 10 min.	66	225
	.01	.02	65°, 5 min.	69	225
10.8	.10	.02		65	230
11.4	.10	.02		71	225
12.3	.10	.02		76	225
12.3	.10	.02	65°, 5 min.	76	
7.0	0.10	0.0008 Dodecyl sulfate		64	230
7.0	0.10	0.009 Decyl sulfate		69	230
7.0	0.02	0.01		67	
7.0	.02	.04		76	
7.0	.02	.05		77	
9.6	0	5.7 Guanidine		99	214
9.6		9.0 Urea		99	212
7.0		5.3 LiBr		60	225
..		90–10% (v./v.) 2-chloroethanol–H_2O		39	275

[a] All optical rotation measurements were performed between 22 and 25° on solutions containing about 1% protein. Specific rotations have been corrected by the Lorentz equation to that obtained in water.

somewhat larger values of the specific rotation were obtained as reported in Table II. However, these values were no greater than those found at *p*H 12.3 and still represent only about 1/3 the change observed in strong hydrogen-bond-breaking solvents.[7] On removal of detergent by dialysis the specific rotation returned to its initial value.

In contrast to the rather meager increases in levorotation noted in aqueous solutions, major changes were found in urea and guanidine solutions. In both of these solvents the specific rotation values were of the same magnitude as have been reported for other unfolded proteins[7] or random-coil polyelectrolytes.[8] It is of interest to note that native thyroglobulin had a relatively low value of λ_c compared to other native proteins and that λ_c remained essentially unaltered under all conditions where water was the solvent.[9] However, a significant though small decrease in λ_c was observed in concentrated urea and guanidine solutions (*cf.* Table I). When urea was removed by dialysis the

(7) C. Schellman and J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **30**, 21 (1956).

(8) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).

(9) B. Jirgensons, *Arch. Biochem. Biophys.*, **85**, 532 (1959).

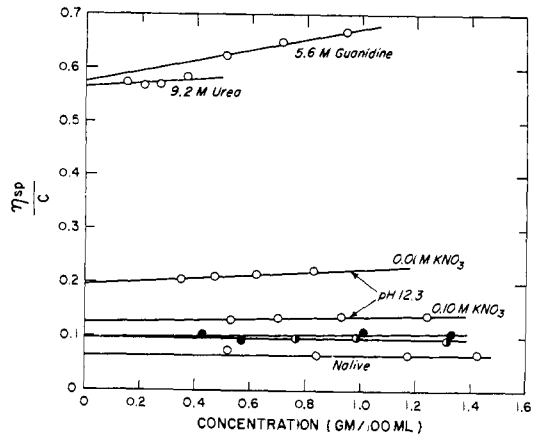

Fig. 4.—Viscometric properties of thyroglobulin in several solvents: O, native thyroglobulin at *p*H 9.6 in 0.01 *M* KNO_3, 0.02 *M* glycine; ●, native thyroglobulin (same *p*H and salts) heated at 65° for 10 minutes; ◑, native thyroglobulin (same *p*H and salts) heated at 53° for 15 minutes. Guanidine and urea solutions were at *p*H 7, *T* 28.1°.

optical rotation returned to the value characteristic of the native form of thyroglobulin.

Two solvents were studied which have been shown to produce appreciable reductions in levorotation in some proteins and polypeptides.[10,11] The optical constants of thyroglobulin in 5.3 *M* LiBr, which is the highest concentration of LiBr in which a 0.5% solution of thyroglobulin was soluble, was only slightly decreased from that found in water. However, no further change in optical rotation occurred on heating to 65° for 10 minutes in 5.3 *M* LiBr. In the second solvent, 90–10% 2-chloroethanol–water solution, a marked decrease in levorotation and increase in λ_c was found. These changes resemble those reported for other native proteins in 2-chloroethanol solutions.[11]

Viscosity.—The reduced specific viscosities of thyroglobulin in several solvents are illustrated in Fig. 4. Native thyroglobulin at *p*H 9.6 in 0.01 *M* KNO_3 had an intrinsic viscosity of ~0.06 dl./g. When this solution was heated for either 30 minutes at 53° or 10 minutes at 65° essentially the same value of 0.10 dl./g. was obtained for the intrinsic viscosity. At *p*H 12.2 in 0.01 *M* KNO_3 the intrinsic viscosity increased to 0.20 dl./g. Increasing the KNO_3 concentration to 0.10 *M* at *p*H 12.2 reduced the intrinsic viscosity to 0.12. The variability of viscosity with ionic strength suggests that the structure of the alkali-denatured thyroglobulin is, at least in part, in a flexible coil form.

It is evident from Fig. 4 that the modifications in viscosity induced by heat or alkali are modest when compared with those achieved in concentrated urea and guanidine solutions. Very similar values of the intrinsic viscosity were found in either 5.6 *M* guanidine or 9.2 *M* urea at *p*H 9.6 in 0.01 *M* KNO_3. Raising the KNO_3 concentration to 0.10 *M* in 9.2 urea had very little effect on the viscosity.

(10) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **30**, 21 (1956).

(11) K. Imahori, E. Klemperer and P. Doty, quoted by P. Doty in "Biophysical Science," ed. by J. L. Oncley, *et al.*, John Wiley and Sons, Inc., New York, N. Y., p. 112, also R. E. Weber and C. Tanford, *J. Am. Chem. Soc.*, **81**, 3225 (1959).

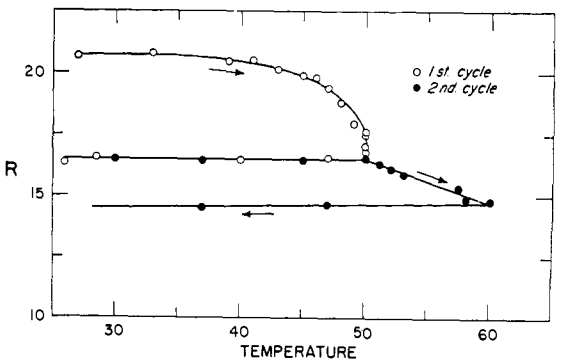

Fig. 5.—Effect of two cycles of heating and cooling on the light scattering of a single solution of thyroglobulin at *p*H 9.6, 0.01 *M* KNO_3, 0.02 *M* glycine. Ordinate is reduced intensity in arbitrary units. Arrows indicate the direction of the temperature change. Thyroglobulin concentration was 0.026%.

When the *p*H of a 5.6 *M* guanidine solution of thyroglobulin was raised to *p*H 12.2 the intrinsic viscosity declined to 0.33 dl./g. A value of ~0.48 dl./g. was found in 9.0 *M* urea at *p*H 12.2 and 0.10 *M* KNO_3.

Light Scattering.—Fig. 5 shows the effect of temperature on the reduced intensity (*R*) of a *p*H 9.6 thyroglobulin solution. The solution was warmed slowly to 50° and maintained at this temperature until no further decrease in reduced intensity occurred. On cooling slowly to 26° no change in scattering occurred. Reheating the same solution to 50° also had no effect on *R*. Between 50 and 60° a small further fall in *R* was recorded. When this solution was cooled slowly again no change in reduced intensity was noted.

The light scattering data are consistent with the ultracentrifuge data in showing that no reassociation of components occurs when heated thyroglobulin solutions are cooled. The reduced intensity decreases noticeably only above 40° which also agrees with the ultracentrifuge data.

Spectrometric Titration of Tyrosine.—It was established almost twenty years ago by Crammer and Neuberger[12] that the ionization of the tyrosine hydroxyl groups in proteins can be determined from the shift of its ultraviolet spectrum to longer wave lengths. A number of recent studies have illustrated the usefulness of this method in analyzing for abnormally ionizing tyrosine groups in proteins.[13,14] Since the iodinated tyrosines appear to be the precursors of the hormones thyroxine and triiodothyronine,[15] the behavior of the tyrosyl residues in thyroglobulin may be of import in understanding the mechanism of formation of these hormones.

The ultraviolet absorption peak of native thyroglobulin at *p*H ~7 occurs at 281 mμ. The absorption curve extends to wave lengths above 330 mμ in

(12) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(13) D. Shugar, *Biochem. J.*, **52**, 142 (1952); C. Tanford, J. D. Hauenstein and D. G. Rands, *J. Am. Chem. Soc.*, **77**, 6409 (1955).

(14) Federation Proceedings, Abstracts of Papers, Vol. XIX (1960); L. B. Smillie and C. M. Kay, p. 337; W. A. DaCosta, J. R. Sparks and F. Friedberg, p. 339.

(15) J. Robbins and J. E. Rall, *Physiol. Revs.*, **40**, 415 (1960).

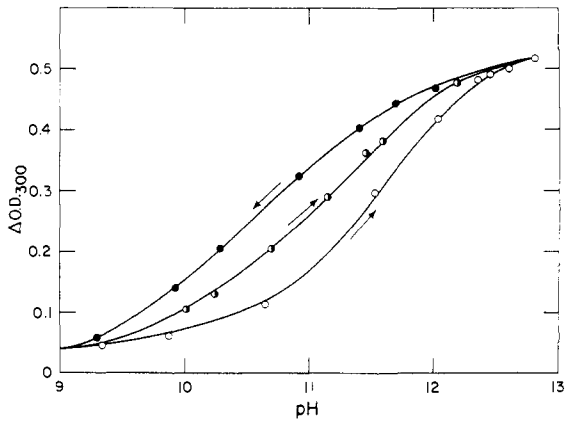

Fig. 6.—Spectrophotometric titration of phenolic hydroxyl groups of thyroglobulin (0.15 *M* KCl, 0.05 *M* glycine): O, first forward curve (adding base); ●, reverse curve from *p*H 12.5 (adding acid); ◑, second forward curve —obtained on neutralized (*p*H 9.3) alkaline denatured thyroglobulin. Thyroglobulin concentration was 0.12%.

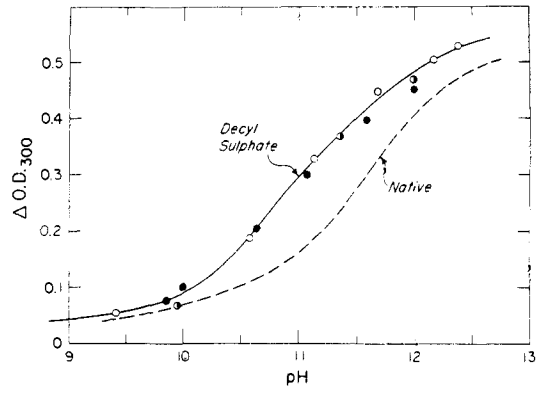

Fig. 7.—Spectrophotometric titration of phenolic hydroxyl groups of thyroglobulin in 0.033 *M* sodium decyl sulfate, 0.13 *M* KCl, 0.043 *M* glycine. Thyroglobulin concentration = 0.12%; O, first forward curve (adding base); ●, first reverse curve from *p*H 12.5; ◑, native thyroglobulin acidified to *p*H 2 in detergent and then titrated with base. Dotted line is the titration curve of native thyroglobulin (*cf.* Fig. 6). Thyroglobulin concentration was 0.12%.

dilute solutions.[16] As the *p*H of thyroglobulin solutions was increased, the absorption maxima of the difference curves (the reference cell contained a *p*H 7 solution of thyroglobulin at the same concentration) shifted gradually from approximately 310 mμ between *p*H 8 and 10 down to 295–300 above *p*H 12. This distinctive spectral behavior is mostly accounted for by the presence of several types of iodinated amino acids which absorb at higher wave lengths than tyrosine.[16]

The increase in optical density (Δ *O.D.*) at 300 mμ of a 0.12% thyroglobulin solution in 0.15 *M* KCl is shown in Fig. 6 when compared with a *p*H 6.5 solution of thyroglobulin at the same concentration. On back-titrating from *p*H 12.5 with 1 *M* HCl a reversal curve was obtained which was considerably less steep than the forward curve and displaced by 0.8 *p*H unit in the acid direction at the mid-point (Δ *O.D.* = 0.28) of the two curves.[17] A second forward curve was secured on the acidified solution (*p*H 9.3) and fell approximately between the other two[18] (*cf.* Fig. 6).

The above set of experiments were repeated with a thyroglobulin solution (*p*H 7.3, 0.15 *M* KCl) which had been denatured by heating at 65° for 10 minutes. The initial forward curve was similar in shape and slightly less steep than the native curve and was displaced to lower *p*H values by 0.2 *p*H units at the midpoint. The reversal curve also formed a hysteresis loop which was 0.45 *p*H units from the forward curve at their midpoints. The second forward curve was shifted only by 0.1 *p*H units in an acid direction from the first one.[19]

Very little difference was found in both the forward and reversal curves from the behavior of native thyroglobulin when a solution (*p*H 6.5, 0.15 *M* KCl) was heated at 53° for 45 minutes.

The data obtained for thyroglobulin in 0.035 *M* sodium decyl sulfate (0.14 *M* KCl) is reproduced in Fig. 7. The back-titration points fell on the curve formed by the forward titration data. Titration of a fluorescent conjugate of thyroglobulin in detergent solution from *p*H 7 to 12.5 had almost no effect on its polarization.[20] When a neutral solution was first acidified to *p*H 2 and then titrated with base the points also fitted on the same curve. In detergent solutions therefore the phenolic hydroxyl groups of tyrosine appear to be titrated reversibly.

Spectrophotometric titration data in 8.0 *M* urea in 0.05 *M* KCl and 0.15 *M* KNO_3 are reproduced in Fig. 8. Several points obtained by acidifying a *p*H 12.5 solution fell on the forward curve. The midpoint of the curve occurred at *p*H 10.90. Donovan, *et al.*,[21] have reported that the *pK* of phenol in 8 *M* urea is about 10.5. Since the titration curve is somewhat less steep than the mass action law for a univalent ion would predict, the difference in *pK* values may be related to a small electrostatic effect. Most, if not all, of the phenolic hydroxyl groups are therefore abnormal in native thyroglobulin and are normalized in 8 *M* urea.

The midpoint of the spectrophotometric titration curve in 5.0 *M* guanidine occurred at *p*H 10.3. Donovan, *et al.*,[21] have reported a *pK* value of 10.15 for phenol in this solvent. 5.0 *M* guanidine there-

(16) C. L. Gemmill, *Arch. Biochem. Biophys.*, **63**, 192 (1956).

(17) A gradual increase in the *O.D.* at 300 mμ occurs between 7.5 and 9.5 in aqueous media corresponding to about 10% of the total spectral change in alkali. This value is approximately equivalent to the iodinated tyrosine and thyronine content of thyroglobulin.[15] We have therefore assumed that the ionization of tyrosine begins at *p*H 9.5 in dilute salt solution.

(18) When the latter experiment was conducted on a sample which had been allowed to stand for three hours at *p*H 9.3, the titration curve was displaced by 0.15 *p*H units, at the midpoint, to the right of that obtained on immediate back-titration with base.

(19) When this experiment was performed in 0.01 *M* KCl the displacement between the thermally denatured (65°, 10 min.) and the native forward curves was 0.45 *p*H units. The displacement, at the midpoint, between the denatured forward and reverse curves was now only 0.20 *p*H units. It would appear therefore that heat denaturation in 0.01 *M* KNO_3 results in the normalization of a greater percentage of the abnormally titrating phenolic hydroxyl groups as compared to similar treatment in 0.15 *M* KNO_3. It should be noted in this connection that much less dissociation of S-19 into S-12 and S-17 occurs with heating in 0.15 *M* KNO_3 (*cf.* Table I).

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

(21) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Mol. Biol.*, **1**, 293 (1959).

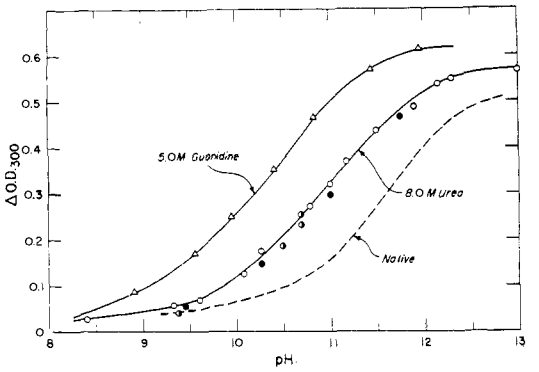

Fig. 8.—Spectrophotometric titration of phenolic hydroxyl groups of thyroglobulin in 5 *M* guanidine and in 8 *M* urea in 0.05 *M* KCl, (○) and in 0.15 *M* KNO_3 (●); reverse curve from *p*H 12.5 (◑) in 0.05 *M* KCl. Thyroglobulin concentration was 0.12%.

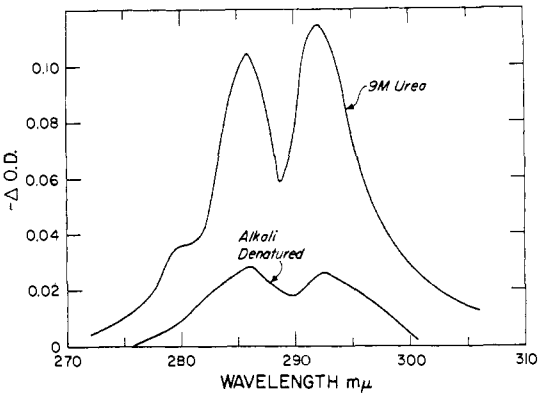

Fig. 9.—The ultraviolet difference spectra of thyroglobulin at *p*H 7.0 in (0.05 *M* phosphate buffer) 9 *M* urea and brought to *p*H 12.5 *in water* for 3 minutes and reacidified to *p*H 7.0. Thyroglobulin concentration was 0.19%.

fore also succeeds in normalizing the phenolic residues. The higher salt concentration in this solvent leads to a smaller electrostatic term and closer agreement between pK values.

Ultraviolet Difference Spectra at Neutral *p*H.—Since the phenolic hydroxyl groups in thyroglobulin show large hysteresis effects in aqueous media and titrate reversibly in 8 *M* urea, this contrast in behavior should be evident in the absorption spectrum of these groups in these two solvents. A shift to shorter wave lengths (blue shift)[22] was observed in 9.0 *M* urea in phosphate buffer at *p*H 7.0. The difference spectra, reproduced in Fig. 9, showed two principal peaks at 286 and 292.5 mμ and a minor peak at *ca.* 279.5 mμ. In 0.025 *M* decyl sulfate three peaks at closely similar wave lengths (*i.e.*, 279, 286 and 293.5 mμ) and magnitude were observed. The higher wave length peak is presumably due to tryptophan and the lower peaks to tyrosine.[23] A much smaller difference curve was obtained for alkali denatured thyroglobulin (*p*H 12.5) which had been reneutralized to *p*H 7.0. When a difference curve was measured for thyroglobulin in 50% sucrose only a small shift to longer wave lengths was evident. Hence changes in the refractive index of the solvent cannot account for the marked blue shift seen in concentrated urea or detergent solutions.[23]

Discussion

The Properties of Thermally Dissociated and Denatured Thyroglobulin.—A solution of thyroglobulin that had been heated at 53° for 45 minutes (*p*H 9.6, 0.10 *M* KNO_3) was comprised of 35% S-12, 35% S-17 and 30% S-19 as determined by measuring the areas of each component in four sedimentation experiments and extrapolating to zero concentration. The direction of the changes in areas was in accord with expectations from the Johnston–Ogston effect. A similar analysis which was performed on a 65° denatured thyroglobulin solution yielded 70% of S-12 and 30% of S-17. The viscosities of these two preparations were the same within experimental errors and were only slightly greater than that of native thyroglobulin. The S-12 component was therefore formed from S-19 without much change in frictional ratio (f/f_0). As has been shown in paper I, these two properties, *i.e.*, a sedimentation constant of 12.0 and a frictional ratio similar to native thyroglobulin, is compatible only with S-12 having a molecule weight very close to half that of the native molecule; hence the S-12 particle formed at elevated temperatures must either be identical or very closely resemble in size and shape the molecule formed in dilute alkali at room temperatures with a similar sedimentation constant.

The molecular weight of a sedimentable particle, whose form can be approximated as an ellipsoid of revolution, can be estimated from viscosity and sedimentation data by utilizing the Simha equation[24] to ascertain the axial ratio (a/b) from the intrinsic viscosity and the Perrin equation[25] to obtain the frictional ratio from a/b. If the S-17 component has the same molecular weight as native thyroglobulin, then one can calculate that it should have a f/f_0 value of 1.7. This value is quite compatible with the increase in viscosity observed in thyroglobulin solutions heated at 53 or 65° if the increase is principally due to the S-17 component.

Light scattering data on thyroglobulin solutions at *p*H 9.6 in 0.01 *M* KNO_3 showed only a small dependence of reduced intensity on concentration below 0.04%. The data reported in Fig. 5 show about a 25% decrease in scattering intensity. Other experiments conducted on solutions which had been heated to 65°, cooled to room temperature and then centrifuged for $^1/_2$ hour at 20,000 × *g* gave a similar percentage decrease in the extrapolated value of the reduced intensity. If S-12 is a molecule of half the mass and S-17 has the same mass as S-19, then a decrease in intrinsic scattering intensity of 35% would be expected from a 70–30 mixture of S-12 and S-17, respectively. The difference between the observed change of 25% and the expected is almost within the experimental errors of the measurements. The discrepancy could be readily accounted for if a very small amount of aggregation occurred on heating.

It seems not implausible that the S-17 component may be formed by dimerization of two S-12 parti-

(22) C. C. Bigelow, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **31**, 305 (1960).

(23) C. C. Bigelow and I. I. Geschwind, *ibid.*, **31**, 283 (1960).

(24) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(25) F. Perrin, *J. Phys. Radium*, **7**, **7**, 1 (1936).

cles, resulting in a slightly more asymmetric particle than S-19, since S-17 appears simultaneously with the formation of the S-12 molecule. Moreover only marginal changes in specific rotation and in the ionization behavior of the tyrosyl groups accompany the appearance of S-12 and S-17 at temperatures below 54°.

The failure of the products of thyroglobulin decomposition to reform the native molecule on lowering the temperature is indeed surprising. A constant mixture of components was reached after heating for several hours at any particular temperature below ~54°. Since no change in either sedimentation or light scattering properties occurred on cooling or dilution, we must assume that the reaction is irreversible. Native thyroglobulin may therefore consist of an assembly of molecules with similar or identical sedimentation coefficients but possessing a distribution of energies. This may be a consequence of the fact that a distribution of iodinated amino acids exists in the thyroglobulin molecules obtained from each animal or there may be differences between animals. If the degree of iodination and thyroxine formation affected the thermal stability of thyroglobulin then an explanation of the data would be at hand.

In paper I it was reported that in dilute alkali solutions, which contained a mixture of S-19 and S-12 (and S-17 at higher *p*H values), the composition was apparently independent of protein concentration as measured either by velocity sedimentation or light scattering. In addition, components were completely resolved under high speed centrifugation. It would appear therefore that a similar situation as is reported above with temperature existed when high *p*H was used to cleave thyroglobulin.

The results cited for compositional heterogeneity in thyroglobulin bear a definite resemblance to a similar type of heterogeneity in DNA. Doty, *et al.*,[26] have demonstrated that the thermal transition temperatures (T_M) in DNA preparations from different sources is related to their guanine plus cytosine content. In addition within a given preparation from a particular source they were able to show by viscosity and absorbance–temperature profiles that a distribution of stabilities with respect to temperature existed.

The Properties of Alkali Denatured Thyroglobulin.—From the value of the intrinsic viscosity of 0.12 obtained for thyroglobulin at *p*H 12.3 in 0.10 *M* KNO_3, a frictional ratio of 1.65 may be derived from the Simha and Perrin equations. When this value of f/f_0 was combined with the sedimentation constant value of 8.9 in the Svedberg equation, a molecular weight of $\sim 250 \times 10^3$ was computed. Since thyroglobulin solutions are heterodisperse at this *p*H, this molecular weight represents only an approximate average mass of the particles present. Moreover the Simha equation is not strictly applicable to a molecule possessing internal degrees of rotational freedom.

When the *p*H of a thyroglobulin preparation (in 0.10 *M* KNO_3) which had been briefly brought to *p*H ~12.3 was reduced, the denatured molecules regained their solubility. A sedimentation diagram of thyroglobulin renatured at *p*H 7.0 showed about 60% S-19, 20% S-12 and 20% of a fast shoulder on the S-19 boundary. The S-19 boundary was now broader and less symmetric than the pattern for the native molecule.

Evidence of molecular recombination of denatured molecules is also available from polarization of fluorescence measurements.[20] About a 12% decrease in polarization occurs between *p*H 10 and 12.5. When the *p*H was brought back to 10 with acid the original polarization was almost recovered. However, the forward curve was not retraced since a definite hysteresis effect occurred. The latter may reflect the time dependence seen in the recovery of the solubility properties of denatured thyroglobulin.

In addition to the recovery of solubility and molecular weight properties certain other features of the internal organization are largely regained. Seventy five per cent or more of the increase in levorotation that occurs at *p*H ~12 is regained on acidification. The extent of the recovery in optical rotatory properties depends on the alkalinity employed for denaturation and the time the solution remained in the alkaline environment. The time required to achieve complete resolubilization of denatured thyroglobulin is also sensitive to the latter factors.

The ultraviolet difference spectrum, measured at *p*H 7.0, of reconstituted thyroglobulin was less than 1/3 as large as that seen in either 9.0 *M* urea or 0.025 *M* sodium decyl sulfate. Since the maximum increase in Δ *O.D.* at 300 mμ observed in strong alkali was approximately the same in aqueous, urea and detergent solutions, the much smaller difference spectrum that occurred in water when the solution was returned to neutral *p*H values presumably originated from a partial reformation of the phenolic hydroxyl interactions.

The Properties of Thyroglobulin in Concentrated Urea and Guanidine Solutions.—Whereas only a relatively small increase in intrinsic viscosity and specific levorotation was produced by either thermal denaturation at *p*H 9.6 or by alkaline denaturation at *p*H 12.3, major modifications in these parameters were noted in both concentrated urea and guanidine solutions at neutral *p*H. The value of the specific rotation was now in accord with those found for other highly unfolded proteins.[7] The profound change in the behavior of one particular kind of residue, *i.e.* the tyrosyl group, in these two solvents is illustrated by the normalization of the phenolic hydroxyl ionization curve. The ultraviolet difference spectrum in 9.0 *M* urea also attests to the notable difference in the environment of these residues in strong urea solution as compared with aqueous media.[23]

Thyroglobulin solutions are quite stable at high concentrations of urea or guanidine at neutral *p*H values. However, when the *p*H is increased to ~12 further changes in the effective rotational kinetic unit occurs as revealed by polarization (of a fluorescent conjugate) measurements on thyroglobulin.[20] Upon examination by hydrodynamic methods of

(26) P. Doty, J. Marmur and N. Sueoka, *Brookhaven Symposia in Biology*, **12**, 1 (1959); S. A. Rice and P. Doty, *J. Am. Chem. Soc.*, **79**, 3937 (1957).

alkalinized urea and guanidine solutions both the intrinsic viscosity and sedimentation coefficient were found to decrease significantly from their values in neutral solution. The latter parameters will usually move in opposite directions if configurational modifications occur without mass changes. When both constants decline simultaneously a reduction in particle size can normally be assumed. It seems evident consequently that a further decrease in the average molecular weight of thyroglobulin subunits occurs in urea and guanidine solution when the *p*H is raised to ~12.

Comments

Dissociation of thyroglobulin into S-12 subunits either by raising the *p*H (<11) or the temperature (<54°) to values below that causing denaturation results in only a minimal perturbation in its secondary or tertiary structure.[27] Further increase in either *p*H or temperature leads to denaturation by first order kinetics involving activation energies typical of denaturation phenomena. Denaturation in alkaline solutions appears to result in somewhat greater structural disorganization than that produced thermally as judged by viscosity, specific rotation, sedimentation and polarization of fluorescence.[20] However, the chemical and physical behavior of both forms of denatured thyroglobulin still does not represent that of a random-chain polypeptide cross-linked by disulfide bridges. The latter molecular state is more closely approximated in concentrated solutions of urea and guanidine. Notwithstanding the major configurational changes induced by these solvents, it is doubtful whether all the secondary and tertiary bonds have been destroyed. As is described elsewhere[20] the polarization in these two solvents is still much too high to suggest that the smallest effective rotational unit is that of a fluorescent dye linked to an amino acid in a randomly-kinked polypeptide chain. The fact that reducing agents which destroy disulfide bridges also fail to produce this level of the polarization is also indicative of residual intramolecular interactions inhibiting complete reactivity and uncoiling. This structure-less state may, however, be approached when thyroglobulin is allowed to further degrade at *p*H ~12 in urea or guanidine solutions.

The configurational changes elicited by detergents in thyroglobulin have been previously described.[6] As revealed by viscosity, sedimentation, ultraviolet difference spectra and polarization studies major structural alterations occur in fairly dilute solutions of sodium decyl and dodecyl sulfate. The degree of unfolding is considerably greater than occurs in thermal or alkali denatured thyroglobulin but appears to be less extensive than in urea and guanidine solutions. In spite of the large changes found in frictional coefficient and relaxation time, there is only a relatively small increase in specific levorotation. It would appear therefore that detergent can destroy the hydrophobic or van der Waals interactions without extensively affecting the secondary structure (*i.e.*, α-helical) of the molecule.

Acknowledgment.—The authors wish to express their indebtedness to Mr. Roland E. Lippoldt for providing excellent assistance.

(27) For a recent discussion of the significance of these terms, see article by K. U. 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.

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

The Properties of Thyroglobulin. VI. The Internal Rigidity of Native and Denatured Thyroglobulin

BY R. F. STEINER AND H. EDELHOCH

RECEIVED JULY 28, 1960

The rotational relaxation time of native thyroglobulin has been evaluated from polarization measurements of thyroglobulin coupled to a fluorescent dye. By treatment with detergent or urea the relaxation time of thyroglobulin was markedly reduced. The original polarization was recovered on dilution out of either reagent. The disruption of the internal structure produced by detergent was evident by the ability of sulfhydryl reagents to decrease further the relaxation time whereas they are without effect in the absence of detergent. Similarly a time-dependent decrease in polarization was evident in alkali when 8 *M* urea or 5 *M* guanidine was present. In the absence of the latter reagent only a relatively small change in relaxation time occurred in alkali. The changes in polarization have been compared with hydrodynamic methods of ascertaining configurational modifications. It is apparent that polarization measurements can provide additional insight into the degree of disorganization of the internal structure of a protein molecule.

Earlier papers in this series have revealed a complex pattern of behavior for thyroglobulin under various environmental conditions.[1,2] The molecular state, as reflected by molecular kinetic and optical rotation data, is profoundly influenced by *p*H, ionic strength and temperature, as well as by the presence of several familiar protein denaturants.[1-4] The reactions which have been observed reflect the occurrence of two distinct kinds of processes, which are rather difficult to resolve. The first of these involves changes in molecular size, while the second is concerned with the cleavage of internal bonds.

At low ionic strengths,[1,3] *p*H's in the vicinity of 11,[1] or in very dilute detergent,[2] there occurs a

(1) H. Edelhoch, *J. Biol. Chem.*, **235**, 1326 (1960).
(2) H. Edelhoch and R. Lippoldt, *ibid.*, **235**, 1335 (1960).
(3) H. Lundgren and J. Williams, *J. Phys. Chem.*, **43**, 989 (1939).
(4) H. Edelhoch and H. Metzger, *J. Am. Chem. Soc.*, **83**, 1423 (1961).