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 pH is raised to $\sim$12.

Comments

Dissociation of thyroglobulin into S-12 subunits either by raising the pH ($<$11) or the temperature ($<$54°) to values below that causing denaturation results in only a minimal perturbation in its secondary or tertiary structure.[27] Further increase in either pH 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.

(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.

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 pH $\sim$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.

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

BY R. F. STEINER AND H. EDELHOCH

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 pH, 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] pH'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).

partial splitting of native thyroglobulin ($S^0_{20,w}$ = 19.4) into subunits of half the molecular weight of the intact molecule ($S^0_{20,w}$ = 12). This dissociation can be largely reversed by readjusting to more neutral conditions, i.e., pH $\sim$7 and 0.1 M KNO_2.

Under more drastic conditions the S-12 submolecules undergo a further fragmentation into subunits of $S^0_{20,w}$ = 8.9. However, this only occurs under conditions where processes of the second kind are occurring. These latter correspond to varying degrees of disruption of the internal fine structure of the molecule and occur at extremes of pH and at high concentrations of urea, guanidine or detergent. The actions of these agents, which are by no means equivalent, are invariably reflected by some degree of inflation of the molecular domain of the particular subunit present. as indicated by an increase in viscosity and frictional ratio. In the case of detergent, this inflation is ionic strength-dependent.

However, on a submolecular level, the apparent degree of attack upon the α-helical content proceeds to markedly different levels for the various cases. Thus only a relatively small change in optical rotation occurs at saturating levels of detergent or at high pH. In contrast, the action of urea or guanidine results in a profound alteration of optical rotation, reflecting presumably drastic changes in the fractional helical content.[5,6]

The complicated nature of the molecular transitions of thyroglobulin obviously makes it desirable to examine these processes by as many criteria as possible. In particular some more direct measure of the internal rigidity of the submolecules was sought in order that this aspect of the problem could be studied independently of factors related to the over-all size and shape parameters of the molecular domain. The method of fluorescence polarization appeared to provide one approach to this problem.[7-10]

This technique, which depends upon measurements of the polarization of the emitted radiation of fluorescent protein conjugates has been shown to yield a harmonic mean of the characteristic rotational relaxation times of the protein conjugate (approximated as an ellipsoid). If the latter is completely rigid and devoid of internal degrees of rotational freedom the mean relaxation time thereby computed corresponds to that of the translational kinetic unit and may, in principle, be employed to compute the parameters characterizing the molecular domain. However, if the molecule does possess internal degrees of freedom then the effective rotational kinetic unit will be smaller than the translational kinetic unit by a factor which is a measure of the loss of internal rigidity of the molecule.

The polarization of fluorescence of a labeled protein is described by equation (1)

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

(6) C. Schellman and J. Schellman, Compt. rend. trav. Lab. Carlsberg, Ser. Chim., **30**, 463 (1958).

(7) G. Weber. Biochem. J., **51**, 145 (1952).

(8) G. Weber, ibid., **51**, 155 (1952).

(9) G. Weber, in "Advances in Protein Chemistry," Vol. VIII, Academic Press, Inc., New York, N. Y., 1953.

(10) R. Steiner and A. McAlister, J. Polymer Science, **24**, 105 (1957).

$$\left(\frac{1}{P} + \frac{1}{3}\right) \Big/ \left(\frac{1}{P_0} + \frac{1}{3}\right) \equiv R = 1 + \frac{3\tau}{\rho_h} \quad (1)$$

where P = polarization of fluorescence
P_0 = limiting value of P at very high values of ρ_h
τ = excited lifetime of fluorescent residue
ρ_h = harmonic mean of characteristic relaxation times of the protein molecule approximated as an ellipsoid

$$\frac{1}{\rho_h} = \frac{1}{3}\left(\frac{1}{\rho_1} + \frac{1}{\rho_2} + \frac{1}{\rho_3}\right) \quad (2)$$

where ρ_1, ρ_2, ρ_3 are the three relaxation times corresponding to the three axes of the ellipsoid. (For an ellipsoid of revolution, $\rho_2 = \rho_3$)
If the protein possesses spherical symmetry, $\rho_1 = \rho_2 = \rho_3$ and equation 1 may be written

$$R = 1 + \frac{R'T\tau}{\eta V} \quad (3)$$

where R' = gas constant
η = solvent viscosity
V = molar volume of protein
T = temperature

The ratio $\rho_h/(3\eta V/R'T)$ ($\equiv\beta$) is a measure of the departure of the protein from the limiting case of a completely rigid, unhydrated sphere. Values of β greater than unity arise from a departure of the molecule from spherical symmetry or from hydration. Values of β less than unity can only reflect a loss in internal rigidity.

It is the purpose of the present paper to extend and supplement the observations upon thyroglobulin obtained by other means. Particular attention will be paid to the loss in rigidity brought about by the various denaturants.

It is worthy of mention that any freedom of rotation of the fluorescent residue plus the side chain to which it is attached should not influence the computed value of the relaxation time, inasmuch as the relaxation time of any rotation of the side chain itself must be much too small to be influenced by any change in solvent viscosity over the normal range of viscosities studied.[9] As the method depends upon the change in polarization with solvent viscosity (as affected by changes in temperature) any rotation of the side chain should influence the apparent value of P_0 but not that of ρ_h. This point has been discussed by Weber.[7,9]

Experimental

Materials.—Thyroglobulin was prepared by the method of differential centrifugation which is described elsewhere.[1] The preparations used in these studies consisted of 90-95% native thyroglobulin (referred to as S-19) and 5-10% of a more rapidly sedimenting component, having an $S^0_{20,w}$ equal to $\sim$30. In the case of the preparation used for making the conjugated TG-CN-IV the S-30 component was eliminated by the methods described in paper I.[1]

The urea used was recrystallized at least once from ethanol. The guanidine was decolorized by Norite treatment prior to recrystallization. Concentrations of solutions of urea and guanidine hydrochloride were determined from their refractive indices. Purified preparations of sodium decyl and sodium dodecyl sulfate were obtained as a gift from the Du Pont Company, Wilmington, Delaware. The Trimethyldodecylammonium chloride was obtained from Armour and Company, Chicago, Illinois. Mercaptoethanol was obtained from the Fisher Scientific Company, Silver Spring, Maryland. The salts used were reagent grade. All water was redistilled in an all-glass unit.

Preparation of Conjugates.—Ten ml. of a 2% solution of thyroglobulin in 0.1 M $NaHCO_3$ (pH 8.5) was chilled to 0°. To it was added with stirring 1 ml. of an aqueous suspension of 1-dimethylamine-naphthalene-5-sulfonyl chloride (DNS).[7] The latter suspension was prepared by adding 1

ml. of a 2% solution of DNS in acetone to 4 ml. of water. The mixture was allowed to stand at 0° overnight. Any DNS remaining in suspended form was removed by centrifugation for 1 hr. at 20,000 r.p.m. in a Spinco Model L preparative ultracentrifuge.

The thyroglobulin conjugate was precipitated by the addition of an equal volume of saturated KH_2PO_4–K_2HPO_4 (pH 7) at 3°. The precipitate was collected by centrifugation, redissolved in 0.01 M KNO_3 to its initial volume and reprecipitated as before. The collected precipitate was redissolved in 0.01 M KNO_3 to its initial volume and dialyzed for 48 hr. at 3° versus 0.01 M KNO_3. It was finally centrifuged at 20,000 r.p.m. for 1 hr. and stored at 3°.

Measurement of Polarization.—The degree of polarization was measured by an adaptation of the Phoenix light scattering photometer. Unpolarized incident light was used, a polaroid analyzer being inserted before the photomultiplier tube. The instrument had previously been checked for linearity of response of the photocell, using neutral filters of known transmission. The photomultiplier showed no variation in response with the plane of polarization. The fluorescent radiation was isolated from any scattered or reflected light by a pair of complementary filters. A Corning 5970 filter intercepted the incident beam and a Corning 3385 filter was inserted before the entrance slit of the photocell.

The cell containing the fluorescent solution was immersed in a larger square cell which was equipped with glass coils through which water from a constant temperature bath could be circulated. In this manner the temperature could be controlled to within 0.3° over a range from 7 to 50°.

The fluorescent intensity was measured for vertical and horizontal orientations of the plane of polarization of the analyzer. The degree of polarization was given by

$$P = \frac{I_V - I_H}{I_V + I_H} \tag{4}$$

In order to compute $\rho_h{}^{25°}$ from equation 1, the polarization was measured as a function of temperature and P_0 was obtained by extrapolation to $T/\eta = 0$. The values of P ranged from 0.1 to 0.23. Because of the high polarizations normally observed the precision was high, P being obtainable to ± 2%.

Excited Lifetimes.—The technique employed in measuring lifetimes has been reported previously.[10] The method depends upon the fact that if a fluorescent solution is excited by a beam of light which is sinusoidally modulated in intensity, the fluorescent radiation is itself modulated and its phase lags that of the exciting radiation by an angle φ. The apparatus to be described is essentially a device for measuring this phase lag.

Excited lifetime measurements were made with the apparatus described in detail in an earlier publication.[10] In brief, light from an AH-4 mercury arc, run from a 200 volt d.c. power supply, is collimated and passed through an X-cut quartz crystal, both ends of which are polished to optical flatness. The beam is then focused to a line image by a second lens.

The beam is modulated at 10 mc. by driving the crystal at 5 mc. with an ARC-5 transmitter. A 10 mc. harmonic appears, to which the phase-measuring unit is sensitive. Electrical contact to the crystal is attained by coating its sides with a lacquer containing a fine silver suspension and mounting it between two copper–beryllium spring contacts.

The line image is focused upon a stop consisting of a vertical line of graphite on a quartz plate. When the crystal is not being driven the latter is just sufficient to block the beam. When the transmitter is turned on, the crystal acts as a diffraction grating with the higher order spectra appearing and disappearing at a 10 mc. rate. The resultant blurring and widening of the line image permits passage of the modulated beam, while the unmodulated zero order spectrum is blocked by the graphite stop.

The modulated beam then passes successively through two square glass cells, each of which is adjacent to a 6217 photomultiplier tube. The first cell always contains a reference scattering solution of Ludox. The second contains either Ludox or the fluorescent solution. In the latter case appropriate complementary filters are used to separate the exciting and fluorescent wave lengths. A variable aperture before the first photocell serves to balance the outputs.

The outputs of the two photocells are coupled into 50 ohm RC-58U transmission lines. The latter are terminated in a nominal 50 ohms at the input of a mixer circuit, which serves

to combine the two signals. The mixed signal is coupled into the 80 ohm input of a Navy RB-C receiver.

The receiver serves as a sensitive and selective output detector. The receiver output meter is used as a null indicator. The output of the first photomultiplier tube is fed through a phase shift unit consisting of a multiswitch system permitting known lengths of transmission line to be thrown in or out of the circuit. The phase shift can be computed from the length of line L added to, or subtracted from, the circuit by the equation[10–12]

$$\varphi = 2\pi f \epsilon^{1/2} L/c \tag{5}$$

where f = frequency, ϵ = dielectric constant of line dielectric and c = velocity of light in vacuo.

The technique of measurement was as follows. With Ludox in both cells the phase shift unit was adjusted until a minimum output was observed for the mixed signal. Then the fluorescent solution was placed in a cell before the second photocell, and the phase was readjusted until a new null was obtained. From the length of line required to compensate for the phase lag introduced by the finite excited lifetime of the fluorescent conjugate, the phase lag itself was computed from equation 5. The excited lifetime is related to the phase lag by[10–12]

$$\tau W = \tan \varphi$$

where $W = 2\pi f$

In the case of solvents 8 M urea and 5 M guanidine hydrochloride, direct measurements of the excited lifetime were supplemented by indirect measurements using the method of Förster to compute the lifetime relative to that in water[13] (Table I). Absorption and emission curves were obtained with a Cary recording spectrophotometer and an Aminco spectrofluorometer, respectively.

TABLE I

Medium	Excited lifetimes, ($\times 10^{-8}$)	Method
H_2O	1.2	Direct
GHCl, 5 M	0.79	Forster
Urea, 8 M	.82	Forster
Urea, 8 M	.80	Direct

Extent of Conjugation.—The ultraviolet spectra of the thyroglobulin conjugates (0.01 M NaOAc, pH 6.5) were measured at concentrations close to 0.1% in a Cary recording spectrophotometer. In addition to the usual protein peak close to 280 mμ a subsidiary band arising from the naphthalene derivative occurred at 330 mμ.

The degree of conjugation was computed from the absorbancies at 280 and 330 mμ.[7,10] The molar absorbancy for the protein was taken as 7.0×10^5. That for the naphthalene residue was taken as 4.3×10^3. The degrees of conjugation for the various preparations are cited in Table II. Account was taken of the absorption of the protein at 330 mμ in determining the extent of conjugation.

TABLE II
DEGREE OF LABELING OF THE THYROGLOBULIN CONJUGATES

Preparation	No. residues per native molecule
TG-CN-II	5.7
TG-CN-III	10.4
TG-CN-IV	4.7
TG-CN-V	1.1

Values of P_0.—The value of P_0 for native thyroglobulin was found to be close to 0.23 for all preparations. This is close to the values reported for other protein conjugates and is only slightly different from the value expected for the immobilized residue itself.[9] It is thus very unlikely that any freedom of rotation of the side chain bearing the conjugate is contributing to the observed depolarization as such a factor would be reflected by an apparent drop in P_0.[7–9]

In the case of thyroglobulin denatured by detergent or urea an appreciable apparent increase in P_0 up to 0.27 was

(11) E. Bailey and G. Rollefson, J. Chem. Phys., **21**, 1315 (1953).

(12) C. Ravilious, R. Ferrar and S. Liebson, J. Opt. Soc. Am., **14**, 238 (1954).

(13) T. Forster, "Fluoreszenz Organischer Virbindungen," Gottingen, 1951.

TABLE III

RELAXATION TIMES OF THYROGLOBULIN UNDER VARIOUS CONDITIONS

Preparation	Protein concn. (g./l.)	Solvent, M	pH	Denaturant	Concn., M	$\rho_h^{25°}$ ($\times 10^8$)	β
TG-CN-IV	1.3	0.1 KNO₃	7.0	None		55.0 ± 10	
TG-CN-III	4.1	.01 KNO₃	7.0	NaC₁₂SO₄	0.001	32.5 ± 5	1.03[c]
TG-CN-III	4.1	.10 KNO₃	7.0	NaC₁₂SO₄	.001	41.0 ± 6	0.87[d]
TG-CN-II	6.1	.1 KNO₃	12.2	None		11.1 ± 0.5	(0.83)[b](.55)[e]
TG-CN-II	6.1	.01 KNO₃	7.0	NaC₁₂SO₄	.0029	7.2 ± 4	
TG-CN-III	2.5	.01 KNO₃	7.0	TDAC	.04	3.3 ± 0.2	.12[a]
TG-CN-IV	1.3	.001 KNO₃	7.0	NaC₁₂SO₄	.018	3.3 ± 0.2	.12[a]
TG-CN-III	2.1	.01 KNO₃	7.0	NaC₁₀SO₄	.035	3.0 ± 0.2	.11[a]
TG-CN-III	2.5	.01 KNO₃	7.5	Urea	8.0	2.4 ± 0.2	(.16)[b]
TG-CN-IV	2.8	.01 KNO₃	7.0	Urea	8.0	2.4 ± 0.2	(.16)[b]
TG-CN-III	2.5	.01 KNO₃	11.7	Urea	8.0	1.4 ± 0.2	
TG-CN-V	1.6	H₂O	6.9	GHCL	5.0	1.3 ± 0.3	

[a] Computed assuming $M = 3.3 \times 10^5$ (ref. 1, 2, 4, 15); [b] assuming $M = 1.6 \times 10^5$; [c] assuming $M = 3.8 \times 10^5$ (from fractional S-12 ($M = 3.3 \times 10^5$) and S-19 ($M = 6.7 \times 10^5$) content); [d] assuming $M = 5.5 \times 10^5$ (from fractional S-12 and S-19 content); [e] assuming $M = 2.4 \times 10^5$ (experimental value)[4] may be too high.

observed. No explanation is available at present for this anomalous effect. It is possible that it is an artifact arising from a slight curvature of the curves of R versus T/η at lower T/η values.

Results

A. The Effect of Individual Agents. Native Thyroglobulin.

—The relaxation time of native thyroglobulin (mol. wt. 6.7×10^5) was so large that only a slight temperature dependence of its polarization was observed for fluorescent conjugates of this excited lifetime; about 5% for a 20° change in temperature was found. Thus only a quite approximate value of $\rho_h^{25°}$ is computable. This is cited in Table III. Within experimental uncertainty, it is in the range to be expected for a *rigid* particle of this size.

The Effect of Thermal Treatment.

—Exposure of thyroglobulin (0.01 M KNO₃, pH 9.8) to a thermal cycle (5 minutes at 70°) results in only a marginal (2%) drop in polarization at 25°.

Thermally denatured solutions contain two sedimenting components: approximately 70% S-12 and 30% S-17.[14] The observed decrease in P is too small to account for the formation of 70% of a subunit of half the molecular weight of native thyroglobulin. If, however, as is proposed elsewhere,[4] the S-17 component is a dimer of S-12 with greater asymmetry than native thyroglobulin (S-19) then the polarization of this molecule should be even greater than that for S-19 and hence could cancel most of the decrease expected in P due to the S-12 molecule. The small change in P should also rule out the possibility that S-17 is an unfolded form of S-19. It is clear that thermal denaturation under these conditions has not resulted in any important loss in internal rigidity.

The Effect of Alkaline pH.

—The complex sequence of events occurring at alkaline pH has been the subject of other papers in this series. The earliest observable effect is a dissociation of native thyroglobulin into a split product (S-12) and a smaller amount of a second component (S-17). Above pH ~11.4 further breakdown occurs, leading to a slower sedimenting component, *i.e.* S-9. This latter component is probably not a discrete

molecular entity in view of the heterodispersity revealed by boundary spreading and asymmetry of its sedimentation pattern.[15]

The latter phase of molecular fragmentation is accompanied by moderate changes in internal structure, as reflected by alterations in solubility, viscosity, ultraviolet spectrum and optical rotation. These latter phenomena are largely reversible when the pH is reduced.

Titration of the thyroglobulin conjugate to alkaline pH's resulted in a gradual drop in polarization, beginning at about pH 10, as Fig. 1 shows. The data were obtained immediately after the adjustment of the pH to the value cited. If the conjugate was back titrated from pH 12.3, a definite hysteresis appeared, reflecting the incomplete (or time-dependent) reversibility of the process. However, the recovery of polarizations close to the initial values indicates that the rigidity of the native molecule was largely regained.

If the measurements of polarization are extended over a range of temperatures (Fig. 2) at pH 12.2, the mean relaxation time may be computed (Table III). The value computed for $\rho_h^{25°}$ under these conditions is 1.1×10^{-7}. The ratio of this quantity to $3\eta V/RT$ is about 0.83 (or 0.55, depending upon the value chosen for M). Inasmuch as the probable deviation of the shape of the molecular domain from spherical symmetry, as well as the occurrence of appreciable hydration would tend to increase this value above unity, it is highly likely that this value reflects the introduction of internal degrees of rotational freedom. In other words, some of the rigidity characteristic of the intact molecule has been lost. However, the effect is slight in comparison with that accompanying the action of detergents or of urea, as will be discussed subsequently. The above observations are in harmony with the fact that, at pH's about 12, the intrinsic viscosity of thyroglobulin is ionic strength-dependent.

The Effect of Detergents.

—The addition of either sodium dodecyl (C₁₂) or decyl (C₁₀) sulfate to a thyroglobulin conjugate at neutral pH and 26° resulted in a rapid drop in polarization (Fig. 3).

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

(15) Unpublished observations of H. Metzger, R. E. Lippoldt and H. Edelhoch.

Fig. 3.—Variation of relative polarization with detergent concentration for TG-CN-II (6.1 g./l.) in 0.01 M KNO_3, pH 7.1, 25.1°: ⊙, Na decyl sulfate and ●, Na dodecyl sulfate. The vertical dashed lines indicate the reversal occurring upon thirtyfold dilution with 0.01 M KNO_3.
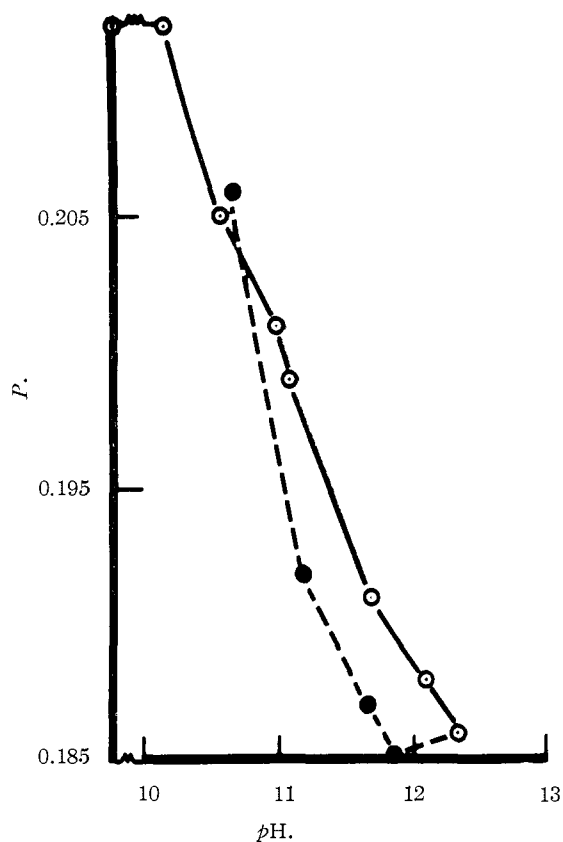

Fig. 1.—Variation of polarization (relative to pH 7) as a function of pH at 26°, TG-CN-II, 5.7 g./l., in 0.1 M KNO_3: ⊙, forward curve and ●, reverse curve.
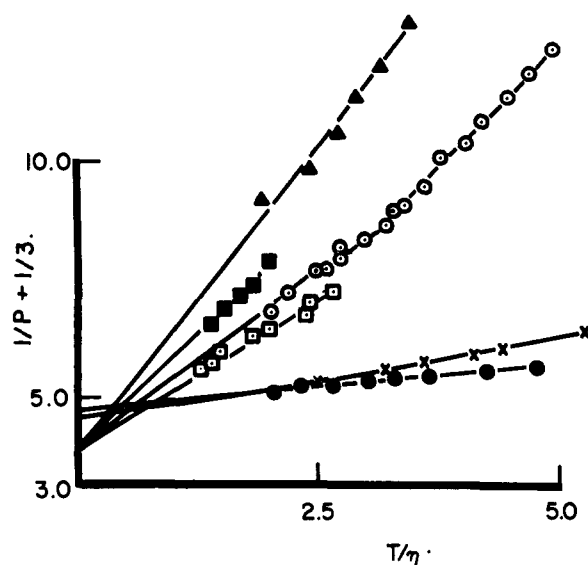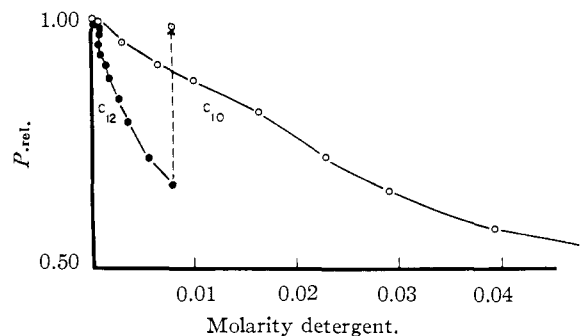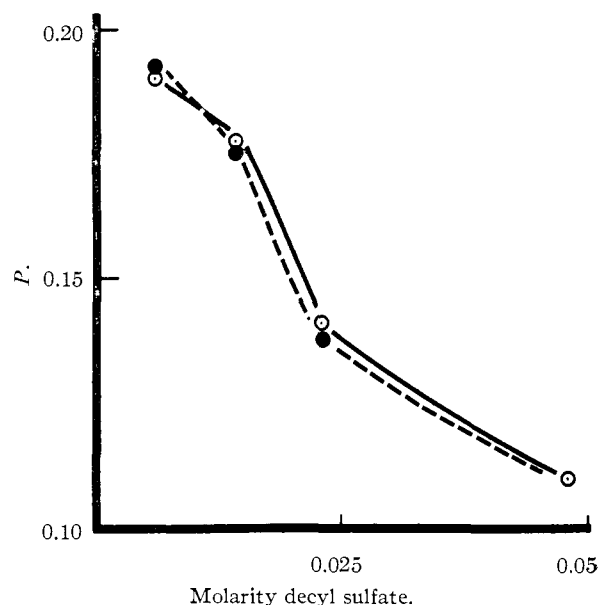

Fig. 2.—Variation of $1/P + 1/3$ with temperature for a range of conditions: ●, TG-CN-III, 4.1 g./l., 0.001 M Na dodecylsulfate, 0.1 M KNO_3, pH 7.0; X, TG-CN-II, 5.7 g./l., 0.1 M KNO_3, pH 12.2; ▣, TG-CN-IV, 2.5 g./l., 8.0 M urea, 0.01 M KNO_3, pH 7.5; ■, TG-CN-III, 2.5 g./l., 8.0 M urea, 0.01 M KNO_3, pH 11.7; ⊙, TG-CN-III, 2.1 g./l., 0.045 M Na decylsulfate, 0.01 M KNO_3, pH 7.0; ▲, TG-CN-V, 0.71 g./l., 0.037 M Na decylsulfate, 0.01 M glycine, pH 9.7; 1.85% mercaptoethanol (v./v.).

Fig. 4.—Reversibility of detergent action for TG-CN-III (1.1 g./l.) in 0.01 M KNO_3, pH 7.0, 26°: ⊙, forward curve and ●, reverse curve (by dilution at constant protein concentration).

The effect was much more dramatic than that observed at alkaline pH as a comparison of Figs. 1 and 3 shows. A considerable quantitative difference occurred between decyl and dodecyl, the latter being much more effective on a mole-for-mole basis.[16]

Figure 4 shows the reversibility of the process. For a particular value of the detergent concentration and of the detergent-protein ratio essentially the same polarization was attained irrespective of the direction from which the given molecular state was approached. The reverse curve was obtained by dilution with a non-conjugated thyroglobulin solution. Alternatively, a hundredfold dilution with solvent of the protein-detergent mixtures resulted in the recovery of a polarization close to the initial value (Fig. 3). These results do not, of course, necessarily indicate that the initial fine structure is completely regained upon dilution

(16) H. Edelhoch, J. Phys. Chem., 64, 1771 (1960).
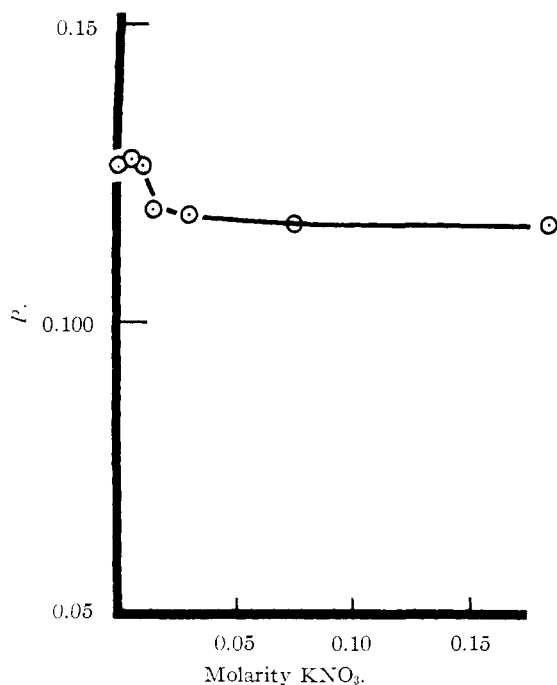
Fig. 5.—Variation of polarization with ionic strength for TG-CN-II (2.2 g./l.) in 0.028 M Na decyl sulfate, 26°.

out of detergent but only that sufficient reformation of internal structure occurs to bring about a recovery of internal rigidity to a level approximating that of the original intact molecule.

In 0.001 M sodium dodecyl sulfate, at a protein concentration of 4.1 g./l., parallel sedimentation measurements indicated that thyroglobulin was partially split into the S-12 components, the degree of splitting being dependent upon the ionic strength. Table III cites values of $\rho_h^{25°}$ and β for two ionic strengths under these conditions. The values of β are sufficiently close to unity to indicate that the native thyroglobulin structure is essentially completely rigid and that the splitting into S-12 components under these conditions is not accompanied by any important loss in rigidity. This result is consistent with sedimentation and viscosity data which indicate no significant change in frictional coefficient at this level of detergent.[2]

At high detergent levels the polarization and hence the relaxation time was almost invariant to ionic strength (Fig. 5). This is in remarkable contrast with the behavior of such measures of the effective volume as the reduced specific viscosity and the frictional coefficient which increase very pronouncedly with decreasing concentration of electrolyte.[2] The marginal change in polarization below 0.02 M KNO$_3$ would formally correspond to a slight increase in rigidity with decreasing ionic strength.

This divergence serves to underline the difference between viscosity and fluorescence polarization as a criterion of molecular state. The viscosity is dependent upon the shape and porosity of the molecular domain and hence is sensitive to the electrostatically (or osmotically) induced inflation occurring at low ionic strengths. The polarization depends only upon the size of the effective rotational

kinetic unit and hence upon the number of internal bonds and will depend therefore upon the parameters of the over-all molecular domain only if the latter can be equated to the rotational unit.

Computation of the limiting values of $\rho_h^{25°}$ in excess detergent revealed an almost tenfold drop over the value for the intact S-12 unit of thyroglobulin. The very low values of β point to an extensive loss of internal rigidity. The limiting values of $\rho_h^{25°}$ were almost identical for decyl and dodecyl sulfate (Table III).

The addition of the cationic detergent trimethyldodecylammonium chloride (TDAC) to thyroglobulin at neutral pH resulted first in a slight fall in P, then in precipitation of the protein, followed by a redissolution at higher levels of detergent. The limiting value in 0.04 M TDAC of $\rho_h^{25°}$ was close to that for the two anionic detergents (Table III).

It thus appears that a similar molecular state is approached in excess detergent for all three cases, at least by the criterion of internal rigidity.

The parallel optical rotation studies discussed in paper V of this series may be profitably reconsidered in connection with the present results. Only a relatively small change in rotation occurs in excess detergent. If current ideas on the subject are valid this suggests that only a modest change occurs in the α-helical content despite the important changes in over-all molecular configuration. Thus it appears likely that the action of detergents is confined largely to the tertiary structure. It further follows tht the tertiary structure must be important in maintaining the internal rigidity of the molecule.

Formally, the limiting value of $\rho_h^{25°}$ in excess detergent would correspond to a (spherical) subunit of molecular weight 3 × 10^4. Almost certainly, however, the remaining rigid regions span a wide range of sizes and this figure represents some kind of poorly defined average. That it is not unduly biased by preferential labeling of some particular part of the molecule is rendered likely by the equivalence of values of $\rho_h^{25°}$ obtained for several conjugates of different degrees of labeling.

The Effect of Urea and Guanidine.—The action of urea upon thyroglobulin is complicated indeed. Superimposed upon the complex splitting into subunits is a continuous alteration in internal structure, as reflected by changes in viscosity and optical rotation.[15]

In conformity with the above, the addition of urea to a thyroglobulin conjugate at neutral pH was reflected by a continuous drop in polarization (Fig. 6). There was no sign of any discontinuous transition, in harmony with the gradual character of the variation of viscosity and optical rotation.[15]

Furthermore, when the urea concentration was subsequently reduced by dilution, the original curve of polarization versus urea concentration was almost retraced (Fig. 6). Thus the loss in internal rigidity brought about by exposure to high concentrations of urea was essentially reversible.[17]

(17) Although the polarization and optical rotation return to close to the values observed for the native molecule on removal of urea the sedimentation and phenolic titration properties are not recovered. The latter show irreversible changes[15] which apparently are not reflected in the polarization of optical rotation data.
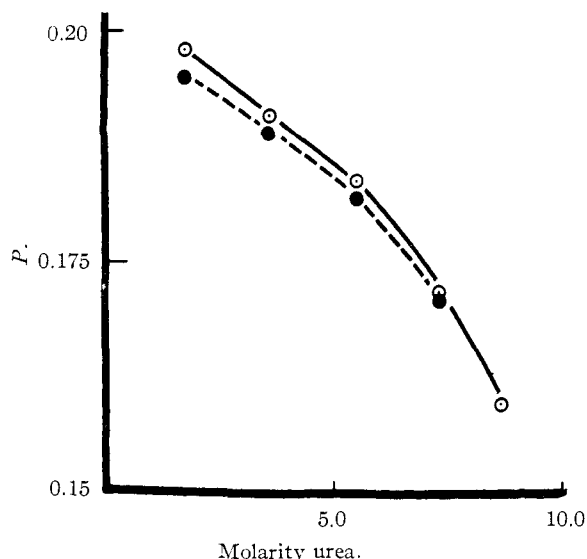
Fig. 6.—Reversibility of action of urea for TG-CN-III (0.6 g./l.) in 0.01 M KNO$_3$, pH 7.8, 26°: ○, forward curve and ●, reverse curve (by dilution). The data are uncorrected for the increase in viscosity with urea concentration.

The effect of 5 M guanidine hydrochloride was even more profound than that of 8 M urea, as Table III shows. This is in accord with the greater effectiveness of guanidine in unfolding proteins and denaturing enzymes, on a molar basis.[18,19]

B. Effect of Reagents in Combination. Effect of Urea and Detergent.—As illustrated in Fig. 7 when thyroglobulin solutions, in varying concentrations of urea, were brought to a given level of detergent concentration, the polarization was further reduced in all instances, except at the highest urea concentration studied, i.e., 9 M. If we can assume that urea has no direct influence on the stability of hydrophobic bonds and that detergent is ineffective against hydrogen bonds, then the disruption of the hydrogen bonded groups in thyroglobulin by 9 M urea leads to the cleavage of most, if not all, of the hydrophobic bonds simultaneously.

The converse is, however, certainly not true since the optical rotation results indicate that a considerable amount of secondary structure remains in thyroglobulin unfolded by excess detergent. It is likely, however, that detergent does succeed in destroying the hydrogen bonds formed between amino acid residues in some cases, if we can generalize from the behavior of the phenolic hydroxyl groups. The latter have been shown to titrate reversibly in detergent solutions.[4] Moreover, the ultraviolet difference spectrum elicited by detergent is almost identical to that observed in 8 M urea. It appears therefore that certain portions of the α-helical backbone are independently stable and do not require any support from the tertiary structure.

In 9 M urea the polarization was almost invariant to the addition of detergent (Fig. 7). If there existed a class of non-covalent bonds resistant to urea, but labile to urea and detergent in

(18) J. Schellman, R. Simpson and W. Kauzmann, J. Am. Chem. Soc., **75**, 5152 (1953).

(19) H. Edelhoch, ibid., **80**, 6648 (1958).
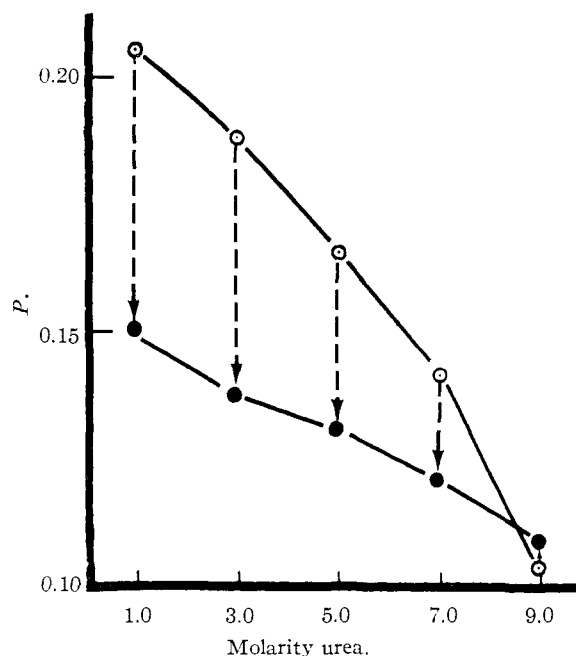
Fig. 7.—Comparison of effects of urea alone (○) and with 0.0025 M sodium dodecyl sulfate (●) for TG-CN-II (2.4 g./l.) in 0.01 M KNO$_3$, 26°. Both curves are corrected for the increase in viscosity with urea concentration, using equations 1 and 3.

combination, it would be expected that the combined action of these reagents would reduce ρ_h to a lower value than 9 M urea alone. Since this does not occur, it appears likely that no such class of bonds exists.

Examination of the temperature dependence of polarization in 8 M urea revealed that $\rho_h{}^{25}$ was greatly reduced from its value for the intact molecule and was, in fact, even smaller than the limiting value found in excess detergent (Table III).[20] The difference between the two values is, however, somewhat smaller than would be expected from the major differences in optical rotation in the two media.

Effect of Detergent Plus Reducing Agent.— As Fig. 8 shows, the polarization of the thyroglobulin conjugate was essentially unaffected by the addition of sulfhydryl reagents in the absence of detergent.[21] Nor was any effect observed at pH 7, with or without detergent. However, in the presence of detergent at slightly alkaline pH (9.6) a definite effect was observed, and the polarization fell to a level which was dependent upon the detergent concentration. The polarizations recorded in Fig. 8 were measured immediately after the addition of the sulfhydryl reagent. The oc-

(20) It is worthwhile to mention that in addition to its direct effect upon the molecular properties of thyroglobulin, the presence of urea or guanidine in high concentrations also considerably reduces the excited lifetime (Table II) and increases the solvent viscosity. Both these effects serve to increase P at constant temperature and hence to counter the effects of the molecular changes discussed above. The data of Fig. 6, 7 and 9 are uncorrected for these factors, which partially mask the full extent of the effect. The cited values of $\rho_h{}^{25}$ have, of course, been computed with due regard to these factors.

(21) This does not, of course, preclude the possibility that an effect might be observed at neutral pH at sufficiently high levels of sulfhydryl reagent or for sufficiently long reaction times.

TABLE IV

EFFECT OF REDUCTION

Preparation	Conc. (g./l.)	Medium	Reducing agent	$\rho_h{}^{25°}$ $\times 10^8$	$S_{20,w}$
TG-CN-V	0.71	0.01 M Glycine, 0.037 M Na decyl sulfate, pH 9.7	....	3.9	
TG-CN-V	0.71	.01 M Glycine, 0.037 M Na decyl sulfate, pH 9.7	Mercaptoethanol, 1.85%	1.72	
Unlabeled	8.0	.01 M Glycine, 0.01 M KNO$_3$, 0.01 M Na decyl sulfate, pH 9.7	....		9.3 (67%) / 12.5 (33%)
Unlabeled	8.0	.01 M Glycine, 0.01 M KNO$_3$, 0.01 M Na decyl sulfate, pH 9.7	Mercaptoethanol, 1.0%		8.45 (83%) / 11.6 (17%)

currence of intermediate polarizations at low levels of reducing agent is probably to be attributed to a slow reaction.[22]
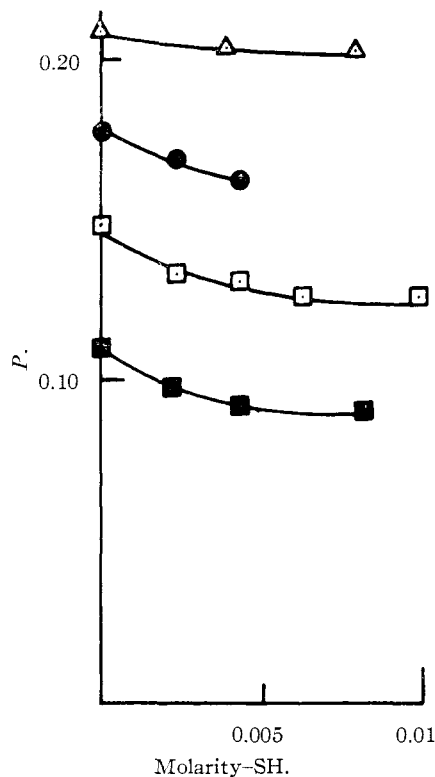
Fig. 8.—Variation of polarization with molarity of mercaptoethylamine for TG-CN-III (1.1 g./l.) in 0.01 M KNO$_3$, 0.02 M glycine, pH 9.7, 26°: △, no detergent; ●, 0.008 M Na decyl sulfate; ⊡, 0.015 M Na decyl sulfate; ■, 0.026 M Na decyl sulfate. Measurements were made immediately after addition of mercaptoethylamine.

The occurrence of a definite alteration in molecular properties upon reduction in the presence of detergent at pH 9.6 was confirmed by ultracentrifugal measurements (Table IV). In 0.037 M Na decyl sulfate the area of the faster peak was reduced by 50%. Furthermore the sedimentation coefficients of both components were reduced by about 10%. There also occurred an appreciable alteration in the appearance of the slower peak, which showed an increase in boundary spreading upon reduction.

From the preceding it follows that the prior action of detergent renders thyroglobulin sensitive

(22) The disulfide bridges of thyroglobulin may also be reduced by sulfhydryl reagents in urea solutions. The reaction, however, is more complex since a rise in polarization occurred with increasing concentrations of reducing agent subsequent to an initial decline.

to the action of reducing agents, presumably as a consequence of the exposure of –S–S– bridges. The question of whether this reflects an actual splitting into smaller kinetic units or a further loosening of the molecule must be left open for the moment.

Among the sulfhydryl reagents which were active in the presence of detergent were thioethanol, mercaptoethanolamine and sodium thioglycollate. The limiting polarizations attained in excess reagent were similar in each case. Table IV compares the values of $\rho_h{}^{25°}$ before and after reduction.

The Combined Effect of Detergent and Alkaline pH.—In the presence of excess detergent the variation of polarization with pH in the alkaline region was very slight (Fig. 9). This is in profound contrast to the cases of urea and guanidine to be discussed subsequently.
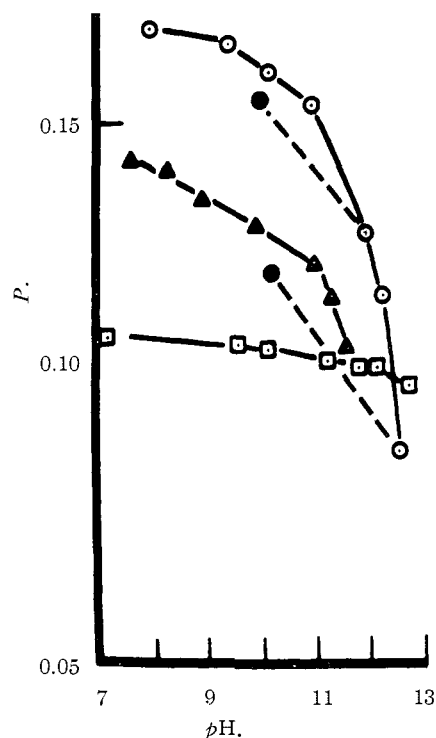
Fig. 9.—Variation of polarization (relative to pH 7) as a function of pH at 26°: ○, TG-CN-III, 2.4 g./l., in 0.01 M KNO$_3$, 8.0 M urea; ●, same, reverse; ▲, TG-CN-III, 2.0 g./l., 5.0 M GHCl; ⊡, TG-CN-III, 0.95 g./l., 0.047 M Na decyl sulfate, 0.01 M KNO$_3$. Data are uncorrected for the increase in viscosity with urea or guanidine (GHCl) concentration.

If detergent was added to a conjugate which had completed a cycle to pH 12.5 and back to
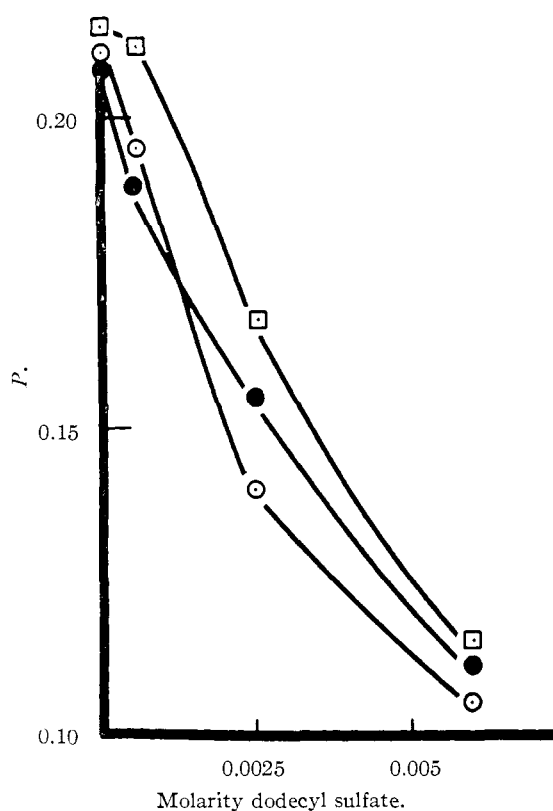
Fig. 10.—Action of detergent on TG-CN-II (2.4 g./l.) in 0.01 M KNO$_3$, 0.02 M glycine, pH 9.8, 26°: ⊙, native; ●, thermally denatured (5 min. at 70°); ▫, alkali denatured (titrated to pH 12.35 and back).
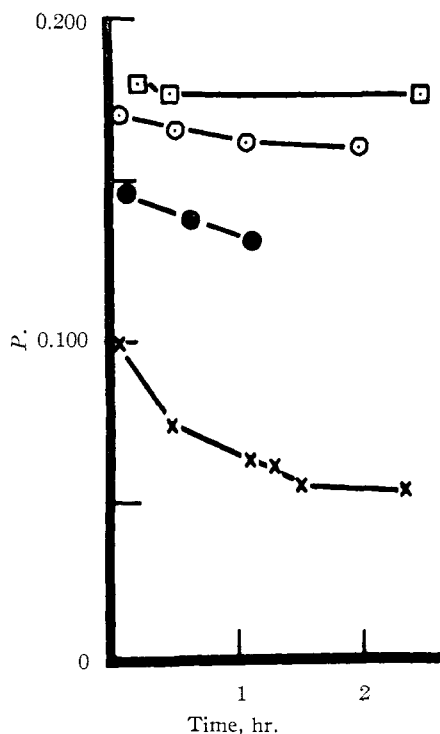

Fig. 11.—Decrease in polarization with time for TG-CN-III (1.5 g./l.) at 26° and pH 12.2 for varying concentrations of guanidine hydrochloride: ▫, 1.25 $M;$ ⊙, 2.50 $M;$ ●, 3.50 $M;$ ×, 5.0 M.

neutrality the variation of polarization with concentration of detergent roughly paralleled that for native thyroglobulin (Fig. 10). Thus exposure to an alkaline cycle does not appear to enhance the susceptibility of thyroglobulin to detergent. This result is not unexpected, in view of the reversible nature of the alkali denaturation.

The Combined Effect of Urea or Guanidine and Alkaline pH.—Even in 5 M guanidine hydrochloride thyroglobulin has apparently not lost all its internal structure at neutral pH. Thus the polarization in 5 M guanidine or 8 M urea drops rapidly at alkaline pH and the relative extent of the variation is much more pronounced than in the case of the native molecule.

The data of Fig. 9 were obtained immediately after adjustment of the pH to the value cited. Upon standing a quite slow further drop in polarization occurred with time (Fig. 11) at pH's above 12 in the case of 8 M urea and above 11 in the case of guanidine. This subsequent drop resulted ultimately (after 24 hr.) in the attainment of polarizations so low as to preclude the existence of rotational kinetic units greater than about 10^4 in apparent molecular weight.

At pH's above 12 in 8 M urea and above about 11 in 5 M GHCl the drop in polarization became largely irreversible, as Fig. 9 shows, even if reneutralization was carried out immediately (within 1 minute) after adjustment of the pH to the given alkaline value. Back titration to pH 10 from pH

12 (in 8 M urea) results in reversal of most of the change in P. Back titration from pH 12.5 results in only about a 50% recovery. It is unlikely that simple alkaline degradation is solely responsible for the irreversibility and for the time effects observed in this region, in view of the absence of these effects at still higher pH's in the presence of detergent.

The products of the combined action of urea, or guanidine, and alkali are too heterodisperse by ultracentrifugal examination to invite more detailed study at this time.[15] It is probable that both a further molecular splitting and an additional loss of structure are occurring.

It is unlikely that this further loss in structure is the result of electrostatic repulsion solely in view of its occurrence at very high ionic strength in 5 M guanidine hydrochloride. It is somewhat more likely that it reflects a loss in residual hydrogen bonding under the conditions. Both the ϵ-amino and tyrosine groups are titrated in this range.[4] In particular the ionization of tyrosine, as followed spectrally, occurs over the same pH range as the drop in mean relaxation time. However, the ionization of tyrosine appears to be essentially normal under these conditions.

Combined Effect of Urea and Acid pH.—In water thyroglobulin is denatured rapidly at pH's below 4.6.[23] In 8 M urea and 0.01 M KNO$_3$ the polarization of labeled thyroglobulin was, however, almost invariant to pH in the acid range. Thus, only a marginal (1%) decrease in polarization was

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