THE MOLECULAR WEIGHT AND ISOELECTRIC POINT OF THYROGLOBULIN

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Although thyroglobulin is the principal protein elaborated in the thyroid gland, and is possibly the actual thyroid hormone, it has not been subjected to study by the more recent physical and chemical methods available for protein research. This has been due in part to the greater interest aroused by its crystalline degradation product, thyroxine, and in part to the classical methods of preparation which failed to remove entirely impurities with undesirable properties or resulted in a more or less denatured product. It is now possible, however, to prepare large amounts of thyroglobulin, free from denatured material and nucleoprotein (1). The thyroglobulin so obtained shows the properties of a pseudoglobulin[1] and a detailed study of it has been undertaken and will form the subject of a series of communications.

The present paper deals with the molecular weight and isoelectric point of thyroglobulin,[2] two fundamental properties which are of interest both from the standpoint of protein chemistry and from that of hormone chemistry and physiology.

EXPERIMENTAL

1. Thyroglobulin Preparations.—The thyroglobulin preparations used were (*a*) a dialyzed portion of lot J 13 B_2 described in (1); (*b*) a dialyzed fresh sample

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* John Simon Guggenheim Fellow, autumn of 1934.

[1] "Pseudoglobulin" is here used in the sense of a globulin soluble in water. Thyroglobulin activity has recently been stated to be in a euglobulin fraction of high molecular weight (2).

[2] For a preliminary note *cf.* Heidelberger, M., and Svedberg, T., *Science*, 1934, **80**, 414.

isolated according to (1) from hog thyroids obtained in Stockholm through the courtesy of Prof. E. Hammarsten and Dr. E. Jorpes; and (*c*) a dialyzed sample of human thyroglobulin prepared according to (1) from a normal thyroid made available through the kindness of Dr. Wm. Barclay Parsons. (*a*) Contained 0.53 per cent of iodine and 0.02 per cent of phosphorus; (*b*) showed 0.58 per cent of iodine and 0.02 per cent of phosphorus; and (*c*) contained 0.70 per cent of iodine and 0.06 per cent of phosphorus. (*a*) Also showed 1.1 per cent of serum proteins (quantitative analyses by the precipitin method by Mr. H. E. Stokinger).

2. Specific Volume of Hog Thyroglobulin.—A 10 cc. pycnometer was used with a 2 per cent solution of thyroglobulin and a 20 cc. pycnometer with a 1 per cent solution. Estimation of the thyroglobulin content of the solutions by the micro-Kjeldahl method (thyroglobulin contains 15.8 per cent N (1)) resulted in a value of 0.71 for the specific volume, while 0.72 was found when the protein content of the solution was estimated from the dry residue (const. wt. at 105–15°, cooled over P_2O_5). The value 0.72 was taken.

3. Isoelectric Point of Hog Thyroglobulin.—The electrophoretic mobility of hog thyroglobulin was studied in different buffer solutions by the method of Tiselius (3). Acetate buffers (0.02 M NaOAc + x M HOAc), in which x varied from 0.003 to 0.5, and phosphate buffers with constant ionic strength, ($\mu = 0.02$) were used. Before each run the protein solution was made up with the buffer to be used and was dialyzed against the buffer solution for about 15 hours at room temperature. The Swedish hog thyroglobulin was used in all the experiments in a concentration of about 0.3 per cent. Chlorine and bromine light filters were used as in the velocity runs. The temperature was in all cases 20.00 ± 0.02°C.

In Table I and Fig. 1 are given the values found for the mobility in the different buffers. It is apparent from the figure that the points (circles) representing cathodic migration do not lie on the same smooth curve as the points for the anodic-migrating native thyroglobulin. Since earlier work (1) had indicated that thyroglobulin was rapidly denatured in acetate buffers below pH 4.8 experiments were carried out in which thyroglobulin was first exposed to acid acetate buffers of different pH for 1 day. After this the protein solution was dialyzed against distilled water in order to remove most of the acid acetate buffer and finally against the more alkaline phosphate buffer to be used for the electrophoresis experiment. The results are given in Table II and Fig. 1.

It is evident that the new values (crosses) found in this way for the anodic migration correspond much better with the points representing cathodic migration. It is therefore probable that thyroglobulin undergoes an irreversible electrochemical change (denaturation) in

TABLE I

Electrophoretic Mobility of Hog Thyroglobulin

Run No.	pH	Buffer	Migration	$u \cdot 10^5$
12	3.27	Acetate	Cathodic	14.6
5	3.47	Acetate	Cathodic	13.5
6	3.96	Acetate	Cathodic	10.4
11	4.24	Acetate	Cathodic	8.2
3	4.93	Acetate	Anodic	4.1
17	5.13	Acetate	Anodic	5.5
10	5.30	Acetate	Anodic	7.7
9	5.46	Phosphate	Anodic	9.4
4	5.49	Acetate	Anodic	9.3
1	5.95	Phosphate	Anodic	12.3
13	6.36	Phosphate	Anodic	15.5
18	7.02	Phosphate	Anodic	16.1
2	7.45	Phosphate	Anodic	18.1
19	8.71	Phosphate	Anodic	19.6

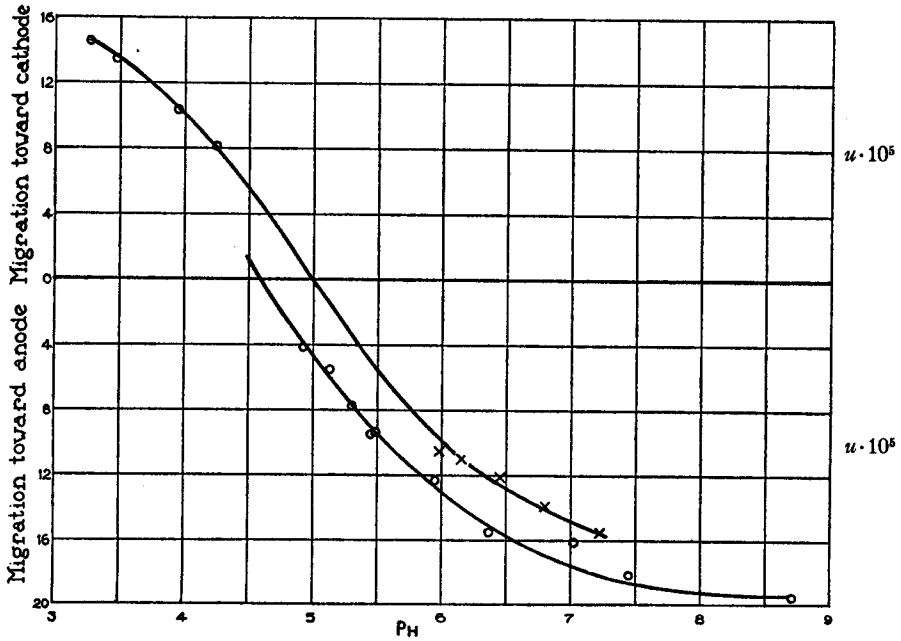

FIG. 1. Electrophoresis of thyroglobulin. o = points from Table I; x = denatured thyroglobulin (Table II).

the more acid acetate buffers, so that the curve formed by the crosses and the original points representing cathodic migration describes the electrophoretic behavior of denatured thyroglobulin. In this connection one of us (4) has found that the isoelectric point is generally shifted toward the alkaline side in the denaturation of proteins.

Denaturation of thyroglobulin also occurs at room temperature in citrate-hydrochloric acid buffers at pH 3.5 (the solution remains clear), 3.9, 4.5, and 4.8 (very slowly), so that the effect is not a specific property of the acetate ion but is probably due to hydrogen ion.

There is also another difference between native and denatured thyroglobulin. The native protein is soluble in acetate buffers down to about pH 4.8, below which denaturation occurs. Denatured thyro-

TABLE II

Denaturation of Hog Thyroglobulin

Run No.	pH I	pH II	$u \cdot 10^5$
8	*ca.* 4	5.98	10.5
7	*ca.* 4	6.15	11.0
16	4.4	6.46	12.1
14	4.2	6.80	13.9
20	5.3*	7.02	16.5
15	3.3	7.23	15.5

* Phosphate.

globulin is practically insoluble in acetate buffers from 4.3 to about 5.5, and in phosphate buffers as far as pH 5.9. The isoelectric point of native thyroglobulin is at pH 4.58 and the slope of the curve at the isoelectric point is $\left(\frac{du}{d\text{pH}}\right)_o = 11 \times 10^{-5}$. For the denatured thyroglobulin the corresponding values are pH = 5.0 and $\left(\frac{du}{d\text{pH}}\right)_o = 11 \times 10^{-5}$. Both the native and the denatured thyroglobulin were electrochemically homogeneous.

4. Sedimentation Constant of Thyroglobulin.—By means of the ultracentrifugal method (5) the rate of fall of the molecules can be determined. The sedimentation constant is the velocity in unit field

$$s = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \qquad (1)$$

TABLE III

Sedimentation Velocity of Thyroglobulin

Run No.	Thyroglobulin concentration	Solvent			pH of solution	$S_{20} \times 10^{13}$
		M	M	M		
		1. Hog thyroglobulin prepared in the United States in 1932				
	per cent					
12	0.16	NaCl, 0.043	NaOAc, 0.057	HOAc, 0.041	4.77	20.2
10	0.16		Na_2HPO_4, 0.006	KH_2PO_4, 0.094	5.76	19.3
5	0.16		Na_2HPO_4, 0.05	KH_2PO_4, 0.05	6.75*	18.5
1	0.15	NaCl, 0.1				18.8
8	0.16		Na_2HPO_4, 0.095	KH_2PO_4, 0.005	8.01*	19.3
9	0.16		$Na_2B_4O_7$, 0.05		9.03	18.9
11	0.16		Na_2CO_3, 0.038	$Na_2B_4O_7$, 0.012	9.83	20.2
		2. Freshly prepared Swedish hog thyroglobulin				
4	Total protein 0.9 Thyroglobulin 0.7	NaCl, *ca.* 0.1				15.5
18	0.33	NaCl, 0.1		HOAc, 0.133	3.02	(10.6)
14	0.17		Na_2HPO_4, 0.05	KH_2PO_4, 0.05	6.8†	19.1
17	0.50		Na_2HPO_4, 0.05	KH_2PO_4, 0.05	6.8†	18.2
15	0.17		NaOH, 0.015	Na_2HPO_4, 0.042	11.33	18.6
20	0.17	NaCl, 0.083	NaOH, 0.012		12.02	12.4 and 9.2
16	0.17		NaOH, 0.012		12.05	(6.9)
		3. Human thyroglobulin				
2	0.36	NaCl, 0.1				19.7
6	0.36		Na_2HPO_4, 0.05	KH_2PO_4, 0.05	6.75*	18.4
7	0.36		Na_2HPO_4, 0.095	KH_2PO_4, 0.005	8.01*	18.3

* pH of solvent.

† Approximate calculated pH.

where $\frac{dx}{dt}$ = observed rate of fall, ω = angular velocity, and x = distance from center of rotation.

The data for the runs made are summarized in Table III and Fig. 2. Practically all of the runs were carried out at speeds of about 40,000 R.P.M., corresponding to centrifugal forces of about 100,000 times gravity. Under these conditions, and throughout the pH stability range it was found convenient to take photographs every 10 minutes.

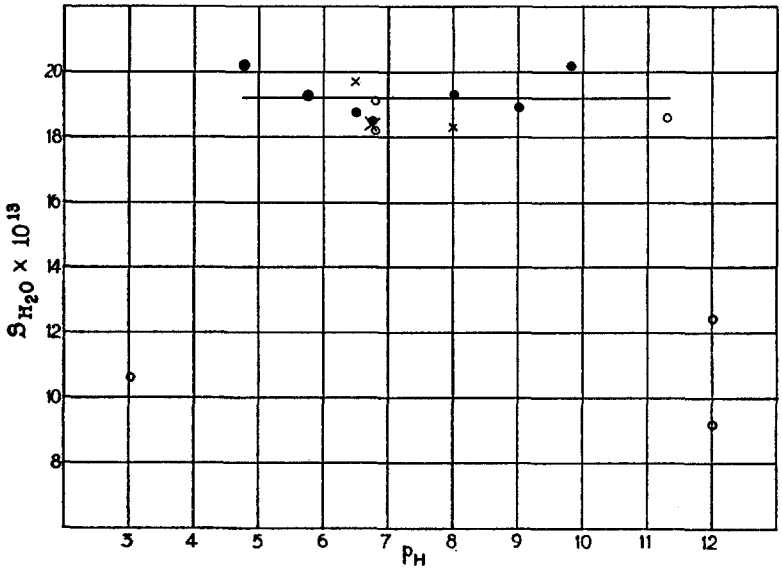

FIG. 2. Sedimentation constant of thyroglobulin. ● = U. S. hog thyroglobulin; ○ = Swedish hog thyroglobulin; x = human thyroglobulin. Line indicates mean value of 19.2.

Thyroglobulin absorbs ultraviolet light very strongly, so that it was possible to use very dilute solutions. The thickness of the column of solution varied from 1.5 to 6.0 mm., depending on the concentration used. The source of light was a mercury lamp, with chlorine and bromine filters interposed, so that the light used was of wave length below about 270 mμ. In the case of the crude hog thyroid extract, run 4, the opacity of the extract to ultraviolet light rendered the use of the refractometric method worked out by Lamm (6) obligatory.

The experiments were carried out at about 25°C., but the sedimentation constant was corrected for the density and viscosity of the salt solution to a basis of sedimentation in pure water at 20.0°C. according to the formula

$$s_{20^0} = s \frac{\eta}{\eta_o} \frac{(1 - V \rho_o)}{(1 - V \rho)} \tag{2}$$

where η = viscosity of solvent, η_o = viscosity of water at 20°C., V = partial specific volume of solute, ρ = density of solvent, ρ_o = density of water at 20°C.

The sedimentation curves show that the main part (4/5 or even more in the concentrated solution, *e.g.* run 17, Table III, Fig. 3) consists of particles probably of the same size. However, all the curves indicate the presence of varying amounts of particles with both higher and lower sedimentation constants. The two runs 14 and 17 made at the same pH, but at concentrations of 0.17 and 0.5 per cent, respectively, seem to indicate that most of the lower molecular components present are formed by dissociation of the principal molecular species. This is well illustrated by the differences in the lower portion of the photometer curves for these two runs given in Figs. 3 and 4.

In run 16, at pH 12, insufficient electrolyte was present. Owing to the Donnan effect an apparent single sedimentation constant of $6.9 \cdot 10^{-13}$ was obtained. On the other hand run 20, in which sufficient electrolyte was present, showed that at pH 12 the molecules with the sedimentation constant $19.2 \cdot 10^{-13}$ had disappeared and were replaced by two new molecules with the sedimentation constants 12.4 and $9.2 \cdot 10^{-13}$. Sedimentation constants of nearly these values have many times been found in Upsala especially in pathological sera (von Mutzenbecher (7) and McFarlane (unpublished)). In run 15, at pH 11.3, about 1/3 probably consisted of two lower molecular components, but the curves were difficult to analyze. In run 18, at pH 3, there were at least two molecular species present (probably, too, with sedimentation constants of about 9 and 12.5), but the curves were very difficult to analyze, and therefore only the mean value $10.6 \cdot 10^{-13}$ is given.

5. Molecular Weight of Thyroglobulin from Sedimentation and Diffusion Constants.—Svedberg (8) has shown that in cases in which it

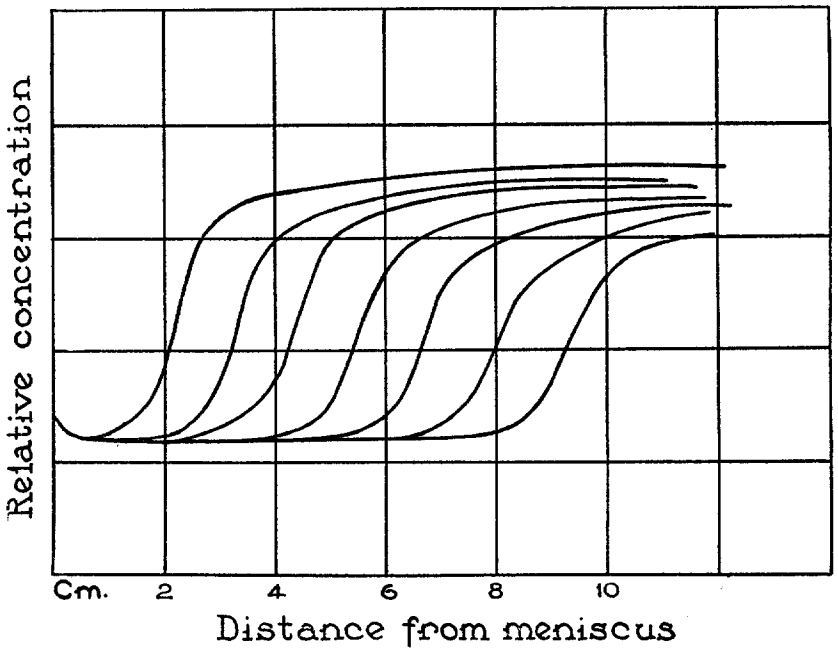

FIG. 3. Photometer curve of run 17.

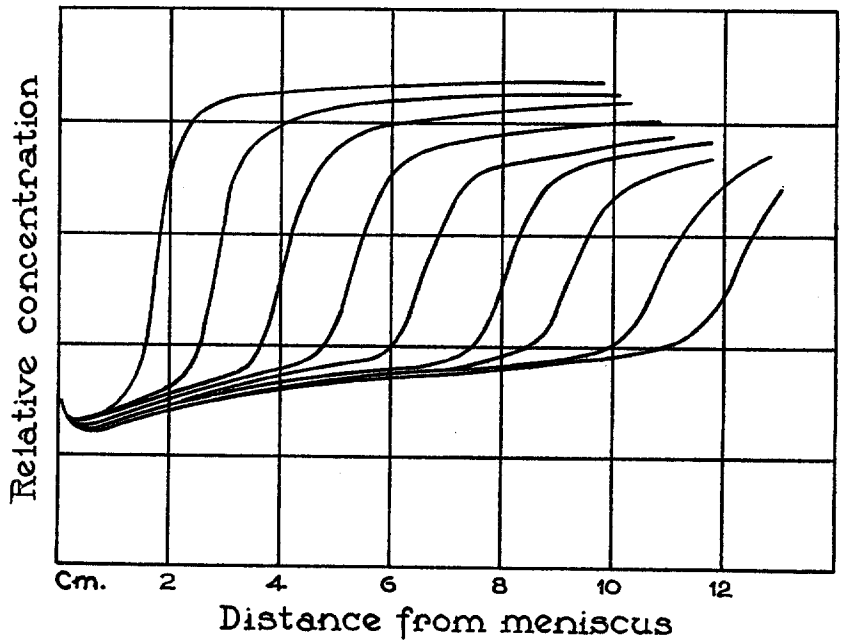

FIG. 4. Photometer curve of run 14.

could be assumed that the frictional coefficient is the same for a sedimenting particle as for a diffusing particle the molecular weight may be calculated by means of the following formula, independently of the shape of the particle:

$$M = \frac{RT \cdot s}{D(1 - V\rho)} \tag{3}$$

in which s is the sedimentation constant and D the diffusion constant at the temperature T (abs.), R the gas constant, V the specific volume, and ρ the density of the solution.

Mr. A. G. Polson, who is carrying out a systematic series of diffusion determinations on different proteins, has made some preliminary diffusion experiments with hog thyroglobulin and kindly put his values at our disposal. These experiments were performed in the apparatus used by Tiselius and Gross (9) but the measurements were made by means of the refractometric method (6) which Lamm has recently adapted for diffusion experiments.

The diffusion measurements were made with a concentration of thyroglobulin of 1 per cent and at a pH of about 5.5. The temperature was 20.0°C. and the values obtained in the salt solutions were corrected to water at 20.0°C. The diffusion curves were not quite ideal, indicating also that thyroglobulin is not monodisperse; if the diffusion constant is calculated as for a monodisperse substance, a value of $2.0 \cdot 10^{-7}$ cm.^{2}/sec. is found. Mr. Polson has, however, analyzed the curves from point to point and found that most of the material has a diffusion constant of $2.39 \cdot 10^{-7}$, but that particles are present with both lower and higher diffusion constants. By formula (3) the latter value and $19.2 \cdot 10^{-13}$ for the sedimentation constant lead to a molecular weight of about 700,000 (actually 696,000).

6. Sedimentation Equilibrium Runs.—The ultracentrifugal technique allows of a direct determination of the molecular weight by means of sedimentation equilibrium measurements (5). Six equilibrium runs were made with different concentrations of hog thyroglobulin and different buffers. The speed varied between 2,500 and 3,300 R.P.M. Equilibrium was attained after 3 to 5 days.

The measurements of the concentration gradient were made by means of the refractometric method (Lamm (6)), but calculation of

the molecular weight from the measurements was carried out by a new and as yet unpublished method worked out by the junior writer in which the following expression is used:

$$M = \frac{RT\frac{dc}{dx}}{(1 - V\cdot\rho)\omega^2\cdot x\cdot c} \tag{4}$$

in which ω is the angular velocity of the centrifuge and $\frac{dc}{dx}$ and c are the concentration gradient and the concentration at the distance x from the center of rotation. Now according to the refractometric method (4) the measured displacement of the lines, Z, is determined by

$$Z = k\cdot\frac{dn}{dx} = k\cdot\alpha\cdot\frac{dc}{dx} \tag{5}$$

in which k is an apparatus constant depending on the thickness of the cell, the distance from the scale to the cell and the photographic enlargement used, $\frac{dn}{dx}$ is the refractive index gradient, and α is the refractive index increment. By introducing this expression containing Z in place of $\frac{dc}{dx}$ in formula (4) we get

$$M = \frac{RT\cdot Z}{(1-V\cdot\rho)\omega^2 x\cdot c\cdot k\cdot\alpha} \tag{6}$$

In order to evaluate c it is assumed that the total amount of protein (substance) in solution at the start is still present in solution when the equilibrium is measured. It is then possible to determine the concentration at different points in the cell, for instance, by a modification of the method used by one of us (10).[3] If some of the substance present at the start is precipitated this method will give somewhat low values. Details of the method will be published elsewhere.

In these runs measurements (photographs) were taken with the Hg line 436 mμ and with a "Lifa" filter No. 216 (630–685 mμ), except in the first run. The refractive index increments were in part deter-

[3] Pedersen, K. O., *Z. phys. Chem., Abt. A*, 1934, **170**, 52.

mined by Mr. Kjell Andersson. For $\lambda = 436\ m\mu$, $\alpha = 192.8 \cdot 10^{-5}$ and for $\lambda = 656\ m\mu$, $\alpha = 186 \cdot 10^{-5}$. The calculated values for violet and red light and for different scale distances agreed fairly well.

Phosphate buffers were used in a concentration of 0.1 to 0.15 molar total phosphate, so that the Donnan effect may be neglected.

Below is given a summary of the results. The concentrations are given in grams per 100 ml. solution.

RUN I

c at Start = 0.335
Rev. Per Sec.: 55.0
pH = 6.8

x	*c*	*M*
5.60	0.160	554,000
5.65	0.197	565,000
5.70	0.245	587,000
5.75	0.307	606,000
5.80	0.388	619,000
5.85	0.493	623,000

RUN II

c at Start = 0.335
Rev. Per Sec.: 53.9
pH = 6.1

x	*c*	*M*
5.60	0.164	470,000
5.65	0.195	495,000
5.70	0.234	521,000
5.75	0.284	556,000
5.80	0.352	598,000
5.85	0.443	647,000

RUN V

c at Start = 0.970
Rev. Per Sec.: 42.5
pH = 6.6

x	*c*	*M*
5.55	0.520	667,000
5.60	0.599	656,000
5.65	0.690	649,000
5.70	0.799	653,000
5.75	0.926	684,000
5.80	1.092	(790,000)
		662,000

RUN VI

c at Start = 0.970
Rev. Per Sec.: 49.5
pH = 6.6

x	*c*	*M*
5.55	0.421	686,000
5.60	0.513	657,000
5.65	0.623	638,000
5.70	0.755	627,000
5.75	0.915	630,000
5.80	1.115	661,000
5.85	1.390	(772,000)
		650,000

Runs III and IV are omitted since they yielded the most uncertain values. In the other cases the error is probably about 10 per cent, and is largely due to the particles of higher molecular weight present,

which made the calculation of the concentration at the bottom of the cell difficult.

From these equilibrium runs it is obvious that the thyroglobulin has a very strong dissociation tendency in more dilute solutions (below 0.5 per cent). This agrees very well with the results of the sedimentation velocity runs. In the more concentrated solutions the dissociation of the thyroglobulin is not very marked, but just as in the dilute solution there are some particles of higher molecular weight present.

7. Molecular Frictional Constant.—The molecular frictional constant may be calculated according to Svedberg (11) from the following formula.

$$f = \frac{M(1 - V\rho)}{s} \tag{7}$$

If 675,000 be taken as the best value for the molecular weight of thyroglobulin, this leads to $9.9 \cdot 10^{16}$ for the molecular frictional constant. For a spherical particle the molecular frictional coefficient is determined by the formula

$$f_o = 6\pi\eta N \left(\frac{3MV}{4\pi N}\right) \tag{8}$$

in which N is the Avogadro number and η is the viscosity of the solution. For $M = 675{,}000$, $f_o = 6.58 \cdot 10^{16}$. Since the disymmetry number is defined as $\frac{f}{f_o}$ its value for thyroglobulin is 1.50.

DISCUSSION

The principal component of thyroglobulin is characterized by a high sedimentation constant, $s_{20} = 19.2 \cdot 10^{-13}$. Of the proteins of the higher animals already studied only a small serum globulin fraction with approximately the same s has been reported (7). From run 4 it would appear that thyroglobulin is actually present in the gland in such large molecules, for the original crude extract showed a sedimentation constant consistent with the findings on the purified protein. The somewhat lower value of s found was doubtless in large measure due to the high concentration of protein in the crude extract (*cf.* Reference 4). If, as appears probable, thyroglobulin is the actual thyroid hormone,

it is a hormone of molecular weight 675,000. This is twenty times as large as that found for insulin (12), so that there is a large gap between the two protein hormones of which the molecular weights are known. It is perhaps too early to judge the physiological significance of the high molecular weight of thyroglobulin except insofar as it would render difficult of acceptance any theory of thyroid hormone action based on the direct diffusion or penetration of the hormone into the cell, assuming the protein to be the actual hormone. It is, of course, possible that a large protein molecule such as thyroglobulin could be deposited on the surface of a cell, there either to exert its action directly by means of the two or three thyroxine groupings present per molecule, or to be broken down into reactive fragments by the proteolytic enzymes of the cell. Possibly these processes would be furthered by the tendency of thyroglobulin to dissociate in dilute solution.

Chemically there is much of interest in the high molecular weight of thyroglobulin. Outside the domain of the respiratory proteins it is perhaps the most easily accessible protein of its size and is therefore readily available for studies on the problems arising in connection with large molecules not containing metallic groups. Hog thyroglobulin is also characterized by a far lower isoelectric point, at pH 4.58, than those of the known serum globulins. While this may be a peculiarity of the animal from which the protein was derived it is evident that in the comparatively alkaline body fluids thyroglobulin would be highly ionized. Thyroglobulin exhibits a pH stability range similar to that of other proteins, but its dissociation tendency on dilution seems to be much greater. It also resembles the serum proteins in that its molecule is not spherical. The low specific volume, however, is unusual.

Although thyroglobulin which has once been dried is difficult to redissolve, the purified protein is remarkably stable in solution. Thus a preparation kept in solution for 2 years showed the same sedimentation constant for its principal component as did a freshly prepared portion. This component was, however, present in smaller amount. It is also noteworthy that the chief component of human thyroglobulin showed essentially the same sedimentation constant as did hog thyroglobulin, so that the molecular weights of the two proteins may be considered as not greatly different.

SUMMARY

1. The sedimentation constant of hog thyroglobulin is $19.2 \cdot 10^{-13}$. That of human thyroglobulin is essentially the same.

2. The specific volume of hog thyroglobulin is 0.72.

3. The isoelectric point of native hog thyroglobulin is at pH 4.58, that of denatured thyroglobulin at pH 5.0.

4. The molecular weight of hog thyroglobulin is, in round numbers, 700,000, as calculated from the sedimentation and diffusion constants, or 650,000, as calculated from the sedimentation equilibrium data.

5. The thyroglobulin molecule deviates markedly from the spherical.

In conclusion the senior writer wishes again to express his hearty thanks to Professor The Svedberg for his generous extension of the hospitality of his laboratory and his freely given counsel, and to the other members of Professor Svedberg's staff for their assistance and many courtesies.

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