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The Properties of Thyroglobulin. XVIII. Isolation of Thyroglobulin Subunits*

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ABSTRACT: After mild reduction of bovine thyroglobulin with dithiothreitol at alkaline pH, followed by alkylation, two new, slower sedimenting species are observed by centrifugation. These species have been isolated in purified form by sucrose gradient centrifugation and found to have one-fourth to one-half the molecular weight of the native protein. Cleavage with cyanogen bromide of the isolated subunits, after extensive reduction and alkylation, revealed no differences by disc electrophoresis between these two species and native thyroglobulin. The number of disc electrophoretic bands observed was close to half the number of methionine residues present in thyroglobulin and implies that the different chains of thyroglobulin must exist in identical pairs.

The slowest sedimenting component therefore appears to be a mixture of two different subunits with very similar molecular properties. The faster sedimenting component(s) appears to be an unfolded form of the 12S dissociation product of thyroglobulin.

The effects of pH, urea, and mercaptoethanol on the extent of disulfide-bond reduction of 19S thyroglobulin have been reported recently (de Crombrugghe *et al.*, 1966). The molecular properties of two, new, slower sedimenting components observed at alkaline pH in dilute urea were evaluated by sedimentation velocity and viscosity. From these measurements it was suggested that the slower component was about one-half the size of 12 S and that the faster one was probably a dimer formed by association of the slower one. It has been shown that the 12S species is a subunit of 19 S observable under a variety of conditions where covalent bonds are unlikely to be broken, *i.e.*, low ionic strength, alkaline pH, in detergent, in urea, and in guanidine solution (Edelhoch, 1960; Edelhoch and Lippoldt, 1960, 1964).

It is the purpose of this communication to describe the isolation of the slower sedimenting subunit, to characterize some of its physical properties, and to compare its chemical properties with those of the native protein.

Materials and Methods

Preparation of Partially Reduced and Alkylated Thyro-

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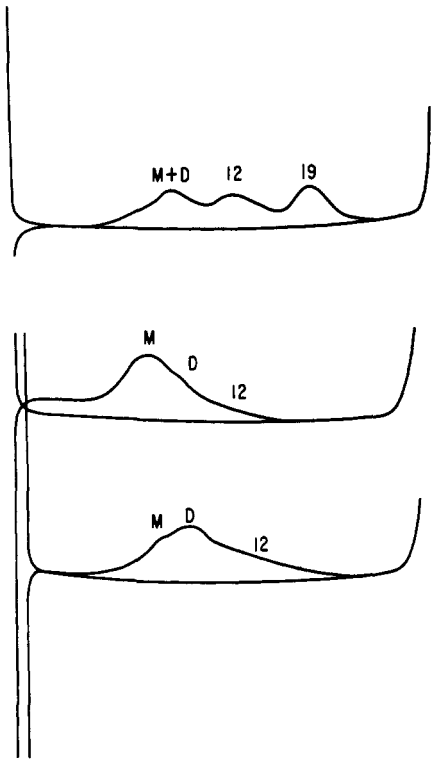

FIGURE 1: Ultracentrifugal patterns of partially reduced, alkylated thyroglobulin (0.6%) in pH 10.0, 0.05 M KCl–0.05 M glycine buffer. Upper half: before differential centrifugation; lower half: after differential centrifugation. Wedge cell (middle): protein from first 5-ml fraction; standard cell (bottom): from second 5-ml fraction. Revolutions per minute = 56,000; exposures at 40 min after reaching full speed. The components in each frame are identified by either their sedimentation constants or by the symbols M and D, as defined in the text.

globulin. Purified bovine thyroglobulin was prepared by a differential centrifugation procedure which has been described elsewhere (Edelhoch and Lippoldt, 1964). There are 101 disulfide (Edelhoch and Rall, 1964) and a few SH groups (Pitt-Rivers and Schwartz, 1967) in native bovine thyroglobulin. The disulfide groups were reduced with dithiothreitol at pH 10.0 (either in 0.05 M glycine–0.05 M KCl or 0.01 M NaF–0.02 M Na_2CO_3–0.016 M $NaHCO_3$) at a molar ratio (reducing agent to thyroglobulin disulfide groups) of 1. The same buffer was used throughout the purification procedure. Dithiothreitol was dissolved in the pH 10.0 buffer and added to a 0.6% solution of thyroglobulin in the same buffer while the pH was maintained constant by the careful addition of 2 M KOH. After 0.5 hr at 25° the pH was reduced to 8.5 with HCl. The SH groups of the protein and unreacted dithiothreitol were alkylated using a molar ratio of iodoacetic acid to added dithiothreitol of 3.0. The pH was maintained at 8.5 by adding 2 M KOH during the addition of the neutralized iodoacetic acid solution. After 30-min reaction at 25° the protein solution was dialyzed against the pH 10 buffer at 4° for 24 hr to eliminate uncombined reagents.

Preliminary Purification of Thyroglobulin Subunits. The above preparation of lightly reduced and alkylated thyroglobulin at pH 10.0 in glycine or bicarbonate buffer (see Figure 1) was centrifuged at 30,000 rpm, for 5.5 hr at 4° in a type 30, fixed-angle rotor in the Beckman Model L-2 ultracentrifuge. The top 2 ml was removed and discarded and the remaining contents of the tube were recovered in 5-ml aliquots. The upper two 5-ml aliquots were relatively enriched in the slower sedimenting components (Figure 1) and were concentrated by vacuum dialysis using a Sartorius membrane filter (collodion bag). The bottom two 5-ml aliquots were mixed, diluted to 0.6%, and recentrifuged in the type 30 rotor. The top 2 ml was discarded and 5-ml fractions were collected from the remainder of the tube. The first two 5-ml fractions were concentrated, added to the material from the first differential centrifugation, and used for subsequent sucrose gradient centrifugation.

Further Purification of Subunits. Sucrose gradient density centrifugation was performed in a Beckman SW 25.1 swinging-bucket rotor at 25,000 rpm for 44 hr at 4° with a 5–28% gradient in the pH 10.0 glycine or bicarbonate buffer. Not more than 10 mg of protein was layered on each tube. Fractions containing 25 drops were collected and diluted with 1 ml of the same buffer. The relative concentration of protein was determined by fluorescence measurements with an Aminco-Bowman spectrofluorometer. Solutions in quartz cuvets were excited at 280 mμ and their emission intensities were observed at 340 mμ. The sucrose gradient centrifugations were repeated on appropriate fractions for further purification of the subunits.

Preparation of Cyanogen Bromide Fragments. Cyanogen bromide cleaves peptide bonds adjacent to methionine residues in a polypeptide chain, converting methionine to homoserine (Gross, 1968). Native and subunit fractions of thyroglobulin were strongly reduced and alkylated before cyanogen bromide treatment. The procedure, in general, followed that of Waxdal *et al.* (1968). A 0.4% solution of protein in 6 M guanidine hydrochloride, 0.5 M Tris, and 0.002 M EDTA (pH 8.5) was placed in a screw-top vial. The vial was flushed with nitrogen, capped, and placed in a 50° water bath for 30 min to denature the protein fully. Dithiothreitol in 6 M guanidine hydrochloride at pH 8.5 (50 moles/mole of protein disulfide) was added; the vial was then flushed with nitrogen, recapped, and maintained at 50° for 4 hr. The solution was cooled to room temperature and iodoacetic acid in 6 M guanidine hydrochloride at pH 8.5 (150 moles/mole of protein disulfide) was added. The alkylation was carried out for 30 min at 25°.

The protein solution was first dialyzed against 6 M guanidine hydrochloride–0.50 M Tris–0.002 M EDTA at pH 8.5 for 24 hr at 4° and then against 70% formic acid for 24 hr at 4°. Cyanogen bromide was added to the 70% formic acid solution of protein to give a molar ratio of cyanogen bromide to protein methionine residues of 150. The reaction proceeded for 20 hr at 25° in a tightly stoppered flask and was terminated by lyophilization after addition of a tenfold excess of distilled water.

Disc Electrophoresis. The disc electrophoresis experiments follow the procedure of Reisfeld and Small (1966). The buffer (pH 8.9) in the upper tray consisted of 5.16

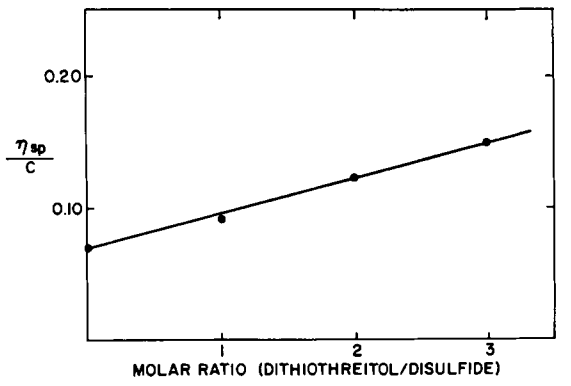

FIGURE 2: The effect of dithiothreitol (expressed as the molar ratio of dithiothreitol to protein disulfide bonds) on the reduced specific viscosity of thyroglobulin in pH 10.0, 0.05 M KCl–0.05 M glycine buffer. Each point represents a separate preparation of reduced and alkylated protein, prepared as described in the text. Protein concentration was 0.39%. T = 25.00°.

g of Tris, 3.48 g of glycine, and of 7 M urea to make 1 l. The buffer (pH 8.1) in the lower tray consisted of 14.5 g of Tris, 60 ml of 1 N HCl, and water to make 1 l. The lower and upper gel stock solutions containing the ingredients described by Reisfeld and Small were brought to volume with 7 M urea or water rather than 10 M urea. An 8% gel was prepared by increasing the amount of the acrylamide in the lower gel. Native thyroglobulin and subunits were run in a 4% gel in water. The cyanogen bromide fragments were run in an 8% gel using 7 M urea in the upper buffer and gels. A current of 2–3 mA/tube was applied and electrophoresis was carried out at 25° until the bromophenol blue tracking dye had moved to the bottom of the gel, usually 1.5–2 hr. The protein bands were precipitated in the gel with 12.5% trichloroacetic acid and stained with 0.01% coomassie blue in 12.5% trichloroacetic acid. The gels were then destained with 12.5% trichloroacetic acid.

Sedimentation Velocity. The Spinco Model E centrifuge was used with double-sector cells. A relatively high ionic strength buffer was used (0.04 M NaF, 0.04 M Na_2CO_3, and 0.033 M $NaHCO_3$) in order to diminish the primary charge effect in measuring the sedimentation coefficients of the subunits at pH 10.0. Standard procedures were used to calculate sedimentation values.

Sedimentation Equilibrium. The Spinco Model E ultracentrifuge equipped with interference optics was used. A cell containing a three-channel centerpiece was employed. The meniscus depletion procedure of Yphantis (1964) was followed. Calculations were made of data collected after about 24 hr. Longer times of sedimentation did not affect the concentration gradient established after 24 hr. A speed of 15,000 rpm was used in the experiments with the slowest sedimenting species. The temperature was controlled at 25°.

Amino acid analysis was performed on 1–2 mg of protein by Dr. E. Miller (National Institutes of Health) following the automatic, accelerated single column procedure of Miller and Piez (1966). Hydrolysis was carried out in 6 M HCl at 100° for 24 hr under nitrogen in a sealed tube.

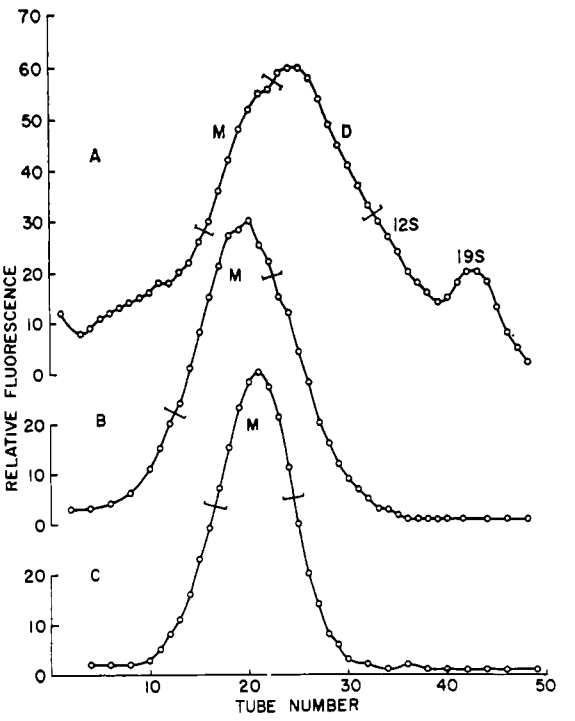

FIGURE 3: Successive sucrose gradient centrifugations showing the purification of M from the differential centrifugation preparation. Gradient, 5–28%; time, 44 hr; 25,000 rpm; 4°. Buffer: 0.01 M NaF–0.02 M Na_2CO_3–0.016 M $NaHCO_3$ (pH 10.0). (A) First gradient analysis of partially reduced and alkylated thyroglobulin from differential centrifugation. (B) Second gradient analysis of the contents of tubes 16–22 in A. (C) Third gradient analysis of contents of tubes 13–22 in B. The contents of tubes 17–24 in C were used for characterization of M.

Viscosity. Viscosity measurements were made in a circular Ostwald capillary viscometer. The flow time for water was 172 sec. The temperature of the bath for the viscometer was controlled at 25.000 ± 0.001°. The reduced specific viscosity, η_{sp}/c, is defined by the ratio $(t - t_0)/(t_0 c)$ where t and t_0 are the flow times of the solutions and solvent, respectively, and c is the concentration of protein in g/100 ml.

Reagents. Dithiothreitol (Calbiochem) and cyanogen bromide (Eastman) were used directly. Iodoacetic acid (Eastman) was recrystallized twice from 2,2,4-trimethylpentane. Guanidine hydrochloride was a product of Aldrich. Urea (Fisher) was made up to a 7 M solution and treated with the H^+ and OH^- forms of Dowex ion-exchange resin (2 g/l.) to lower the conductivity prior to use in disc electrophoresis. Glass-distilled water was used throughout. Reagent grade salts were used.

Results

I. Purification of Reduced and Alkylated Subunits. A. PARTIAL REDUCTION OF DISULFIDE BONDS. Low molar ratios of dithiothreitol to protein disulfide bonds were used in an attempt to split preferentially the interchain disulfide bonds. If few interchain bonds are split one could hopefully obtain globular subunits which are soluble in water and more closely related to their structure

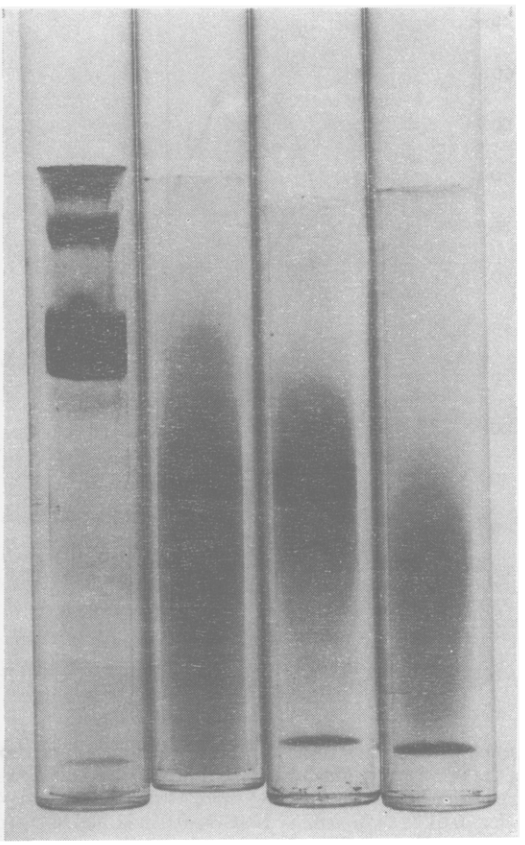

FIGURE 4: Disc electrophoresis patterns of native and purified fractions of reduced and alkylated thyroglobulin in a 4% polyacrylamide gel. Each tube contained about 0.2 mg of protein. From left to right: (1) native thyroglobulin; (2) contents of tubes 22–23 (Figure 2A) after first sucrose gradient centrifugation; (3 and 4) the D and M fractions after the third sucrose gradient centrifugation. The band at the bottom of the gel is the salt front.

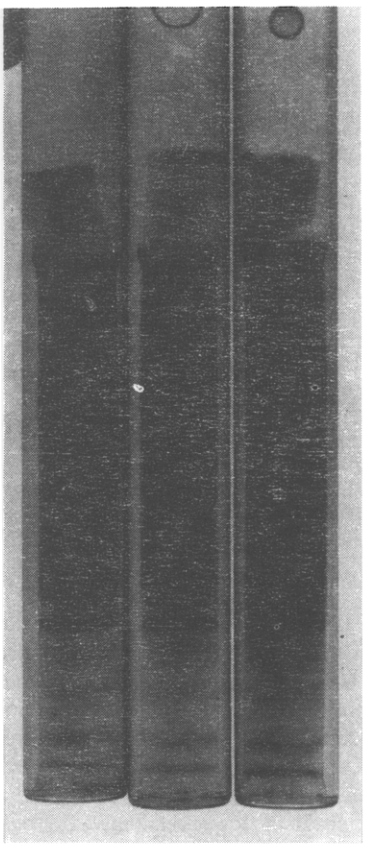

FIGURE 5: Disc electrophoresis in 8% polyacrylamide gel in 7 M urea of thyroglobulin fragments produced by cyanogen bromide. Each tube contained about 0.15 mg of protein. From left to right, tubes contained fragments derived from native, D, and M species, respectively. The band at the bottom of the gel is the salt front.

in the native form of thyroglobulin. The ultracentrifugal pattern of bovine thyroglobulin after reduction (and alkylation) at a molar ratio of 1.0 is illustrated in Figure 1. The four components which are evident have been designated as 19 S, 12 S, D, and M in harmony with an earlier description (de Crombrugghe *et al.*, 1966).

A molar ratio of 1.0 was selected since lower ratios produced smaller yields of subunits while larger ratios had a greater effect on the form of the components than on the composition. The effect of increasing the molar ratio of dithiothreitol to protein disulfide bonds on the viscosity of thyroglobulin is reported in Figure 2. Light-scattering data were consistent in showing that the major decrease in turbidity occurred at molar ratios between 1 and 2. Higher concentrations of reducing agent however accelerated the rate of reduction and final values of turbidity were reached after 30 min instead of times greater than 1 hr which were found at molar ratios of 1 to 2.

B. ISOLATION OF M AND D SPECIES. Since the sucrose gradient centrifugation which was needed for the final purification of the subunits can only accommodate small amounts of protein, the M and D species were partially separated from the 19S and 12S species by a preliminary differential centrifugation, as outlined in the Materials and Methods section. The relative amount of M plus D in the solutions collected from the differential centrifugation experiment was about 70% which represented slightly better than a twofold increase in their concentration. This solution was then purified further by sucrose gradient centrifugation (see Methods). The distribution of protein in the first gradient experiment, as determined by fluorescence measurements, is depicted in Figure 3A. The small leading peak is clearly 19 S. The D and M species comprise the fast and slow sides of the main peak with the 12S species in the lead of the fast slope. The contents of the tubes on the fast (26–30) and slow (16–22) sides of the peak were pooled, concentrated, and rerun separately in the same gradient. The distribution after gradient resolution of the protein in tubes of 16–22 of Figure 3A is shown in Figure 3B. The distribution curve is still asymmetric with evidence of components sedimenting faster and slower than the principal component. The protein solutions in tubes 13–22 of Figure 3B were then pooled and run a second time on the same gradient. The distribution of protein after centrifugation is illustrated in Figure 3C. The contents of tubes 17–24 in Figure 3C were pooled, dialyzed, concentrated, and used for characterization of the M species.

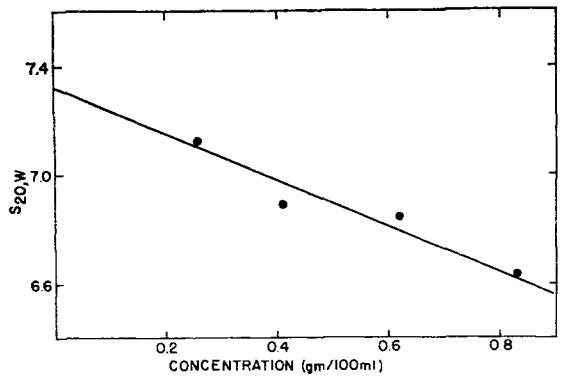

FIGURE 6: The concentration dependence of the sedimentation coefficient of the M species in pH 10.0, 0.04 M NaF–0.04 M Na_2CO_3–0.033 M $NaHCO_3$ buffer.

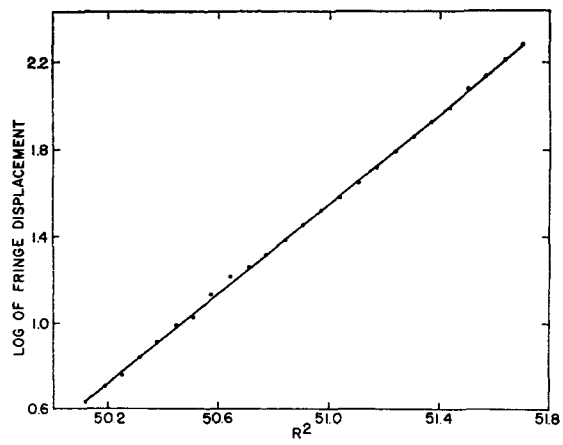

FIGURE 7: The sedimentation equilibrium data of the M species in pH 10.0, 0.04 M NaF–0.04 M Na_2CO_3–0.033 M $NaHCO_3$ buffer. Protein concentration was 0.04%. Data obtained at 15,100 rpm at 25.4°.

Two successive sucrose gradient centrifugations were performed on the protein in fractions 26–30 of the first gradient (Figure 3A) and treated in a similar manner as described for the purification of the M fraction. This fraction then constituted the D component.

II. Characterization of Subunits. A. DISC ELECTROPHORESIS. Gel electrophoresis patterns in water of native thyroglobulin and M and D species are shown in Figure 4. The first tube on the left shows the pattern of native bovine thyroglobulin. The fastest moving band representing the 19S species is broad, perhaps due to the heterogeneity of iodine content of thyroglobulin. The two slower moving bands presumably represent the 27S and 32S components, respectively.

The pattern for the material obtained from the fraction between the M and D regions of the first sucrose gradient centrifugation (tubes 23–25; Figure 3A) is shown in the second tube. It is seen to contain only the M and D species. The third and fourth tubes show the patterns of the D and M species obtained from the third sucrose gradient centrifugation (see Figure 3C). The band representing the D species is sharper than that of the M and migrates more slowly. The D species is contaminated with a small amount of M component whereas the M fraction is free of D. It should be noted that although the M band is diffuse it is never resolved into two distinct bands.

B. AMINO ACID ANALYSIS. Amino acid analysis of the M and D preparations from the second sucrose centrifugations agreed within experimental errors with the composition of the native protein. The extent of reduction of M and D could be approximated from the amount of carboxymethylcysteine observed. Two preparations of M gave 4 and 7 moles while D gave *ca.* 2 moles/mole of each species. The content of tyrosine, monoiodotyrosine, and diiodotyrosine, as determined by spectral analysis (Edelhoch, 1962), revealed no significant differences between the M species and the native protein.

C. CYANOGEN BROMIDE FRAGMENTS. Figure 5 shows the disc electrophoresis patterns for the cyanogen bromide fragments prepared from the native thyroglobulin and the M and D fractions. The latter were obtained from the third sucrose purified gradient centrifugation. The patterns for thyroglobulin and the M and D species were essentially identical and there were approximately 23 bands in each gel. Electrophoresis was also performed in gels about 25 cm in length, where the bands were better separated and were essentially identical with those shown in Figure 5. Amino acid analysis of acid hydrolyzed, cyanogen bromide fragments revealed that 80–85% of the methionines were converted to homoserine or homoserine lactone and 90% of the disulfide groups had been reduced and alkylated.

D. SEDIMENTATION VELOCITY. The sedimentation pattern of the M species (in 0.04 M NaF–0.04 M $NaCO_3$–0.033 M Na_2HCO_3, pH 10.0) at 0.82% revealed only a single, symmetrical boundary. The concentration dependence of its sedimentation coefficient was small (Figure 6) and typical of a globular protein. An extrapolated value ($s^0_{20,w}$) of 7.3 was found which is in good agreement with the value found after mercaptoethanol reduction in 1 M urea (pH 10.2) reported previously (de Crombrugghe *et al.*, 1966).

E. SEDIMENTATION EQUILIBRIUM. The molecular weight of the M species (from the third sucrose gradient) was determined by sedimentation equilibrium. The ionic strength of the standard pH 10.0 bicarbonate buffer (see Methods) was increased more than twofold in order to reduce nonideal behavior due to the moderately high charge density of the protein at this pH. A linear dependence of log fringe number against r^2 was observed (Figure 7) with solutions containing initial concentrations of M of 0.025, 0.040, and 0.075%. Molecular weights of 159,000, 163,000, and 156,000, respectively, were computed from the slopes using a partial specific volume of 0.713 for the protein (Edelhoch, 1960). Doubling the ionic strength of the solution gave essentially the same result except that a small upward curvature was found at higher concentrations.

A fourth sucrose gradient centrifugation was performed on the D fraction since disc electrophoresis after the third centrifugation showed that it was contaminated with M. The protein from the tubes in the middle of the curve were pooled, concentrated, and dialyzed against the bicarbonate buffer used for the equilibrium experi-

ments with M (*i.e.*, 0.04 M NaF–0.04 M Na_2CO_3–0.033 M $NaHCO_3$). A plot of log fringe number against r^2 for a 0.04% solution gave a molecular weight of 320,000 although a few points at the lowest concentration were above the line connecting the rest and suggesting the presence of a small amount of impurity with a lower molecular weight.

Discussion

A molar ratio of dithiothreitol to protein disulfide bonds of 1 was chosen as a compromise in order to obtain the maximum yield of subunits with the minimum amount of structural disorganization. At smaller molar ratios the yield was less while at higher ratios viscosity measurements indicated that significant unfolding had occurred.

The molecular weight of 160,000 obtained for the M species is similar to the values calculated by de Crombrugghe *et al.* (1966) for fully reduced and alkylated bovine thyroglobulin in 5 M guanidine hydrochloride from both sedimentation equilibrium and viscosity sedimentation data by the Flory-Mandelkern equation. This suggests that the subunits produced by mild reduction and alkylation are the same size as those produced by complete reduction and alkylation in 5 M guanidine hydrochloride. However, other workers have obtained molecular weights as low as 80,000 and 113,000–123,000 for fully reduced and alkylated thyroglobulin in 5 and 6 M guanidine hydrochloride and sodium dodecyl sulfate, raising the possibility that the monomeric subunit of thyroglobulin is smaller than a quarter of the 19S molecule (mol wt 660,000) (Pierce *et al.*, 1965; Lissitzky *et al.*, 1968). The results of this report do not shed further light on the size of the smallest subunit of thyroglobulin.

No significant differences were observed between purified M fractions and native thyroglobulin either by amino and iodoamino acid analysis or by disc gel electrophoresis patterns of their cyanogen bromide fragments. Since there are 46 methionine residues/mole of bovine thyroglobulin (Edelhoch and Rall, 1964) and only half this number of bands was found after cyanogen bromide cleavage, all chains must be present in identical pairs. Consequently there should be as many different types of chains as is required to build a molecule of 330,000 from the subunits. Since the molecular weight of the M species is close to 160,000 this preparation should therefore consist of a mixture of two different chains. The size and charge of these two chains should be very similar since they were not resolvable either by sedimentation or gel electrophoresis. It should be emphasized that some of the bands seen in gel electrophoresis may result from incomplete disulfide reduction or methionine conversion. It was, however, shown that the number of gel bands was independent of the iodine content since preparations of human goiter (0.04% iodine) and normal human thyroglobulin (0.62% iodine) gave indistinguishable patterns after reaction with cyanogen bromide.

Further independent evidence that the subunits are not chemically identical has been suggested by fingerprint data of tryptic digests (Lissitzky *et al.*, 1965) and by polyacrylamide gel electrophoresis of fully reduced and alkylated thyroglobulin in 8 M urea (Edelhoch, 1965).

The D preparation has been shown to have a molecular weight about twice as large as the M. Since it sediments only slightly faster than the M species it must have a much larger frictional ratio. It is probably an unfolded form of the 12S molecule since it has fewer disulfide bonds broken than the M species. Since D produced as many fragments as 19 S after cyanogen bromide treatment, it must contain all the chains present in 19 S either as pairs of identical chains or as a mixture of two different 12S molecules.

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