Evidence has been presented that thyroglobulin may be synthesized by way of its subunits (1, 2). When thyroid slices are incubated with labeled amino acids, radioactivity appears in the 12 S and 3 to 8 S fractions of the soluble thyroid protein before it is found in thyroglobulin (19 S). Furthermore, the radioactivity of the 12 S fraction, and to a lesser extent of the 3 to 8 S fraction, can be transferred by unlabeled amino acid into the 19 S protein. Although these labeled proteins can be found in the soluble fraction under the conditions used for analysis, this may very well not represent their actual localizations within the intact cell but rather indicate their relative ease of extraction from particle-bound sites.

There is a lag of 15 to 20 minutes before significant amino acid incorporation into thyroglobulin is found. Iodination of thyroglobulin, however, is a much more rapid process, occurring within a matter of seconds after addition of 125I to incubating thyroid slices. Iodination occurs after formation of the thyroglobulin polypeptide (1, 3-7) and presumably occurs close to the apical end of the follicular cell, perhaps just before secretion of the protein into the colloid, although not all investigators are in agreement as to the exact anatomical site (8, 9). Additional support for the dissociation of iodination and thyroglobulin polypeptide synthesis comes from experiments in which the former is completely blocked by propylthiouracil while the latter proceeds normally (1, 3, 5, 6). From this information it is reasonable to suppose that the intracellular sites of peptide bond formation and iodination might be different. This report will describe experiments in which an effort was made to identify these sites by subcellular fractionation of lamb thyroid slices labeled with 125I or amino acid.

Additional evidence for the heterogeneity of thyroglobulin has come from experiments similar to those described above in which sucrose gradient centrifugation analysis has revealed that both the 125I- and amino acid-labeled thyroglobulin sediment more slowly than the bulk (presumably colloidal) of unlabeled thyroglobulin (1, 2). It appeared worthwhile to establish whether the newly synthesized material differed structurally from the preformed thyroglobulin. Experiments will be described in which it was found that the former was more susceptible to dissociation to its 12 S subunit by detergents and alkaline pH than the latter. Such data are of special interest in view of the possibility that the slice-labeled 12 S protein might result from the degradation of a metastable 19 S protein during isolation (1). This paper will also provide evidence for an immunological relationship between the 12 S protein labeled with amino acid in vitro and thyroglobulin, and will describe some of the properties of the 12 S protein.

EXPERIMENTAL PROCEDURE

Methods used in the incubation of lamb thyroid slices with radioactive amino acids or 125I and in the preparation of labeled thyroid soluble protein for sucrose density gradient centrifugation analysis were as previously described (2) and modified (1). Unless otherwise specified, 1 g of lamb thyroid slices was incubated in 4 ml of Eagle's medium containing 50 μCi of 125I or 100 μg of H-leucine. All other operations were conducted in the cold.

Subcellular Fractionation—Following incubation, thyroid slices were rinsed in four changes of cold Medium A, homogenized with 4 ml of Medium A in a Potter-Elvehjem glass homogenizer, and centrifuged at 900 X g for 10 minutes to remove nuclei, whole cells, and cell debris. The supernatant fluid was centrifuged at 9,000 X g for 10 minutes. Both pellets (900 X g and 9,000 X g) were separately washed five times by gentle resuspension in Medium A and centrifuged at the previous centrifugal forces for 10 minutes. The first through fourth washes of each were pooled, concentrated to a small volume with Carbowax, dialyzed against PBS, and analyzed by sucrose density gradient centrifugation as described for the soluble thyroid proteins (1, 2). The fifth wash was treated similarly. The 9,000 X g supernatant fraction was centrifuged at 105,000 X g for 90 minutes in the Spinco model L preparative ultracentrifuge. The supernatant fluid from the 105,000 X g centrifugation was concentrated and freed of unincorporated label by four precipitations with 50% ammonium sulfate. The final precipitate was dissolved in a minimal volume of PBS and analyzed by gradient centrifugation (2). The 105,000 X g pellet was washed once by gentle rehomogenization in 0.5 ml of Medium A, layering of the suspension on top of a tube containing 20% sucrose in Medium A, and centrifugation for 4 hours at 105,000 X g. The sucrose solution was treated as the wash of the high speed pellet and was analyzed as described for the washes of the slower speed pellets. The 900 X g, 9,000 X g...
a, and 105,000 × g pellets were resuspended separately in 0.5 ml of Medium A and dispersed in a Raytheon sonic oscillator, cooled by ice water, for two separate 15-minute periods. The sonic extract was centrifuged at 25,000 × g for 30 minutes, and an aliquot of the resulting supernatant fluid was put on a 10 to 25% sucrose gradient and analyzed as described (2). Gradient centrifugation at 37,000 r.p.m. was performed for 114 hours. Sonic treatment released 20 to 30% of the total radioactivity of the various pellets.

**Detergent Dissociation and Reversal**—Labeled thyroid soluble proteins in PBS were treated by addition of an equal volume of PBS containing 0.003% SDS for 45 minutes at room temperature. An aliquot of this solution was layered on 10 to 25% sucrose in PBS saturated at 4°C with SDS and analyzed by centrifugation as described (2). The remainder of the solution was dialyzed against large volumes of PBS overnight at 4°C (“reversed”) and similarly analyzed on a gradient not containing SDS. A control protein solution was diluted in half with PBS and then worked up as described for the “SDS-reversed.” Thyroid soluble protein was also treated with 0.3% sodium deoxycholate, allowed to stand at 0°C for 1 hour, and dialyzed overnight against PBS prior to sedimentation analysis.

**Alkaline Treatment of Thyroglobulin**—A solution of labeled thyroglobulin in PBS was brought to pH 11 by addition of 1 N sodium hydroxide, stirred for 5 minutes, and neutralized with 1 N hydrochloric acid before sedimentation analysis. Such treatment has been shown to dissociate thyroglobulin to its 12 S half-molecule with incomplete reversibility upon neutralization (11).

**Preparation and Concentration of Gradient Protein Subfractions**—The labeled thyroid soluble proteins prepared as described (1, 2) were centrifuged preparatively on a 5 to 20% sucrose gradient in PBS at 25,000 r.p.m. for 24 hours in the SW-25 rotor of the Spinco model L ultracentrifuge. Following collection of about 0.5 ml per test tube, the optical density at 280 mg was read and a 0.05-ml aliquot was counted in the Packard Tri-Carb liquid scintillation spectrometer (2). Gradient samples with the desired sedimentation constant were pooled and then concentrated to a small volume with the use of Carbowax. Carrier thyroglobulin (15 mg) was added prior to Carbowax treatment. To reduce its volume, the protein solution was placed in Visking dialysis tubing, which was then covered with dry Carbowax until the desired volume (usually 0.5 ml) was reached. The tubing was retied to a smaller volume, and subjected to dialysis against PBS overnight.

**Preparation of Antithyroglobulin Serum**—Thyroglobulin prepared by the procedure of Edelhoch (11) was further purified by centrifugation in a 5 to 20% sucrose gradient in PBS. Such material was determined to be pure 19 S by ultracentrifugation in the Spinco model E analytical ultracentrifuge. Antisheep thyroglobulin antiserum was prepared in rabbits by three intramuscular injections in the left rear leg of 10 mg of ultracentrifugally pure 19 S thyroglobulin in 1 ml of Freund’s adjuvant per injection. The second injection was made 1 week after the first, and the third 6 weeks after the second. Bleedings through the ear vein were started 10 days after the last injection and continued at 1- to 2-week intervals until four collections of blood had been obtained. Collected blood was left at room temperature for 1 hour, rimmed, and allowed to stand in the cold overnight prior to low speed centrifugation to remove the clot. Immunoelectrophoresis in agar and agar diffusion, only one precipitin line is found when antiserum is tested against either total thyroid soluble protein or purified thyroglobulin. Serum was stored at -20°C.

Antiserum to bovine serum albumin was a gift of Dr. Stanley Yalnizian.

**Antibody Precipitation of Sucrose Gradients**—The amounts of antiserum and corresponding protein varied with the titer of the antiserum, but all precipitation reactions were carried out in the region of antibody excess. After a 0.05-ml aliquot of the sucrose gradient fractions was counted, another aliquot (0.4 ml) of sucrose gradient fractions was first nonspecifically precipitated with 0.05 ml of antiserum to bovine serum albumin in the presence of 28 µg of added carrier bovine serum albumin. Precipitates were centrifuged after incubation for 30 minutes at 37°C and standing in the cold overnight. Aliquots (0.4 ml) of the supernatant fractions were specifically precipitated with 0.05 ml of antiserum to thyroglobulin in the presence of 250 µg of added ultracentrifugally pure thyroglobulin. The samples were incubated and chilled as above before removal of the precipitates. Final supernatant fractions were either counted directly in the Packard Tri-Carb liquid scintillation spectrometer (1) or precipitated with trichloroacetic acid and washed in ethanol-ether (3:1) before counting. All precipitates were dissolved in 1.0 ml of Hyamine and counted in the scintillation spectrometer. All counts were corrected for dilution factors and efficiency of counting and referred back to the original gradient.

**Chemicals**—dl-Leucine-4,5-3H (5.45 curies per mmole) was obtained from New England Nuclear Corporation. Carrier-free NaI from Volk Radiochemical Company. Carbowax 20-M (polyethylene glycol) was from Union Carbide Chemicals Company.

**RESULTS**

**Attempt at Subcellular Localization of Iodination of Thyroglobulin**—Subcellular fractionation of 125I-labeled thyroid slices failed to reveal a particulate fraction enriched in 125I labeled thyroglobulin over that in the 105,000 × g supernatant fraction of the slice (Fig. 1). Particle-bound 125I-labeled protein was solubilized by sonic disruption and analyzed by gradient centrifugation. Whatever 19 S radioactivity could be found in the soluble fraction after sonic disruption of the 105,000 × g, 9,000 × g, or 900 × g pellets could be entirely accounted for by contamination from the original high speed supernatant fluid (as determined by analysis of the pellet washes). This was true for thyroid slices incubated for 5, 20, or 90 minutes with 125I. Sonic disruption did, however, appear to release radioactivity which was very slowly sedimented (Fig. 1). This was not due to breakdown of 125I-labeled 19 S protein by the sonic disruption itself. Therefore, no evidence could be found that newly iodinated thyroglobulin was particle-bound during its formation and susceptible to release by sonic disruption. These data, however, do not eliminate the possibility that iodination is rapidly followed by release of the protein from the particle sites. Amino Acid-labeled Thyroglobulin of High Specific Activity Associated with Particles—In contrast with the above, sonic

2 The term “soluble fraction” is used throughout to refer to the 105,000 × g supernatant fluid resulting from homogenization of the thyroid slice in either PBS or Medium A. It remains to be established whether or not the composition of the soluble fraction is otherwise identical with that of the sap of the intact cell.
is loosely bound to the particles and has been easily extracted into the soluble fraction. Such a possibility is raised by finding an enrichment of the particle washes with respect to 12 S label. On the other hand, other experiments indicate that the 12 S radioactivity is probably denatured to a considerable extent by sonic disruption under conditions in which the 19 S radioactivity is not significantly affected. As seen in Fig. 3, sonic disruption of labeled soluble thyroid protein leads to a significant loss of radioactivity associated with 12 S region of the gradient. Such a loss is not accompanied by the appearance of label in smaller fragments but rather, as seen in Fig. 4, in which the 12 S component was produced by detergent treatment (12), is found in a pellet at the bottom of the gradient. It is likely that the 12 S protein has been selectively denatured and made insoluble by the sonic disruption. Similar results have been obtained upon sonic treatment of 125I-labeled 12 S prepared by dissociation of labeled 19 S protein. This aggregation phenomenon increases in rate with increase in temperature and decrease in pH.

The use of detergents to release 12 S protein from particles is limited by the capacity of detergents to dissociate thyroglobulin to its half molecules (12). Accordingly, as expected, detergent treatment of particles releases 3H-leucine-labeled 12 S protein which probably was derived in large part, if not entirely, from 19 S protein.

Fig. 1. Gradient centrifugation analysis of 125I-labeled proteins associated with subcellular fractions of lamb thyroid slices. Five slices were incubated at 37° in 100% O2 for 5 minutes in 2 ml of Eagle’s medium containing 50 μe of 125I as previously described (2). Cellular fractionation, release of protein from particles by sonic disruption, and sucrose gradient analysis of the solubilized labeled protein were performed as described in "Experimental Procedure" and References 1 and 2. A, sedimentation profile of the 105,000 X g soluble protein precipitable by 70% ammonium sulfate; B, sedimentation profile of the protein solubilized by sonic disruption of the 9,000 X g pellet. A similar picture was found on analysis of the 900 X g and 105,000 X g pellets.

Disruption of similar particles prepared from thyroid slices incubated with radioactive amino acids for 20 and 90 minutes caused the release of 10 S protein of higher specific activity than existed in the soluble fraction (Fig. 2). Labeled 19 S protein specifically attached to particles was found only when slices had been incubated for at least 15 to 20 minutes. After this lag period, the specific activity of the sonically released thyroglobulin increased at a rapid rate. Accordingly, the kinetics of amino acid labeling of thyroglobulin associated with particles resembled that found for labeling of thyroglobulin in the soluble fraction (1, 2). A considerable portion of this lag may be concerned with the assembly of the thyroglobulin molecule from subunits. Polypeptide chain synthesis and aggregation may occur in close proximity on particulate structures, presumably microsomes. Following completion of the 10 S protein, it may be released into the cell sap or may be transported, particle-bound, to the apical end of the cell for extrusion into the colloid. The experimental design employed here would not distinguish between these two possibilities.

It should be noted that at a time (20 minutes of incubation) when 12 S radioactivity exceeds that of the 19 S protein in the soluble fraction, very little if any 12 S label is found to be solubilized upon sonic disruption of particles derived from these slices. This might be due to the fact that the 12 S protein is not significantly affected by sonic disruption. The use of detergents to release 12 S protein from particles is limited by the capacity of detergents to dissociate thyroglobulin to its half molecules (12). Accordingly, as expected, detergent treatment of particles releases 3H-leucine-labeled 12 S protein which probably was derived in large part, if not entirely, from 19 S protein.
The association of labeled thyroglobulin of high specific activity with all three particle fractions may be due to binding to different species of particles or may be due to the presence in each fraction of a common species. The 9,000 × g pellet undoubtedly is heavily contaminated with microsomes, and the 900 × g pellet contains whole cells as well as cell debris and nuclei. Until more precise methods of cell fractionation are used and monitored by electron microscopic examination, elucidation of the nature of the particle involved in thyroglobulin maturation must be deferred, although the data strongly implies the endoplasmic reticulum in this process.

Precipitation of SDS-prepared 12 S Fraction with Antibody—If the 12 S fraction labeled with radioactive amino acid during incubation of thyroid slices could be shown to be immunologically related to 19 S thyroglobulin, the possibility that this fraction is a protein entirely unrelated to thyroglobulin but synthesized by the thyroid would be eliminated. Demonstration of a relationship would represent further support for the possibility of a precursor-product relationship between the two. As a test for the ability of antiserum to thyroglobulin to precipitate 12 S protein, 125I-labeled 19 S protein was (a) purified by sucrose gradient centrifugation, (b) subjected to dissociation to 12 S protein with SDS followed by dialysis (12), and (c) rerun on a sucrose density gradient; (d) individual fractions were allowed to react with the specific antibody after a preliminary precipitation of an unrelated antigen-antibody system (bovine serum albumin and its antiserum). It is expected from the work of Edelhoch and Lippoldt (12) that after exposure to 0.0015 M SDS considerable compositional and configuration reversibility can be obtained by removal of the detergent by dialysis. Such an experiment is shown in Fig. 5. It can be seen that the 12 S 125I-labeled fraction formed by SDS treatment is specifically precipitated by antiserum prepared against purified thyroglobulin. While ultracentrifugally pure 19 S material was used to prepare antibody, it is possible that following its injection into the animal partial breakdown to 12 S protein occurred and antibody was formed against this unit as well as against 19 S protein. Even if this should be so, the conclusion as to whether or not the biologically synthesized 12 S protein is related to 19 S protein would remain valid.

Precipitation of Amino Acid-labeled Soluble Thyroid Proteins with Antibody—In Fig. 6 the ability of antithyroglobulin antibody to precipitate soluble thyroid proteins biologically labeled with 125I was investigated. It can be seen that there is specific precipitation of label in the 19 S and 12 S fractions with antibody, but that label in the 8 to 12 S region is not precipitated. The 12 S protein labeled in shorter incubations behaved similarly with antiserum. The failure of 3 to 8 S protein to precipitate need not mean that it is not related to the other two proteins but may be due to loss of antigenic sites because of a change in the three-dimensional structure of these smaller units. While this type of finding is common with fragments of globular proteins, Metzger, Sharp, and Edelhoch (13) were able to find antigenic activity in tryptic digests of beef thyroglobulin. It should be noted that the biologically labeled 12 S fraction appears not to be precipitated by antibody as completely as the 19 S fraction. This may be because the 12 S, as might be expected, is not as good an antigen for the antithyroglobulin antibody as

Fig. 3. Effect of sonic disruption on 12 S protein labeled in vitro. Soluble thyroid proteins from slices incubated with 200 µc of 3H-leucine for 20 minutes as described in "Experimental Procedure" were sonically disrupted for 2 minutes; an aliquot was removed (O--O), and the remainder (O-----O) was sonically disrupted for an additional 3 minutes. An aliquot that had not been sonically treated (O- - -O) was compared with the sonically disrupted aliquot by sedimentation analysis. Since the optical density patterns were identical, only one is shown.

Fig. 4. Effect of sonic disruption on SDS-dissociated thyroglobulin. A preparation of 19 S protein from a 4½-hour incubation of 20 slices of lamb thyroid in 8 ml of Eagle's medium containing 200 µc of 3H-leucine was isolated, concentrated, treated with 0.0015 M SDS, and dialyzed as described in "Experimental Procedure." This procedure yielded a mixture of labeled 19 S and 12 S proteins (A), half of which was analyzed by gradient centrifugation without further treatment and the other half of which (B) was subjected to sonic disruption prior to centrifugation. The radioactivity which was sedimented to the bottom of the gradients ("pellet") is indicated in the figure. As will be discussed later, SDS treatment has resulted in a greater dissociation of label than of optical density from 19 S to 12 S.
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Fig. 5. Antibody precipitation of SDS-dissociated thyroglobulin labeled with $^{125}$I. Slices were incubated with $^{125}$I for 45 minutes as described in "Experimental Procedure." SDS-labeled 19 S protein was (a) purified by sucrose gradient centrifugation, (b) dissociated with SDS and dialyzed for partial reversal, and (c) rerun on a sucrose density gradient; (d) individual gradient fractions to which 0.4 ml of water was added were precipitated with antithyroglobulin (TG) antiserum (TG-anti TG ppt) after preliminary treatment with antiserum to bovine serum albumin (BSA) (BSA-anti BSA ppt) as described in "Experimental Procedure." The radioactivity in each tube not precipitated by antibody (TG-anti TG supernate) was precipitated with trichloroacetic acid and counted. "Original" represents the total radioactivity initially present in each tube. The position on the gradient of the 19 S optical density peak is indicated by the arrow.

Fig. 6. Antibody precipitation of $^{3}$H-leucine-labeled soluble thyroid proteins. Slices were incubated with $^{3}$H-leucine for 90 minutes as described in "Experimental Procedure." Soluble protein precipitated by 50% ammonium sulfate was prepared for sedimentation analysis on a 5 to 20% sucrose density gradient in a Spinco model L SW-25 rotor. Antibody precipitations and analyses were carried out as described in Fig. 5. The position on the gradient of the 19 S optical density peak is indicated by the arrow. For definition of terms, see legend to Fig. 5.

The 19 S fraction, or it may indicate that the 12 S fraction is not homogeneous.

Detergent- and Alkaline pH-induced Dissociation of 19 S Optical Density and Label—Further confirmation of the known heterogeneity of thyroglobulin has been derived from labeling experiments in which sucrose gradient analysis of the labeled soluble proteins revealed the peak of radioactivity ($^{125}$I or labeled amino acid) to sediment slightly slower than that of the bulk of the 19 S protein as measured by its optical density (1, 2) (Figs. 1A, 2B, 7, and 8). It was thought, therefore, that the extent to which 19 S optical density and radioactivity dissociated to 12 S under various physical conditions might be quite different. It might be expected that less mature thyroglobulin molecules, as represented by the labeled 19 S fraction, would have less compact structures than the preformed thyroglobulin and might be more susceptible to agents producing dissociation. As seen in Figs. 7 and 8, such proved to be the case. In Figs. 7 and 8, isolated $^{125}$I- or $^{3}$H-leucine-labeled 19 S protein was treated with SDS ($0.0015 \text{ M}$), part of which was dialyzed and part of which was not dialyzed prior to gradient centrifugation analysis. It can be seen that with both isotopes, label from 19 S dissociates to 12 S more readily with SDS than does the optical density. Furthermore, reversal of dissociation by dialysis still leaves more radioactivity than optical density in the 12 S region, resulting in an increased specific activity of the 12 S fraction. As previously reported (12), the protein components sediment somewhat more slowly when SDS is present in the gradient owing to changes in the frictional properties of the SDS-bound proteins (Figs. 7 and 8). Sodium deoxycholate (0.3%) similarly breaks down -19 S label to 12 S to a greater extent than optical density.

Treatment of labeled 19 S at pH 11 leads to the same result. Recently, Lissitsky et al. (7) have reported similar results with ammonium hydroxide treatment of thyroglobulin iodinated in vivo.
It should be noted that 19 S protein labeled with radioactive amino acid appears to be more labile to breakdown by detergents and alkaline pH than the 125I-labeled protein. This suggests that iodination occurs on thyroglobulin relatively more mature than thyroglobulin newly synthesized from amino acids and is consistent with current concepts of the stage of thyroglobulin formation at which iodination occurs.

**SDS Treatment of Biologically Labeled 12 S Protein**—As seen in Fig. 9, treatment of a partially purified 3H-leucine-labeled 12 S fraction (containing some labeled 19 S protein) with 0.0015 M SDS leads, as expected (12), to no further dissociation of the 12 S fraction.

**SDS Treatment of Biologically Labeled 3 to 8 S Protein**—Similarly, SDS treatment of the 3H-leucine-labeled 3 to 8 S fraction does not lead to the formation of smaller units (Fig. 10).

**DISCUSSION**

The follicular cell of the thyroid gland is richly endowed with a complex network of endoplasmic reticulum, studded by ribonucleoprotein particles, which extends from the base to the apical end of the cell bordering on the colloid (14). Autoradiographic experiments on rat thyroid glands labeled in vivo with 3H-leucine have disclosed a sequence of protein labeling as follows: (a) polypeptide initiation on the ribonucleoprotein particles, (b) release within the lumen of the cisternae of the endoplasmic reticulum, (c) migration via the lumen of the cisternae (perhaps by way of the Golgi zone, where the carbohydrate moiety may be acquired) to the apical end of the cell, and (d) inclusion within small apical vesicles which empty their protein contents into the colloid of the follicle (8). This entire sequence of events takes about 4 hours, although labeling of the apical vesicles can be found as early as 1 hour after injection of label. The time course followed in the thyroid closely resembles that found in the formation and secretion of the digestive proenzymes by the pancreas (15, 16). While it is tempting to correlate the autoradiographic experiments in vivo with the data derived from gradient centrifugation analysis of protein labeling in thyroid slices, this is probably best left until experiments have been done which simultaneously apply both methods of analysis under identical conditions, i.e., in the same species either in vivo or in vitro. Nevertheless, it is worth noting that the exportable protein, thyroglobulin, or its precursors appear to traverse a series of distinct anatomical loci, each of which might play a special role in maturation of the 19 S protein.

The 15- to 20-minute lag which precedes significant amino acid labeling of thyroglobulin in the soluble fraction of the thyroid slices could be due to (a) compartmental complexities within the thyroid cell which provide barriers to the utilization of entering radioactive amino acid by the microsomes, (b) maturation of thyroglobulin (aggregation from subunits) on the microsomes (or within the cisternal lumen) and subsequent secretion into the cell sap, or (c) maturation of thyroglobulin in the cell sap. The first possibility is unlikely, since other soluble proteins (12 S and 3 to 8 S) become labeled without a significant time lag. Defini-
the particulate fraction raises the possibility of its migration to very heavy structures (>400 S), which, when treated with tritiated leucine for polyribosomal aggregates shows that label is attached to the soluble fraction, suggesting that subcellular structure therein represented remains to be determined, but the apical vesicles, which have been shown by autoradiography of rat thyroid to be labeled late with 3H-leucine before labeling of the colloid, might be involved. In sharp contrast, newly iodinated thyroglobulin was not found to be particle-bound at any time, and presumably iodination occurs close to the colloid.

Proteolytic enzymes digest newly iodinated thyroglobulin more rapidly than that previously iodinated, suggesting that the former is more susceptible to degradative attack because of a less well organized tertiary structure, i.e. greater degree of uncoiling (17). Support for this concept comes from experiments in which the sensitivity of thyroglobulin to proteolysis could be increased by treatment with agents (detergents) expected to disorganize the tertiary structure further (18). It is of interest that preliminary experiments indicate that the amino acid-labeled 12 S protein is more susceptible to degradation by proteolytic enzymes than the label associated with the 19 S protein. The data reported in this paper on the ease of dissociation to 12 S subunits of both 125I- and 3H-leucine-labeled thyroglobulin are consistent with the above, and further document the heterogeneity of thyroglobulin. They also point to the possibility, not yet disproved, that the amino acid labeled 12 S fraction found on gradient centrifugation analysis may result from breakdown during isolation of an especially labile 19 S fraction (metastable 19 S, see Reference 1). The greater ease of dissociation of the amino acid-labeled 19 S by detergent than of the iodine-labeled protein would be consistent with such a possibility. If isolated amino acid-labeled 12 S protein can be shown to be converted directly to 19 S thyroglobulin when added to incubating thyroid slices, one might be on more certain ground in assigning it a precursor role in thyroglobulin biosynthesis.

The demonstrated antigenicity of the 12 S protein for thyroglobulin antiserum established its relationship to the 19 S protein. The biologically prepared 12 S fraction labeled with 3H-leucine appears not to be as effective in the antiserum precipitation reaction as that formed by dissociation of 125I-labeled thyroglobulin. While it is conceivable (and probable) that the two labels are measures of different species of thyroglobulin molecules, the 12 S dissociation products of which differ in exposed antigenic sites, it is expected that the biologically made 19 S protein would differ sufficiently in third-dimensional structure from the SDS-formed 12 S protein to be antigenically not exactly the same. Furthermore, it is probable that the 3H-leucine-labeled 12 S protein is contaminated with lighter proteins. The identification of labeled basic subunits of thyroglobulin within the 3 to 8 S fraction will require the preparation of an antibody against isolated subunits made by complete dissociation of 19 S protein by disulfide bond-breaking agents, urea, etc.

**SUMMARY**

1. After a lag of about 20 minutes, thyroglobulin (19 S) of high specific activity could be released by sonic disruption of particles prepared by subcellular fractionation of lamb thyroid slices incubated with 3H-leucine. The formation of thyroglobulin by aggregation of its subunits may be particle-associated. Under these same conditions, labeled 12 S protein was not found to be solubilized. Sonic disruption, however, appears to lead to the denaturation of labeled 12 S protein but not of labeled 19 S protein.

2. Similar experiments with 125I as the label failed to exhibit the association of 125I-labeled thyroglobulin with particles at a

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**FIG. 10. Effect of SDS on 3 to 8 S protein labeled with 3H-leucine.** A preparation of 3 to 8 S protein from a 30-minute incubation with 3H-leucine was isolated, concentrated, and treated with 0.0015 M SDS as described in "Experimental Procedure" prior to sedimentation analysis.

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3 H. G. Sellin and I. H. Goldberg, unpublished data.
higher specific activity than found in the soluble fraction. This is further evidence that iodination and synthesis of the polypeptide are separate processes and may occur at different sites.

3. The 12 S protein biologically labeled with 3H-leucine is immunologically related to thyroglobulin and is not degraded by 0.0015 M sodium dodecyl sulfate.

4. Detergents and alkaline pH dissociate newly formed thyroglobulin to its 12 S half-molecule to a greater extent than preformed thyroglobulin. Recently synthesized thyroglobulin probably has a less compact tertiary structure than the more mature protein. 3H-Leucine-labeled thyroglobulin is more labile to the action of sodium dodecyl sulfate than is 125I-labeled thyroglobulin. This may be indicative of their relative biological maturities.

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