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Biosynthesis of Thyroid Iodoproteins *in Vivo* and in Tissue Slices*

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SUMMARY

The time course of formation of the thyroid iodoprotein 19 S and its subunits (6 S, 7 S, and 12 S) has been studied after intravenous administration of [^{3}H]leucine to normal rats and guinea pigs and after *in vitro* incorporation in thyroid hemilobes.

The uptake of the [^{3}H]leucine by the rat gland *in vivo* paralleled the disappearance of the radioactivity from the plasma (30 min): the amino acid was rapidly bound to a particulate fraction and was more slowly incorporated into proteins having solubility properties similar to that of thyroglobulin. Sucrose gradient analysis of the newly formed thyroglobulin-like labeled proteins showed the presence of a significant proportion of thyroglobulin and of its half-sized, 12 S subunit even at the earliest labeling interval (10 min). These data suggest that the assembly of the polypeptide chains of thyroglobulin occurs before the newly formed molecules are released from the intracellular membranes.

The 6 S and 7 S labeled intermediates appear to be related to newly formed, unstable thyroglobulin molecules, the equilibrium between these units and the fully assembled molecules being shifted toward the latter with increasing labeling times.

A similar pattern was observed after pulse labeling guinea pigs *in vivo*. In this species, however, there is a larger proportion of 12 S subunits derived from dissociation of newly formed and consequently poorly iodinated thyroglobulin molecules. Both in rats and guinea pigs, the sedimentation rate of newly formed thyroglobulin increased progressively from 14 to 19 S with the labeling time.

After labeling thyroid hemilobes *in vitro*, the disappearance of the labeled intermediates 6 S, 7 S, and 12 S was both delayed and incomplete. It appears that the time-dependent disappearance of the labeled subunits 6 S, 7 S, 12 S, and of the unfolded 14 to 17 S species (prethyroglobulin) is related to later chemical modifications which increase the stability of the newly formed thyroglobulin molecules toward dissociation and unfolding. These later modifications include iodination and its oxidative side effects (oxidation of —SH to —S—S—) and addition of the carbohydrate moiety.

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Studies concerning the biosynthesis of thyroglobulin have been carried out mainly with tissue slices (1–5), thyroid lobes or hemilobes (5–14), isolated thyroid cells and cell-free systems (15–19). Such studies have the disadvantage that some processes, like iodination, thyroxine synthesis, etc., which certainly modify the structure of newly formed thyroglobulin, are either impaired or too slow to be observable in the few hours in which tissue slices can be incubated in culture media.

Relatively few studies have been performed on the biosynthesis of thyroglobulin *in vivo* (20–29). Furthermore, most of the studies have been concerned only with the analysis of the soluble fraction of the thyroid glands labeled with an amino acid. In a previous study from this laboratory (22) we have shown that after *in vivo* labeling of rats and guinea pigs the appearance of a radioactive amino acid in the 19 S thyroglobulin region of sucrose gradients was preceded by its earlier appearance in a slower sedimenting component. Two soluble, biosynthetic precursors of thyroglobulin have been recently isolated and characterized (30).

Since there is good evidence that most glycoproteins are synthesized on membrane-bound polyribosomes (31), it appeared of interest to study the relationship between the uptake of amino acid radioactivity by the thyroid gland, its incorporation into membrane-bound proteins, and the appearance of the soluble precursors previously characterized (30).

The results obtained are consistent with the conclusion that the formation of native (19 S) thyroglobulin molecules consists of the following steps: (*a*) Synthesis of the elementary polypeptide chains; (*b*) assembly of the chains on the membranes of the endoplasmic reticulum to form precursors (6 S, 7 S, 12 S) which are in equilibrium with newly formed thyroglobulin of molecular weight 660,000; (*c*) addition of carbohydrates and iodine to the newly formed molecules with increase in the stabilization of the 19 S form of thyroglobulin.

MATERIALS AND METHODS

Materials

L-[4,5-^{3}H]Leucine, Hyamine hydroxide, and Liquifluor were purchased from the New England Nuclear Co. (Boston, Mass.). Specific activities of the amino acid ranged from 5 to 55 Ci per mmole. Each sample, in 5 ml of 0.01 N HCl, was brought to neutral pH by the addition of small amounts of 0.2 M Na_2HPO_4. Nuclear Chicago Solubilizer (NCS) was purchased from the Nuclear Chicago Co. (Delle Piane, Ill.). All other chemicals were of reagent grade.

Pulse Labeling in Vivo and in Vitro

The animals (male rats, Sprague-Dawley strain, weighing from 100 to 250 g each, and guinea pigs, England strain, weighing from 200 to 250 g each, kept on a Purina laboratory diet containing 1.2 μg of iodine per g of diet) were injected intravenously (rats) or intracardially (guinea pigs) with 0.1 to 2.0 mCi per animal. The animals were killed at various lengths of time after the injection (from 10 min to 15 days) by exsanguination under light ether anesthesia and the thyroid glands removed. In some instances blood samples were withdrawn from the animals just before killing (from 5 min to 2 hours after the injection of the amino acid) and the plasma was separated. The *in vitro* incubations were performed as previously described (30). Up to a maximum of 1.0 mCi of [^{3}H]leucine (60 μM) was added to 3.0 ml of Eagle's medium which was free of the corresponding unlabeled amino acid for periods of time ranging from 10 min up to 6 hours.

Fractionation and Measurement of Incorporated Radioactivity

The thyroid glands (20 to 30 mg per gland) from the *in vivo* and *in vitro* experiments were washed with a cold solution of 0.1 M KCl–0.02 M sodium phosphate, pH 7.2 (standard buffer). They were then frozen and thawed twice, cut into fine pieces with scissors, suspended in twice the volume of standard buffer, and centrifuged at 30,000 × *g* for 10 min. After centrifugation the sediment (particulate fraction) was washed twice with standard buffer and the supernatants collected (soluble fraction). The soluble extract and the insoluble sediment (which was resuspended in standard buffer) were dialyzed against large volumes of standard buffer to remove all the radioactivity due to the free amino acid. The soluble extract was further fractionated by ammonium sulfate precipitation (three times at 4°, pH 6.8, between concentrations of 1.4 and 1.8 M salt). The ammonium sulfate precipitates were dissolved in standard buffer and both precipitates and supernatants were dialyzed against the same buffer to remove the ammonium sulfate. The following measurements of the fractions were performed.

^{3}H Radioactivity of Unfractionated Soluble Extract after Dialysis—Aliquots of the extract were placed in a liquid scintillation vial containing 13 ml of Liquifluor (diluted 1:25 with toluene), 2.0 ml of anhydrous ethanol, 0.2 ml of NCS.

^{3}H Radioactivity and Absorbance at 280 nm or at 210 nm of Extract after Fractionation with Ammonium Sulfate—Aliquots were used in the same scintillation mixture specified above for measuring the radioactivity and other aliquots were used for the absorbance readings. Extinction coefficients (of 1% solution in 1-cm path length) of 10.5 and 207 for the absorbance at 280 nm and at 210 nm, respectively, were used (32).

Particle-bound ^{3}H Radioactivity—The sediment of the 30,000 × *g* centrifugation was dialyzed as described and then incubated overnight with 0.2 ml of NCS at 37°. Aliquots of the solubilized sediment were then placed in a scintillation vial containing all the usual reagents except NCS.

Total ^{3}H Radioactivity Present in Thyroid Gland—This was given by the radioactivity of the total unfractionated soluble extract plus the radioactivity found in the insoluble sediments, each being measured before dialysis.

^{3}H Radioactivity of 25-μl Aliquots of Plasma from Rats—The total plasma volume for a rat weighing 100 g was considered to be about 4.0 ml, according to the literature (33). All the radioactivity measurements were expressed in disintegrations per min (except where otherwise stated) after correction for the quenching of the samples by the external standardization procedure.

Analysis of Purified Thyroid Extracts

The precipitates of the ammonium sulfate fractionation after solubilization and dialysis against standard buffer were analyzed by sucrose density gradient centrifugation, as described (30, 32).

RESULTS

Labeling in Vivo

Rat—After a single intravenous injection of [4,5-^{3}H]leucine (pulse labeling) into normal, fasting rats the label rapidly disappears from the plasma (Fig. 1). The rate of disappearance does not vary appreciably if amounts of labeled L-leucine, ranging from 0.01 to 0.20 μmole per 100 g of body weight, are injected. In all cases about 1% of the administered dose is found in the total plasma 5 min after injection of the labeled amino acid. The concentration of the radioactivity in the plasma thereafter decreases more slowly; the radioactivity within the thyroid gland increases rapidly up to 10 min and stays almost constant at later times (Fig. 1). The concentration of ^{3}H in the gland is higher than in the plasma, even at the earliest sampling interval thus indicating a very rapid uptake of the label by the thyroid tissue.

The nature of the radioactivity present in the thyroid gland was studied at various intervals after injection of the label. At labeling intervals of 10 to 30 min most of the thyroidal radioactivity is represented by the free amino acid, since a large percentage of the ^{3}H was dialyzable from the whole homogenate; at times from 1 to 6 hours the radioactivity bound to protein increases progressively from 20 to 80% of the total.

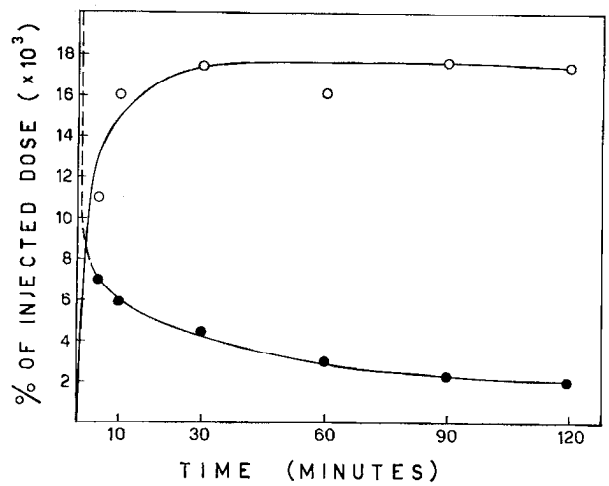

FIG. 1. Time course of plasma and thyroid radioactivity in rats after labeling with [^{3}H]leucine. Six male Sprague-Dawley rats (100 g body weight) were injected intravenously with an exactly measured dose of tritiated leucine, ranging from 1.04 to 1.7 mCi (specific activity 5 Ci per mmole). At various times after the injection one animal was sacrificed and a blood sample collected. Thyroid glands were also excised from each animal and the whole thyroid radioactivity and plasma radioactivity were measured as described in the text. The radioactivity present in the two tissues is expressed as ^{3}H disintegrations per min incorporated per 25 mg of thyroid (○——○) and per 25 μl of plasma (●- - -●), as a percentage of the total injected radioactivity.

A significant proportion of the protein-bound radioactivity is not soluble in aqueous buffer solutions at neutral pH and sediments with the cell particulate fraction at 30,000 × *g*. Part of the soluble fraction precipitates between 1.4 and 1.8 M ammonium sulfate, *i.e.* in the same range of salt concentration in which thyroglobulin is precipitated. Table I shows the distribution of the label in the thyroglobulin-like proteins, in the other water-soluble proteins, and in the particulate fraction, as a function of time after pulse labeling. The radioactivity in the soluble proteins precipitable by ammonium sulfate steadily increases. Fig. 2 shows the time course of incorporation of the tritiated leucine (expressed as counts per min per mg of protein) into the thyroglobulin-like proteins until 8 days after injection of the label. The maximum incorporation is reached 12 hours after the injection. The soluble thyroglobulin-like proteins were further analyzed by sucrose density gradient centrifugation. At early times, from 10 to 30 min, most of the radioactivity is associated with two slow sedimenting proteins (6 S and 7 S); a significant proportion of the total label is associated with faster sedimenting species representing larger sized thyroglobulin molecules (Fig. 3). After 60 min the major radioactive peak sediments at a rate of ~14 S, although radioactivity is still present in significant amounts in the 7 S and 12 S species. At longer time intervals the radioactivity tends to disappear completely from the 6 S, 7 S, and 12 S sedimenting proteins and becomes largely associated with peaks whose sedimentation rates increase from 14 S at 2 hours to about 19 S at 24 hours. At the longest interval studied, therefore, the radioactive peak has the same sedimentation coefficient as stable, previously existing thyroglobulin.

TABLE I

Distribution of the [³H]leucine radioactivity among the protein fractions of the rat thyroid gland after in vivo pulse labeling[a]

Protein fraction	Percentage of total protein-bound radioactivity (in time after pulse labeling)				
	10 min	30 min	60 min	120 min	360 min
	%	%	%	%	%
30,000 × *g* pellet	38	40	47	38	28
Soluble, precipitated between 1.4 and 1.8 M $(NH_4)_2SO_4$	15	18	30	34	46
Soluble, supernatant of 1.8 M $(NH_4)_2SO_4$[b]	47	42	23	28	26

[a] Three animals have been utilized for each indicated time. Each animal received 0.5 mCi of [³H]leucine (specific activity 5 Ci per mmole). Soluble extracts and 30,000 × *g* pellets were prepared as described in the text and exhaustively dialyzed against standard buffer to eliminate the free [³H]leucine.

[b] Since the amount of radioactivity which precipitated at 1.4 M ammonium sulfate was negligible, it was not accounted for in the calculations.

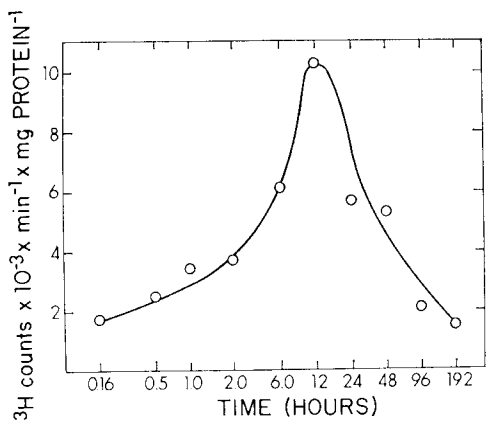

FIG. 2. Time course of labeling of thyroglobulin-like proteins of rats. The animals (one per each experimental point) received a dose of 1.0 mCi of [³H]leucine (specific activity 5 Ci per mmole). The soluble thyroid extracts were precipitated between 1.4 and 1.8 M ammonium sulfate and proteins and radioactivity were estimated as described in the text. The results (representing the average value of six different experiments) have been expressed in terms of counts per min incorporated per mg of protein.

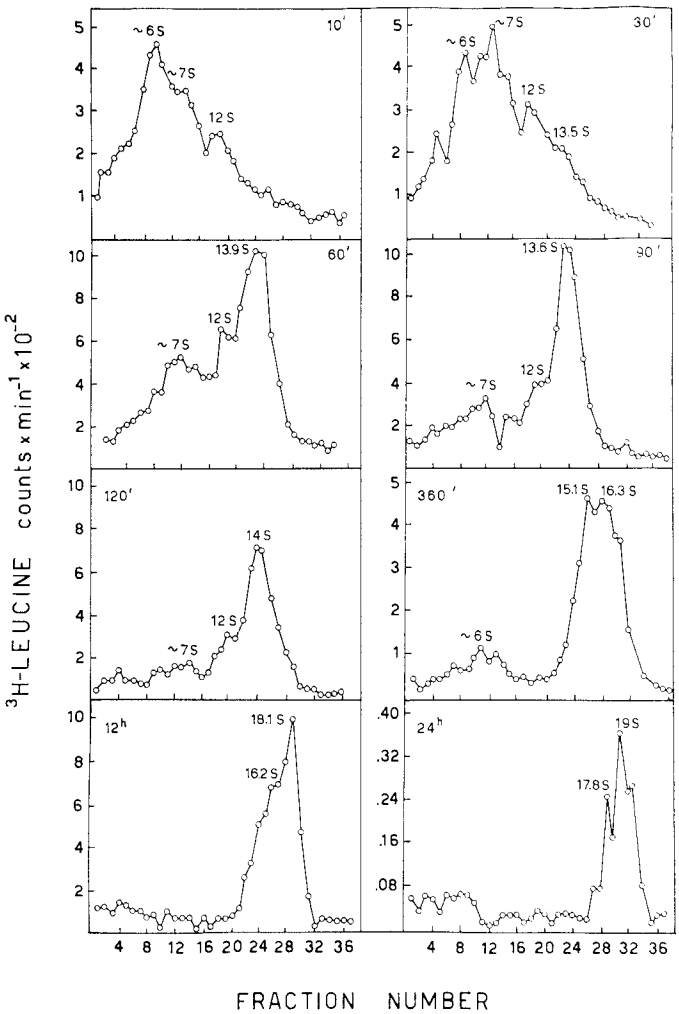

FIG. 3. Sucrose density gradient centrifugation profiles of thyroglobulin-like proteins of rats labeled *in vivo* with tritiated leucine. Each sedimentation profile refers to the proteins obtained from two animals. For the first six intervals of labeling (10 to 360 min) each animal received 0.5 mCi of the amino acid (specific activity 55 Ci per mmole); for the remaining pulse labelings each animal received 150 μCi of tritiated leucine. The protein extracts were purified by ammonium sulfate fractionation and analyzed by sucrose density gradient centrifugation in a 5 to 28% sucrose gradient (w/v) in standard buffer. Centrifugation was in the rotor SW 41 of the Spinco L-2-65 centrifuge. Equivalent time of centrifugation at 4° and at 39,000 rpm was 12 hours. Centrifugation is from *left* to *right*. The sedimentation coefficients were calculated on the basis of the migration of stable 19 S thyroglobulin.

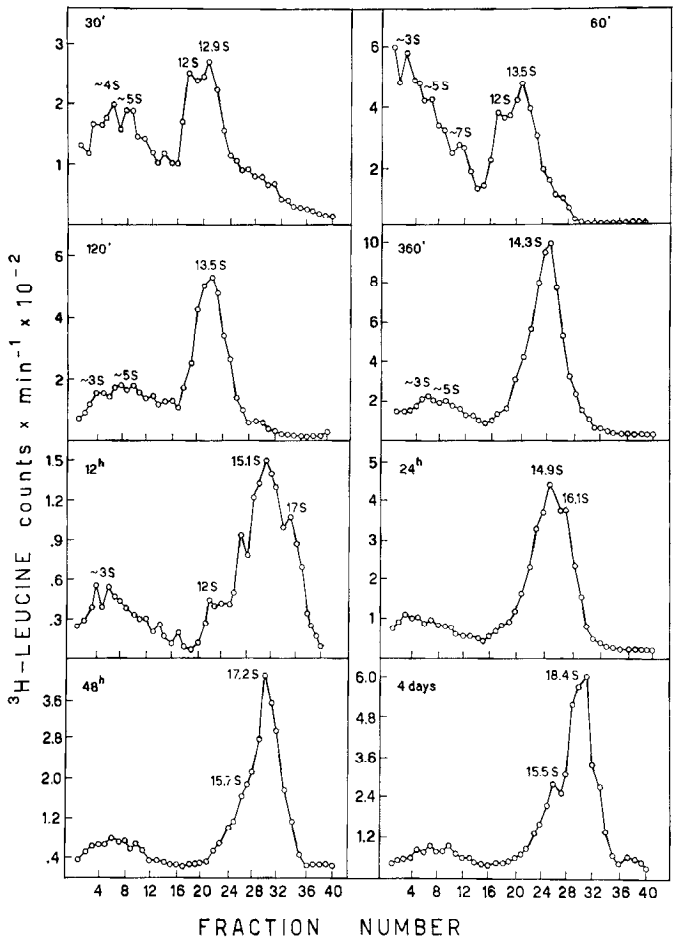

FIG. 4. Sucrose density centrifugation profiles of the thyroglobulin-like proteins of guinea pigs injected with a single dose of tritiated leucine. Each sedimentation profile refers to the proteins obtained from two animals. Each animal received intracardially 0.5 mCi of the amino acid (specific activity 30 Ci per mmole). All conditions of centrifugation were as described in Fig. 3.

Guinea Pig—The radioactivity sedimentation profiles, obtained from guinea pig thyroid extracts after labeling *in vivo* with tritiated leucine, are shown in Fig. 4. Two differences between the rat and the guinea pig patterns are evident: (*a*) high proportions of the radioactivity are associated at early times of labeling (10 to 30 min) with one or more proteins having sedimentation rates of 2 to 4 S, besides the 6 S and 7 S species: (*b*) at the same time intervals after labeling, the faster sedimenting proteins have smaller sedimentation rates than the corresponding species in the rat. The fastest sedimenting protein peak, observable after 24 hours, has a sedimentation rate of 16 S and approaches 19 S only after 4 days. A much longer time was necessary in the guinea pig to observe the radioactive label in the normal form of 19 S thyroglobulin.

Labeling in Vitro

When rat thyroid hemilobes are incubated *in vitro* in the presence of labeled leucine, a 12 S peak is clearly evident at all times of labeling studied (10 to 360 min); it represents a significant proportion of the radioactivity at the early times and tends to disappear only after longer incubation (Fig. 5). This result is in agreement with the original observation made by Seed and Goldberg (1). Furthermore, the sedimentation rate of the fastest sedimenting component does not increase with time, in the manner it does *in vivo*: after 6 hours of incubation there is a much greater increase in the sedimentation rate of thyroglobulin labeled *in vivo* than *in vitro*.

DISCUSSION

Following a single pulse labeling *in vivo* [^{3}H]leucine is rapidly taken up by the thyroid gland. The maximum uptake by the thyroid seems to be reached 10 min after the injection (see Fig. 1).

This information has allowed us to consider the *in vivo* labeling as true pulse-labeling experiments: it is unlikely, in fact, that further changes in the intrathyroidal concentration of [^{3}H]leucine would occur at longer intervals after the injection of the radioactive amino acid. The greatest proportion of the label after 10 min was present mainly in the form of the free amino acid, as it is shown by the fact that more than 80% of the total thyroid radioactivity could be removed by dialysis.

Since the time of protein synthesis has been estimated to be of the order of seconds, it is not surprising that 10 min after labeling, protein-bound radioactivity has been found in the thyroid gland. However, the time course of labeling of the various protein fractions is different.

The first fraction to become labeled is the "particulate" fraction. The labeling of this fraction reaches its maximum value in 60 min (see Table I). It is reasonable to assume that this radioactivity represents labeled proteins which are still attached to the membranes of the endoplasmic reticulum. They are most probably related to thyroglobulin since a precursor-product relationship between these proteins and soluble thyroglobulin appears evident from the results presented in Table I. Furthermore, it has been shown that after their release from the subcellular particles with detergents these labeled proteins exhibit the same sedimentation (2, 25) or electrophoretic (34) properties as the thyroglobulin-like proteins. The soluble fraction contains proteins related to thyroglobulin (those precipitated in the narrow range of ammonium sulfate concentration) as well as proteins not related to thyroglobulin. These latter are soluble in 1.8 M ammonium sulfate and do not react with antisera against thyroglobulin.[1] The specific radioactivity of the proteins of the soluble-ammonium sulfate-precipitable fraction reaches a maximum only 12 hours after the injection of leucine in the rat (see Fig. 2).

The sucrose density gradient profiles of these labeled proteins in the rat show the presence of the following protein species: (*a*) a 3 S protein; we have previously demonstrated that this component is not related to thyroglobulin by immunological and chemical criteria (30); (*b*) two discrete components (6 S and 7 S) which are related to thyroglobulin by both, immunological and chemical criteria (30); (*c*) a 12 S protein; (*d*) a spectrum of molecules whose sedimentation rates increase with the time of labeling from about 13 to 19 S.

If one excludes the low molecular weight 3 S protein, all the other labeled sedimenting species can be interpreted as being precursors of 19 S thyroglobulin since they disappear at long pulse-labeling times. However, all the above sedimenting species could also represent products derived from newly formed thyroglobulin. It has been recently shown that the labeled 12 S

[1] Unpublished results.

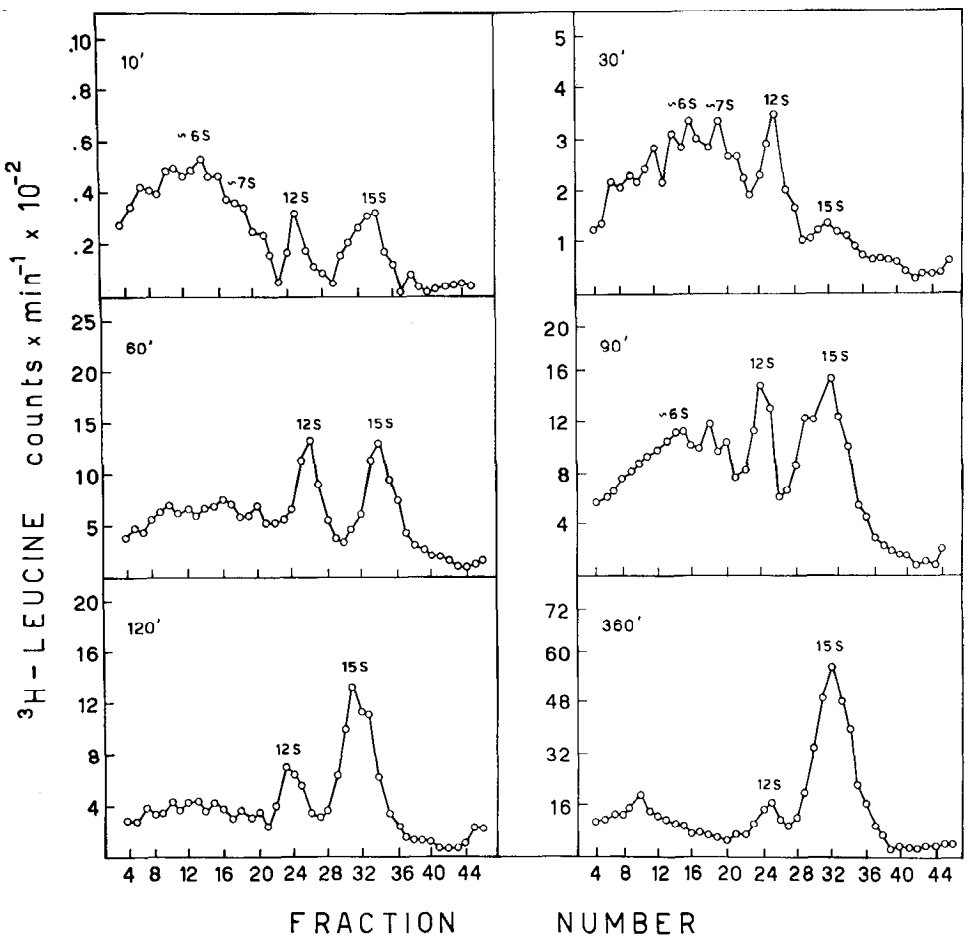

FIG. 5. Sucrose density gradient centrifugation profiles of the thyroglobulin-like proteins obtained from rat thyroid hemilobes incubated *in vitro* in the presence of [^{3}H]leucine. Thyroid glands from 12 rats were excised from the animals, cut in hemilobes, and preincubated for 30 min in 3.0 ml of Eagle's medium free of L-leucine under 95% O_2 and 5% CO_2. Incubation was continued in the same medium after the addition of 57 μCi per ml of [^{3}H]leucine (0.5 μM). At each indicated time eight hemilobes were withdrawn from the incubation medium and utilized for the preparation of the extracts. Conditions of centrifugation were as in Fig. 3.

component is indeed a dissociation product of newly synthesized 19 S which is produced by the low temperatures used for sucrose gradients (5, 35). There is evidence that suggests that the 6 S and 7 S may also be dissociation products of newly synthesized thyroglobulin since they associate to labeled 19 S molecules in the presence of dissociated nonlabeled thyroglobulin (12).

As far as the molecules having sedimentation rates between 13 S and 18 S are concerned, it is interesting to note that molecules having similar sedimentation properties have been known for some time and referred to as prethyroglobulin (3), noniodinated (8), or immature (29) thyroglobulin. Although a process of maturation of thyroglobulin has been previously postulated (8), our results show for the first time that the appearance of 19 S thyroglobulin *in vivo* in two animal species is preceded by the appearance of a spectrum of molecules whose sedimentation rates are slowly shifting with time from about 13 S to about 18 S.

Schneider *et al.* (36) have recently presented evidence that a 15 S species isolated from guinea pig and rat tissue slices when incubated with [^{14}C]valine, results from the unfolding of newly formed thyroglobulin at 2°. As the temperature was increased from 2 to 22° the sedimentation rate increased regularly from 15 to 19 S. Since all sucrose gradient analyses performed in the present work were done at 4°, it is possible that also the molecules observed by us, ranging from 13 to 18 S have a similar origin. If this is so, the increased sedimentation rates observed *in vivo* as a function of time of labeling, would reflect an increased degree of stability of thyroglobulin with the time after labeling. It has been proposed that iodine is one of the factors involved in such stabilization process (3, 8, 29, 37). Schneider *et al.* have shown that the temperature-dependent dissociation of 19 into 12 S (5, 35) or unfolding into 15 S (36) is greater with poorly iodinated thyroglobulin.

Evidence from the present work also suggests that thyroglobulin is stabilized by iodine.

In the guinea pig, in which the rate of iodination is slower (38), the transformation into stable thyroglobulin is also slower. In the *in vitro* experiments, in which the iodination processes are impaired, units with lower sedimentation rates are present in greater amounts than *in vivo* and are still present after relatively long pulse-labeling intervals. The mechanism by which iodine may favor the stabilization process and therefore shift the equilibrium toward the higher polymerized forms may be through the oxidation of some still unoxidized —SH groups of the newly formed thyroglobulin (39).

Other processes, however, like the addition of the carbohydrate moiety, which has been shown to occur in a stepwise manner (34, 40, 41) may also contribute to the increased stability seen with newly synthesized thyroglobulin as a function of time. We have indeed shown previously (30) that the 6 S, 7 S, and 12 S proteins have not yet the complete carbohydrate moiety present in 19 S thyroglobulin.

In conclusion, it seems that after their release from the endoplasmic membranes, the thyroglobulin-like proteins have most of the properties of mature thyroglobulin (immunochemical reactivity and solubility properties in ammonium sulfate solu-

tions), but however, lack some elements of structure. At this stage they migrate into an intracellular compartment where they are extractable by aqueous solvents. Only after 12 hours of labeling in the rat, molecules having a sedimentation rate of 19 S are not unfolded by the low temperatures of the sucrose gradient experiments.

It is tempting to conclude that this time is necessary for the transport of the proteins from their site of synthesis through the Golgi apparatus to the apical cell border where secretion into the colloid takes place. During such transport process late chemical modifications occur, such as the completion of the carbohydrate chains and addition of iodine, which confer the final conformation to the protein.

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