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Identification and Characterization of Two Labeled Intermediates in the Biosynthesis of Rat Thyroglobulin*

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GIANCARLO VECCHIO, M. STELLA CARLOMAGNO, AND EDUARDO CONSIGLIO

From the Centro di Endocrinologia e Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Istituto di Patologia Generale della Universitá di Napoli, Naples, Italy

SUMMARY

Two protein components related to 19 S thyroglobulin and labeled at early times during its biosynthesis have been isolated and characterized. A 3-8 S fraction was prepared from the soluble extract of rat thyroid glands incubated *in vitro* with radioactive amino acids ([^{3}H]leucine and [^{14}C]isoleucine) or carbohydrates ([^{3}H]- or [^{14}C]mannose and [^{3}H]- or [^{14}C]galactose) or both. From this fraction three components were purified. Two of them, homogeneous by ultracentrifugal and electrophoretic criteria, had sedimentation rates of 6 S and 7 S and molecular weights close to 112,000 and 186,000, respectively. The molar ratios of labeled leucine to isoleucine incorporated into the 6 S and 7 S fractions were the same as those found in 19 S thyroglobulin and in its 12 S subunit. Both the 6 S and 7 S protein units were quantitatively precipitated by anti-rat thyroglobulin antibodies. Double labeling with [^{3}H]leucine and ^{14}C-carbohydrates demonstrated that mannose is present in the fully assembled 19 S and 12 S proteins as well as on the slower sedimenting 6 S and 7 S units, whereas galactose is incorporated only after the formation of the fully assembled stable thyroglobulin molecule. It is concluded that the 6 S and 7 S labeled proteins, which contain only part of the carbohydrate moiety of thyroglobulin, participate in the structure of newly formed unstable 19 S molecules and may represent intermediates during the assembly process of the elementary polypeptide chains of thyroglobulin.

Several studies have shown the presence of a labeled slow sedimenting component (usually referred to as 3-8 S) in the crude soluble extracts which are prepared from thyroid slices after incubation *in vitro* with ^{3}H- or ^{14}C-amino acids (1–7). It has been postulated that the *in vitro* labeled 3-8 component includes low molecular weight precursors of thyroglobulin (19 S) (3). However, a precursor-product relationship between 3-8 S and 19 S has been observed only after partial purification of the *in vivo* labeled 3-8 S component (8). The isolation and characterization of the low molecular weight precursors of thyroglobulin has not yet been achieved, although attempts to fractionate the 3-8 S component have been reported from this[1] (6) and another laboratory (9).

In the present work the 3-8 S component has been resolved into three well defined peaks by sucrose gradient centrifugation, two of which, a 6 S and a 7 S species, have been isolated and shown to be related to 19 S thyroblobulin. These protein subunits, containing only part of the carbohydrate moiety of 19 S, have a rather compact structure and a molecular size intermediate between the elementary chains of thyroglobulin and the fully assembled native molecule.

MATERIALS AND METHODS

Preparation of Labeled Thryoid Extracts—Male Sprague-Dawley rats (200 g each), kept on a Purina laboratory diet (1.2 μg of iodine per g of diet), were exsanguinated under light ether anesthesia and the thyroid glands (20 to 30 mg each) were excised. Hemilobes from 10 to 20 animals were incubated in 2 to 3 ml of a modified Eagle's medium containing L-[4,5-^{3}H]-leucine (specific activity 40 to 50 mCi per μmole, final concentration 20 to 100 μM) or D-[1-^{3}H]mannose or D-[1-^{3}H]galactose (specific activities, 2.4 mCi per μmole, final concentrations 83 μM). Unlabeled leucine was omitted in the experiments of labeling with the corresponding tritiated amino acid, and was added at a final concentration of 20 to 100 μM in the other two cases. Glucose was omitted when labeling with the carbohydrates, and unlabeled galactose (5.6 mM) and mannose (5.6 mM) were added when labeling with tritiated mannose and galactose, respectively (5). In experiments containing two isotopes [4,5-^{3}H]leucine (72 μM) + [^{14}C]mannose (specific activity 25 μCi per μmole, final concentration 5.8 mM), or [4,5-^{3}H]-leucine (9 μM) + [U-^{14}C]isoleucine (specific activity 312 μCi per μmole, final concentration 640 μM) were used. In this latter instance the concentration of leucine and its final specific activity in the medium were made equal to those of isoleucine by the addition of unlabeled leucine. In order to increase the incorporation of the labels (and, in the case of the leucine-isoleucine

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[1] G. M. Claar, M. S. Carlomagno, and G. Vecchio, communication presented at the Second meeting of the European Thyroid Association, Marseille, September 1968.

experiment, in order to decrease the intracellular pools of these two amino acids) the incubations with the radioactive compounds were preceded by a 30 to 40 min preliminary incubation in an Eagle's medium free of the two unlabeled amino acid (leucine-isoleucine experiment) or of unlabeled leucine and glucose (all other cases). Preliminary incubations and incubations were carried out at 37° under CO_2-O_2 (5:95). Incubations were for 30 min (except in the case of the leucine-isoleucine experiment which lasted for 60 min). After incubation the glands were rapidly washed in a chilled solution of KCl 0.1 M + sodium phosphate buffer, 0.02 M, pH 7.2 ("standard" buffer) and frozen. The procedure for preparation and purification of the soluble extracts has been described (6, 8).

Sucrose Density Gradient Centrifugations—These were carried out as previously described (10); the rotors SW 25.2 (sucrose gradient 5 to 40% in standard buffer) and SW 41 (sucrose gradient 5 to 28%) of the Spinco L-65 ultracentrifuge were used in the preparation of the labeled thyroglobulin subunits. Sucrose gradient fractions were collected automatically by means of a sucrose gradient fractionator (ISCO) and the absorbance at 280 nm measured through a flow cell-ultraviolet analyzer.

Filtration (Sephadex G-200)—A column of Sephadex G-200 (92 × 1.46 cm, 160-ml bed volume) was prepared according to standard procedures. Reference or labeled proteins were applied in a total volume of 1.0 ml. Elution was carried out with standard buffer at a flow rate of 3.5 ml per hour and 1.0-ml fractions were collected. Elution profiles of the reference proteins were assessed by measuring the absorbance at 280 nm; those of the radioactive proteins by counting aliquots of each fraction.

Polyacrylamide Gel Electrophoresis—This was performed either in a 0.4 M glycine + 0.05 M Tris buffer, pH 8.6 (11), or in a buffer containing sodium dodecyl sulfate (12). In the first instance polyacrylamide concentrations of 3.5% or 5% were used. Five percent polyacrylamide was used with the SDS buffer. The labeled proteins (5,000 to 10,000 cpm) were applied in 50 to 100 μl of standard buffer on the top of the gel columns. Radioactivity was determined (see below) after cutting the gels in 1-mm sections according to the technique used by Iandolo (13).

Immunoprecipitation Experiments—Highly purified rat 19 S thyroglobulin prepared as described (10) and dissolved in standard buffer was injected subcutaneously into male rabbits after emulsification with an equal volume of complete Freund's adjuvant. After three injections of 5 to 10 mg once a week for 3 consecutive weeks the animals were bled, and the sera adsorbed with normal rat serum, and stored in small aliquots at −20°. Of a typical antiserum preparation 100 μl precipitated 400 μg of purified 19 S rat thyroglobulin at the equivalence point. The immunoprecipitation reactions were carried out by incubating 50 to 100 μl of the purified labeled fractions (corresponding to a minimum of 1000 cpm) in standard buffer together with 50 μl of the undiluted antiserum at 37° for 30 min and at 4° for 24 to 48 hours. Due to the small quantities of antigen present these conditions corresponded to antibody excess and were likely to yield, therefore, quantitative precipitation of the labeled antigens. A small but visible precipitate was present in the cases of positive reactions. In every instance the precipitates were washed two times with cold standard buffer and the radioactivity of both supernatants and precipitates was measured. The specificity of the precipitation was tested by adding 2 to 4 mg of unlabeled 19 S rat thyroglobulin to the reaction mixture. When such an excess of unlabeled 19 S was added to the 6 S and 7 S proteins labeled with leucine, isoleucine, or mannose, almost complete inhibition of precipitation of the radioactivity was observed. This indicates that the labeled antigens were precipitated by the same antibodies which are able to precipitate 19 S thyroglobulin.

Radioactivity Measurements—^{14}C and ^{3}H radioactivity was measured in a liquid scintillation counter (Mark 1, Nuclear Chicago Corporation) by standard procedures. Counting efficiencies were 35% and 88% for ^{3}H and ^{14}C, respectively; in the double labeling experiments the ^{3}H efficiency was 32% in its own channel and <0.01% in the ^{14}C channel; ^{14}C efficiency was of 41% in its own channel and 12% in the ^{3}H channel. No quenching corrections were used for sample with exactly identical composition. Quenching corrections were applied when measuring supernatants and precipitates of the immunoprecipitation reaction, by the external standardization procedure. Radioactivity of each millimeter of the gel electrophoresis columns was measured as described by LeBouton (14).

Determination of Apparent Molecular Weights of 6 S and 7 S Proteins by Gel Filtration and Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate—The elution volumes of the proteins chromatographed on the Sephadex G-200 column were accurately determined by weighing each collected fraction. From the elution volumes obtained, k_d values were calculated according to Siegel and Monty (15). A linear relationship existed between the values of $k_d^{1/3}$ and the Stokes radii of a series of reference proteins. From the values of Stokes radii for the 6 S and 7 S proteins, apparent molecular weights were calculated according to the following relation (15, 16): $M = (6\pi\eta Nas)/(1 - \bar{v}\rho)$ where M = molecular weight, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume, η = viscosity of medium, ρ = density of medium. The value used for $\bar{v}$ in the case of both proteins was not that known for bovine 19 S thyroglobulin (0.7135), but a value of 0.726, calculated on the basis of the known amino acid composition of rat thyroglobulin.[2] This value was used because of the low carbohydrate content of the 6 S and 7 S proteins compared with that of 19 S thyroglobulin (see "Results"). The molecular weights of the 6 S and 7 S proteins were also determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate after establishing a calibration curve with proteins of known molecular weight. Electrophoretic mobility was calculated as described by Weber and Osborn (12). All reference proteins were treated with both sodium dodecyl sulfate (1%) and dithiothreitol (1%) in 0.01 M sodium phosphate, pH 7.0, at 37° for 4 hours and the labeled 6 S and 7 S proteins were treated with the same buffer containing 1% sodium dodecyl sulfate but not dithiothreitol.

RESULTS

Fractionation of 3-8 S Component

When rat thyroid hemilobes are incubated for 30 min at 37° in Eagle's medium containing radioactive L-leucine, a variable proportion (usually more than 50%) of the protein-bound radioactivity of the crude soluble extracts is found in the top half of the sucrose gradient centrifuge tube (3-8 S), the remaining label

[2] The value of $\bar{v} = 0.726$ was calculated according to the procedure of Cohn and Edsall (17) from the amino acid composition of rat thyroglobulin reported by Rolland *et al.* (18).

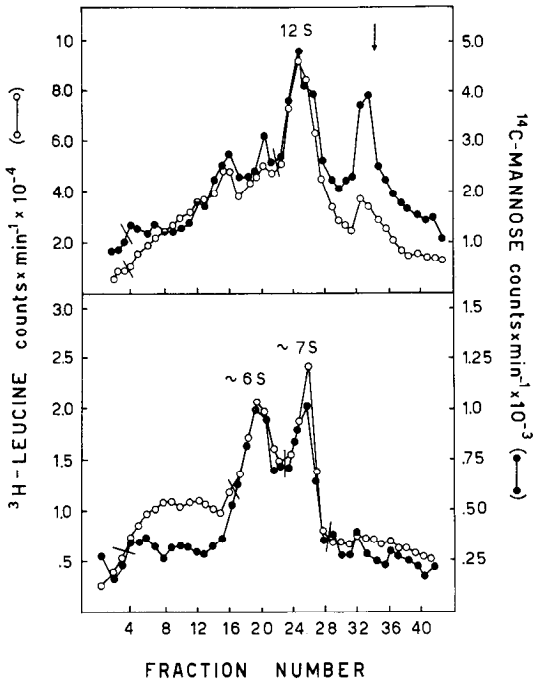

FIG. 1. *Upper panel*, sucrose density gradient centrifugation pattern of a partially purified labeled rat thyroid extract. Rat thyroid glands from 20 animals were previously incubated for 30 min in 3.0 ml of Eagle's medium free of unlabeled leucine and glucose and then incubated for 30 min with 21 nmoles of [4,5-^{3}H]leucine (1.0 mCi) and 18 μmoles of [1-^{14}C]mannose (450 μCi). At the end of incubation the soluble extracts were prepared as previously described (6, 8), layered onto a sucrose gradient tube (5 to 40%, w/v) and run in an SW 25.2 rotor of a Spinco-Beckman centrifuge at 4°. The *arrow* points at the peak migration of 19 S visualized by absorbance at 280 nm. Equivalent time of centrifugation at 23,000 rpm was 34 hours and 36 min. *Lower panel*, sucrose density gradient centrifugation pattern of the Fractions 4 to 21 of the gradient shown in the *upper panel* (see *cuts* on the figure). The fractions were combined, concentrated under reduced pressure, dialyzed against standard buffer, layered onto a sucrose gradient tube (5 to 28%, w/v) and run in an SW 41 rotor at 4°. Equivalent time of centrifugation at 39,000 rpm was 20 hours and 36 min. Sedimentation is from *left* to *right* in both experiments.

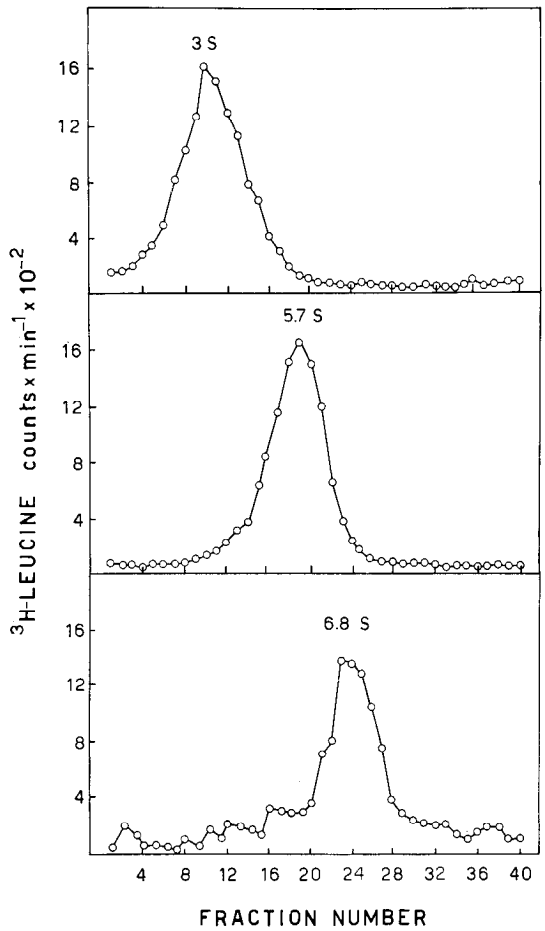

FIG. 2. Sucrose density gradient centrifugation patterns of Fractions 4 to 16 (*upper panel*), 17 to 22 (*middle panel*), and 23 to 28 (*lower panel*) obtained from a centrifugation experiment similar to that shown in the *lower panel* of Fig. 1 (see *cuts* in Fig. 1). The incubation conditions were the same as specified in Fig. 1, except that only [4,5-^{3}H]leucine (22.5 nmoles, 1.1 mCi) was added. After concentration and dialysis the fractions were layered on three different sucrose gradient tubes (5 to 28%, w/v) and run at 4° in the SW 41 rotor. The equivalent time of centrifugation at 39,000 rpm was 19 hours and 12 min. Sedimentation is from *left* to *right*.

being represented by the faster sedimenting 12 S and 17 S components. After three or more precipitations between 1.4 and 1.8 ammonium sulfate, the proportion of the label associated with the 3-8 S component remains constant. In order to compare the incorporation of mannose and leucine into the 3-8 S component, a double labeling experiment (30 min) was performed. The proportion of the [^{14}C]mannose associated with the faster sedimenting fractions (particularly 19 S) is significantly higher than that of [^{3}H]leucine. (Fig. 1, *upper panel.*)

The doubly labeled slow sedimenting component (3-8 S) was separated from the heavier units by a preparative zonal centrifugation in the SW 25.2 rotor (Fig. 1, *upper panel*). After concentration by dialysis under reduced pressure, the 3-8 S was fractionated in the SW 41 rotor. The increased gravitational force and the higher length to diameter ratio of the SW 41 gives better resolution than the SW 25.2. Three ultracentrifugal peaks were thus resolved (Fig. 1, *lower panel*), and the corresponding labeled protein fractions were tested for homogeneity by sedimentation and electrophoretic analysis. The sucrose density sedimentation patterns of the isolated labeled components are presented in Fig. 2. The sedimentation rates calculated, relative to bovine serum albumin, were of 3 S, 5.7 S, and 6.8 S. The 5.7 and 6.8 S peaks were symmetrical, even though the latter still contained a small amount of the 5.7 S material. The 3 S peak was asymmetrical by ultracentrifugation (Fig. 2) and showed heterogeneity also by polyacrylamide gel electrophoresis (not shown). Of the radioactivity of the faster sedimenting components (the 6 S and 7 S), 80 to 90% migrated as single components by polyacrylamide gel electrophoresis (Fig. 3).

Characterization of Labeled Units Isolated from 3-8 S Component

In order to ascertain whether the radioactivity units isolated from the 3-8 S component were related to 19 S thyroglobulin, chemical and immunological characterization of such units were performed.

Chemical Characterization—Double labeling experiments were carried out in order to compare the incorporation of two amino acids, leucine and isoleucine, into each of the sedimenting units isolated from the thyroid hemilobes labeled *in vitro*. On the basis of the total radioactivity incorporated and the specific activities of the added [^{3}H]leucine and [^{14}C]isoleucine, it is possible

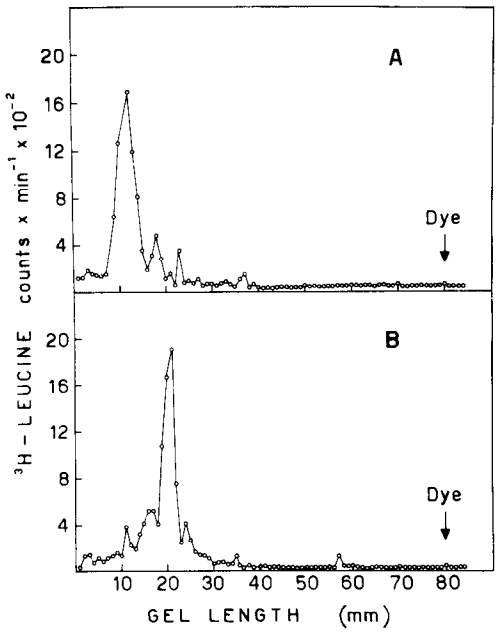

FIG. 3. Electrophoretic migration in polyacrylamide gel of the ^{3}H-7 S (*A*) and ^{3}H-6 S (*B*) proteins purified as described in Figs. 1 and 2. The labeled proteins in 50 to 100 μl of standard buffer were layered on the top of two separate 8-cm long polyacrylamide (5%) gel columns and run for 3 hours (110 volts, 8 ma per gel column) in a buffer containing 0.4 M glycine and 0.05 M Tris, pH 8.6. At the end of the run each gel was cut by means of a gel slicer and the slices (1 mm thick) were solubilized as described by LeBouton (14) and counted in scintillation vials.

to evaluate the total number of residues incorporated into the newly formed protein units provided that the intracellular pool of the two free amino acids is so low as not to appreciably decrease their specific activities. These pools could be largely depleted by previously incubating the thyroid hemilobes for a sufficient length of time in Eagle's medium free of the corresponding amino acids ("starvation"). Preliminary experiments demonstrated that a preliminary incubation of 45 min in Eagle's medium free of both leucine and isoleucine is sufficient to decrease the incorporation of tracer amounts of either amino acid by 95%.

The results of double labeling experiments with [^{3}H]leucine and [^{14}C]isoleucine are reported in Table I; similar results were obtained in two other experiments. The ratio between the number of residues of leucine and isoleucine incorporated in each newly synthesized protein fraction (see Table I) is very similar in the purified 6 S, 7 S, 12 S, and 19 S proteins, while it is definitely lower in the 3 S fraction.

Similar double labeling experiments were carried out with thryoid hemilobes incubated *in vitro* with [^{3}H]leucine and [^{14}C]mannose or [^{14}C]galactose. The results obtained with labeled galactose are in agreement with those recently reported by Herscovics (5); this sugar is not incorporated to any appreciable extent into the slower sedimenting 3 S, 6 S, and 7 S units, but only in the faster sedimenting 12 S and 19 S components. As far as the labeling with mannose is concerned, Herscovics (5), observed that the relative degree of labeling with leucine and mannose of the unfractionated 3-8 S component was similar to that found in the 12 S and 19 S proteins. On the contrary, our results clearly demonstrated that the 3 S is only slightly labeled with [^{14}C]mannose and the ratio of [^{14}C]mannose to [^{3}H]leucine

TABLE I

Incorporation of [^{3}H]leucine and [^{14}C]isoleucine in components isolated from doubly labeled rat thyroid hemilobes

Thyroid hemilobes were previously incubated for 45 min in Eagle's medium free of leucine and isoleucine and then incubated for 60 min at 37° in Eagle's medium containing [4,5-^{3}H]leucine and [U-^{14}C]isoleucine (640 μM each). The final specific activity was adjusted to 312 μCi per μmole for both amino acids.

	Ultracentrifugal fractions				
	3 S	6 S	7 S	12 S	19 S
[^{3}H]Leucine (dpm)...	1460	1290	1120	3010	1720
[^{14}C]Isoleucine (dpm).	750	525	475	1250	670
Leucine (pmoles).....	2.13	1.88	1.63	4.38	2.51
Isoleucine (pmoles)..	1.09	0.76	0.69	1.82	0.98
Ratio of [^{3}H]leucine to [^{14}C]isoleucine...	1.95	2.47	2.36	2.41	2.56

TABLE II

Immunoprecipitation of labeled protein fractions isolated from rat thyroid hemilobes incubated with radioactive L-leucine, D-mannose, and D-galactose

Anti-rat 19 S thyroglobulin serum (50 μl) was added to each labeled fraction (10,000 dpm in 50 to 100 μl of standard buffer). After 30 min at 37° and 48 hours at 4° the mixtures were centrifuged and each precipitate washed two times with standard buffer.

Labeled protein	Radioactivity precipitated (percentage of total)		
	[^{3}H]Leucine	[^{3}H]Mannose	[^{3}H]Galactose
	%	%	%
3 S.....	25	25	27
6 S.....	71	72	37
7 S.....	78	78	18
12 S.....	82	84	82
19 S.....	84	85	86

in the 6 S, 7 S, and 12 S units is about one-half that found in 19 S (see Fig. 1). These results were confirmed by measuring the [^{14}C]mannose to [^{3}H]leucine ratio in the isolated fractions. If the [^{14}C]mannose to [^{3}H]leucine ratio for 19 S thyroglobulin is arbitrarily set to equal to 1.00, the values found for the isolated 6 S, 7 S, and 12 S units are of 0.47, 0.40, and 0.51, respectively.

Immunological Properties—The immunological reactivity of the slower (6 S and 7 S) and faster (12 S and 19 S) sedimenting fractions, labeled either with L-leucine, D-mannose, or D-galactose, was tested by immunoprecipitation with an antiserum against highly purified rat 19 S thyroglobulin. The results (summarized in Table II) demonstrated that the [^{3}H]leucine-labeled 6 S and 7 S units were precipitated by antithyroglobulin antibodies almost to the same extent as the newly formed 19 S and its 12 S subunit. Very similar results were obtained when the 6 S and 7 S intermediates were labeled either with [^{14}C]isoleucine (not shown) or with [^{3}H]mannose, indicating that the incorporation of leucine, isoleucine and mannose is into molecules with antigenic determinants in common with those of native thyroglobulin. On the contrary, the small amount of radioactive galactose

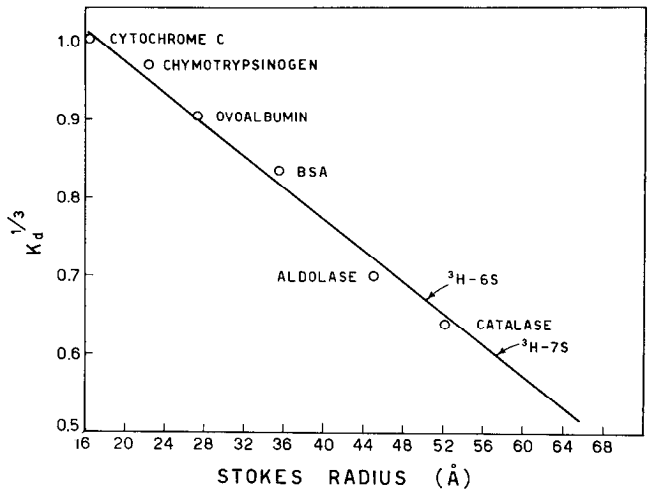

FIG. 4. Relationship between $K_d^{\frac{1}{3}}$ and Stokes radius for a series of reference proteins. Gel filtration was through a column of Sephadex G-200. Column height and inner diameter were of 92 and 1.46 cm, respectively. The values of Stokes radii for the 6 S- and 7 S-labeled proteins have been interpolated. *BSA*, bovine serum albumin.

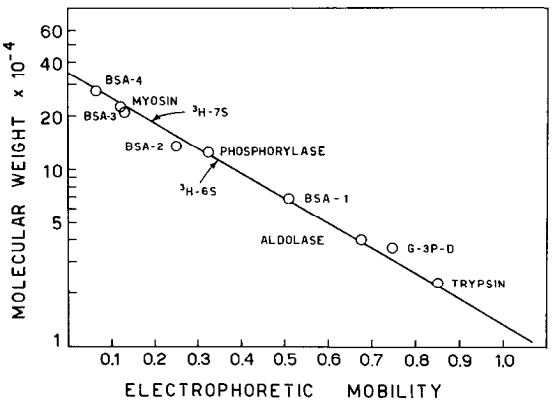

FIG. 5. Relationship between molecular weights and electrophoretic mobility in polyacrylamide gel in sodium dodecyl sulfate for a series of reference proteins (*BSA-1*, *-2*, *-3*, *-4* refer to the monomer, dimer, trimer, and tetramer of bovine serum albumin; *G-3P-D* stands for glyceraldehyde 3-phosphate dehydrogenase). Experimental details were as described by Weber and Osborn (12). The ^{3}H-6 S and ^{3}H-7 S proteins were treated with 1% sodium dodecyl sulfate in 0.01 M sodium phosphate, pH 7.0 for 4 hours before electrophoresis.

TABLE III

Molecular properties of isolated rat 6 S and 7 S labeled proteins

The sedimentation coefficients for the labeled 6 S and 7 S proteins were obtained by sucrose density gradient centrifugation, Stokes radii by gel filtration through Sephadex G-200 and molecular weights by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The values for the 12 S protein are those reported by Aloj *et al.* (20), those for 19 S by Edelhoch (19).

Thyroid protein	Sedimentation coefficient × 10^{13}	Stokes radius	Molecular weight
		A	
Rat 6 S	5.7	50.4	112,000
Rat 7 S	7.1	57.4	186,000
Lamprey 12 S	11.7	67.0	331,000
Bovine 19 S	19.4	86.6	669,000

associated with the slower sedimenting fractions, was not appreciably precipitated by antithyroglobulin antibodies. In every instance, the 3 S component, labeled either with amino acids (leucine and isoleucine) or with carbohydrates (mannose and galactose), had no significant cross-reactivity with native thyroglobulin.

Physicochemical Properties—The 6 S and 7 S labeled units, obtained in high degree of purity, were characterized further.

The sedimentation coefficients of the 6 S and 7 S proteins were evaluated by sucrose density gradient, the Stokes radii by gel filtration and the molecular weights by polyacrylamide gel electrophoresis in SDS. In every instance a series of proteins having known physicochemical parameters were used as references (Figs. 4 and 5). The results obtained, together with those reported for bovine 19 S (19) and lamprey 12 S (20), are summarized in Table III.

DISCUSSION

The results now obtained demonstrate that the labeled 3-8 S component, observed during the biosynthesis of 19 S thyroglobulin in tissue slices (1–7), and the 5 S component detected previously (8) after *in vivo* labeling of rats and guinea pigs, consist of a heterogeneous mixture of proteins. The procedure followed in the present work has allowed a resolution of this mixture into at least three components with sedimentation coefficients of approximately 3 S, 6 S, and 7 S. The labeled 6 S and 7 S have been purified and are largely homogeneous insofar as their sedimentation properties and electrophoretic mobilities are concerned. They are related to thyroglobulin by both chemical and immunological criteria.

Since the 6 S and 7 S proteins were available only in tracer amounts, chemical characterization by methods such as amino acid analysis or proteolytic fragment analysis was not possible. For this reason the following approach was used. It was decided to study the ratio of the incorporation between leucine and isoleucine, two amino acids which are not interconvertible and are present in rat thyroglobulin in relatively high proportions (494 residues of leucine and 185 of isoleucine per molecule of 19 S thyroglobulin (18)). The ratio of two radioactive amino acids incorporated at the same time into thyroglobulin precursors should be the same as the ratio found in thyroglobulin provided that: (*a*) the newly synthesized units contain all the peptide chains of thyroglobulin in the same proportion in which they are present in the native molecule; (*b*) the amino acid incorporation into the polypeptide chains fully synthesized *de novo* is far in excess of the incorporation of the label into the pre-existing unfinished chains. The molar ratio of leucine to isoleucine in rat thyroglobulin is equal to 2.67 according to the amino acid analyses reported by Rolland *et al.* (18) and Hoshino and Ui (21). The value of 2.57 now found for the ratio of [^{3}H]leucine to [^{14}C]isoleucine in 19 S (see Table I) is in very good agreement with the above value. Furthermore, the ratio of leucine to isoleucine found in the 6 S and 7 S proteins is very similar to that in 19 S.

The results of the immunoprecipitation experiments demonstrated that the 6 S and 7 S proteins share a significant proportion of their antigenic structure with native 19 S thyroglobulin.

Since thyroglobulin is a glycoprotein containing from 8 to 10% carbohydrates (22), a further attempt to disclose the chemical relationship between native thyroglobulin and its biosynthetic precursors has been studied by measuring the incorporation of

radioactive carbohydrates. The data suggest the following conclusions. (*a*) Only 10% of the labeled galactose is associated with the slow sedimenting fractions. Furthermore, only one-quarter of this percentage is precipitated by antithyroglobulin antibodies. The incorporation of galactose, therefore, takes place after the assembly of the 19 S molecule. (*b*) Mannose is found on both the slower sedimenting 6 S and 7 S units and the faster sedimenting 12 S and 19 S. The 6 S, 7 S, and 12 S, however, contain only one-half the mannose of the 19 S, thus indicating that not less than 50% of this sugar is incorporated on the fully assembled 19 S.

These results are in general agreement with those reported by Herscovics (5). Our studies have been carried out on the individual fractions isolated from the 3-8 S component. Herscovics (5, 23) and Whur, Herscovics, and Leblond (24) have postulated that, although galactose and fucose are incorporated only on the 12 S and 19 S species, mannose is added on the smaller biosynthetic subunits present within the 3-8 S component. However, it is also possible that all carbohydrates (including mannose) are incorporated during or after the assembly of the 19 S molecule; actually the 12 S subunit derives from the dissociation of newly formed thyroglobulin molecules (7) and it is possible that even the 6 S and 7 S have similar origin (see below). *Carbohydrates are added in a stepwise manner (25–27), and the* addition of mannose precedes that of galactose, which occupies a more external position in one of the thyroglobulin glycopeptides (28). A similar stepwise addition of carbohydrates has been demonstrated in the case of immunoglobulin biosynthesis (29–31). The presence of mannose and the absence of galactose from the 6 S and 7 S subunits can also be explained if the most recently synthesized molecules have not yet acquired the complete carbohydrate moiety and the dissociation constant of 19 S to its subunits is much greater for these molecules than for the older ones.

Unlike the 6 S and 7 S, the 3 S probably represents polypeptide chains of newly formed thryoid proteins not related to thyroglobulin, since no clear chemical or immunological relationship between the 3 S and rat 19 S could be demonstrated. However, the possibility that the labeled 3 S component contains only some of the peptide chains of thyroglobulin with a leucine to isoleucine ratio different from that of the complete molecule, cannot be ruled out. Furthermore, the lack in the smaller biosynthetic units of some antigenic determinants typical of the native molecule would not be surprising. It is evident that when the number and the minimal size of the peptide chains of thyroglobulin will be elucidated, further studies will be necessary to establish the nature of the labeled 3 S material.

The molecular properties of the isolated 6 S and 7 S were studied by methods based on measurements of radioactivity. The molecular weights obtained for these two units by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (112,000 and 186,000, respectively) are in good agreement with those (119,000 and 170,000) calculated on the basis of their sedimentation coefficients, Stokes radii and a partial specific volume of 0.726. Even considering the error inherent in both methods used, it seems unlikely that the 6 S and 7 S are in monomer-dimer relationship. The number and size of the basic chains of thyroglobulin are not yet established: molecular weights ranging from 80,000 to 185,000 have been obtained in denaturing solvents after reduction and alkylation of 19 S (32–35). Preliminary results[3] indicate that if the 6 S protein is treated with an excess of dithiothreitol, the electrophoretic mobility in sodium dodecyl sulfate corresponds to a molecular weight of 42,000. It is, therefore, likely that at least the 6 S intermediate contains polypeptide chains linked together by S—S bonds.

The presence of some organized structure in these units is also indicated by the comparison of their behavior on gel electrophoresis in sodium dodecyl sulfate, density gradient centrifugation, and gel filtration. If the values reported in Table III are used to calculate the frictional ratios (f/f_0) (15, 16) of the 6 S and 7 S proteins, values between 1.5 and 1.6 are obtained for both molecular species. Even though the f/f_0 values thus obtained are approximate, they indicate that the shape of the 6 S and 7 S is not very different from that of native thyroglobulin (for bovine 19 S, $f/f_0 = 1.49$). It seems likely, therefore, that the biosynthetic 6 S and 7 S units have a certain degree of structural organization.

The question arises about the significance of the labeled 6 S and 7 S components. One possibility is that these soluble units accumulate during the biosynthesis of thyroglobulin and represent, therefore, precursors of the fully assembled native molecule. In keeping with this possibility is the precursor-product relationship between these units and the newly formed 19 S postu*lated from experiments both in vivo (8) and in tissue slices (3).* An alternative explanation, however, is that the 6 S and 7 S units derive from, or are in equilibrium with, newly formed and particularly unstable thyroglobulin molecules. In such a case the apparent precursor-product relationship between these units and 19 S would be due to the fact that the proportion of unstable 19 S progressively decreases with the age of the molecule. The greater stability of the older molecules seems to be related to late chemical processes such as stepwise addition of carbohydrates (25), formation of intra- and interchain S—S bonds (36, 37), and incorporation of iodine (38). It has been recently shown that the labeled 12 S and 15 S to 17 S components, formerly considered precursors of thyroglobulin on the basis of a definite precursor-product relationship between them and the native thyroglobulin, actually result, respectively, from dissociation (7) and unfolding (39) of the newly formed and poorly iodinated molecules. Furthermore, it has been previously shown (6) that a preparation containing both the 6 S and the 7 S labeled units associates *in vitro* with disaggregated unlabeled thyroglobulin to form faster sedimenting species such as 15 S and 19 S. Therefore it is not possible from the kinetic data alone to conclude that the 6 S and 7 S components accumulate during the biosynthesis of thyroglobulin. At the present time it would be also difficult to establish a precise quantitative relationship between the labeled 6 S and 7 S proteins now isolated and the native thyroglobulin molecule since the question of the size and number of the polypeptide chains of 19 S is still open.

In conclusion, the labeled 6 S and 7 S components, which have now been isolated and characterized for the first time, are closely related to newly formed thyroglobulin molecules which have not yet reached their most stable configuration. They are probably intermediate in size between the basic polypeptide chains and the fully assembled molecule, and have a degree of structural organization. In the more stable 19 S molecules these units are bound by covalent bonds (most likely S—S) since dissociation of thyroglobulin even by strong denaturing agents does not lead to

[3] G. Vecchio, and E. Consiglio, unpublished data.

subunits smaller than 330,000 molecular weight. The labeled 6 S and 7 S units may derive from dissociation of incomplete and unstable 19 S molecules but, even in this case, they participate after the formation of interchain disulfide linkages in the final structure of thyroglobulin.

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