# BIOSYNTHESIS OF THYROGLOBULIN:
## PARTIAL CHARACTERIZATION OF A LABELED 6 S PRECURSOR

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## 1. Introduction

The biosynthesis of thyroglobulin (19 S), the major thyroid iodoprotein, has been the subject of several recent reports. The incubation of thyroid slices [1–3], hemilobes [4,5], or isolated cells [6,7] with radioactive aminoacids is followed by the incorporation of radioactivity into three components having sedimentation rates (as measured by density gradient) of 3–8 S, around 12 S and from 17 to 19 S, respectively. The intermediate peak (12 S) has been considered to be one of the thyroglobulin subunits, corresponding to one half of the native molecule; kinetic experiments indicated that this labeled component was a relatively short-lived precursor of thyroglobulin (19 S). The slowest sedimenting peak (3–8 S) was heterogeneous and did not disappear even after long times of incubation *in vitro* [1]. For these reasons, neither its nature, nor its relationship with 19 S thyroglobulin was clearly established.

In the present work the slowest sedimenting component was obtained by labeling thyroid glands *in vivo* or *in vitro* with radioactive L-leucine and D-mannose and has been purified and further characterized. It has been shown to partially associate *in vitro* with half molecules of thyroglobulin (12 S subunits) and to react with antibodies against rat thyroglobulin: the degree of the immunological reactivity was slightly greater for the molecules labeled with mannose than for those labeled with leucine.

## 2. Methods

### 2.1. *Preparation of the labeled 6 S component*

Rat thyroid hemilobes from 5 animals were incubated in 95% $O_2$, 5% $CO_2$ at 37°C in 2 ml of leucine- and glucose-free Eagle's medium. After 30 min 0.24 $\mu$moles of 4–5 $^3$H-L-leucine (NENC, Boston, spec. act. = 5 Curies/mMole) and 20 $\mu$Moles of 1-$^{14}$C-D-mannose (Radiochemical Centre, Amersham, UK; spec. act = 0.3 Curies/mMole) were added to each flask and the hemilobes were further incubated for 10′, 30′ or 60′. In experiments performed with a single label, $^3$H-L-leucine or 1-$^3$H-D-mannose (spec. act. 0.7 Curies/mMole) (0.1 and 1.0 $\mu$Moles per flask, respectively) were used. 0.1 $\mu$Mole of non radioactive leucine was added in the flasks containing the radioactive mannose. In some instances the rats were injected intravenously with a pulse label of 4.5 $^3$H-L-leucine (10 $\mu$C per g body weight) and killed 5 min later. In both cases the soluble thyroid extracts were prepared as previously described [8], purified by repeated salting out with $(NH_4)_2 SO_4$ [9] and analyzed by sucrose gradient centrifugation.

In the rat thyroid extract after 10, 30 or 60 min of labeling *in vitro* as well as after 5 min of labeling *in vivo*, a large proportion of the radioactivity was associated with a broad peak, sedimenting between 3 and 8 S [1,9]. The gradient fractions corresponding to the top third of the centrifuge tube were therefore pooled, concentrated by dialysis under reduced pres-

sure and again submitted to sucrose gradient centrifugation. One rather broad radioactive peak ($\sim$ 6 S) was then obtained, most of which was recovered and used for further characterization.

## 2.2. Conversion in vitro of the 6 S component to 19 S

Ultracentrifugally homogeneous preparations of rat 19 S thyroglobulin were obtained [8] and dialyzed for 16 hr at + 4°C against 0.002 M bicarbonate buffer at pH = 10.0. These conditions induced extensive dissociation (80%) of the 19 S into the 12 S subunits, which represent half molecules of the native protein [10]. Such dissociation was largely reversible (more than 70%) by dialysis against a "standard" buffer (0.1 M KCl, 0.02 M phosphate, pH 7.4). The purified $^3$H-6 S component (200,000 dpm) was added to 10 mg of cold rat thyroglobulin: the mixture was then exposed to low ionic strength-high pH buffer (see above), then to the standard buffer and finally analyzed by sucrose gradient centrifugation. In control experiments, the 6 S component was either (a) added to undissociated cold 19 S, the dialysis against the low ionic strength-alkaline medium being omitted, or (b) was submitted to the dissociating and reversal procedures in the absence of cold thyroglobulin.

## 2.3. Immunoprecipitation of the labeled 6 S with thyroglobulin antibodies

Rabbit antiserum against highly purified 19 S rat thyroglobulin was prepared by injecting the antigen emulsified in complete Freund's adjuvant. The immunoprecipitation was performed by standard techniques. $^3$H- or $^{14}$C-radioactivity was measured both in the supernatants and in the specific precipitates; the latter were washed twice with cold saline. In some cases the antigen-antibody complex was co-precipitated by a goat antiserum against rabbit immunoglobulins (Hoechst AG, Frankfurt a.M., W. Germany) according to Roitt et al. [11], in order to obtain visible discrete precipitates.

## 2.4. General techniques

Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge according to standard procedures; density gradient centrifugation in 5–40% sucrose gradient in the SW 25.2 rotor of the Spinco model L2-65 centrifuge. Protein concentration was measured by absorbance measurements at 280 or
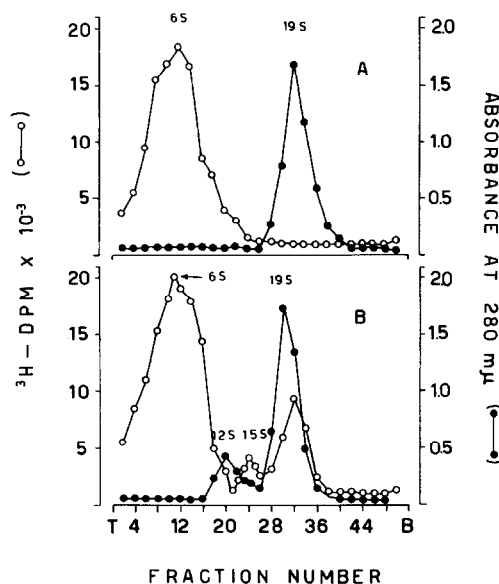


Fig. 1. Conversion in vitro of the $^3$H-leucine labeled 6 S peak to 19 S. A. Sucrose density gradient patterns of the $^3$H-labeled purified 6 S (200,000 dpm) mixed with 10 mg unlabeled rat 19 S thyroglobulin. B. Sucrose density gradient pattern of the same radioactive material (250,000 dpm) mixed with 10 mg unlabeled rat 19 S thyroglobulin and subjected to a dissociation-reassociation procedure (see Methods) prior to centrifugation. Sucrose 5–40%, rotor SW 25.2 of the Spinco centrifuge Model L-2-65. Equivalent time of centrifugation 29 hr at 23,000 rpm. T = top and B = bottom of the centrifu                    fuge tube.

210 mu. $^3$H- and $^{14}$C-radioactivity was determined by a liquid scintillation counter equipped with a three channel analyzer; the counting error was below 5%.

## 3. Results

### 3.1. Conversion of the 6 S component to 19 S in vitro

The sedimentation pattern of the labeled 6 S component, which was isolated from thyroid hemilobes after incubation for 30 min with $^3$H-leucine, did not show any change if the solution was first exposed to low ionic strength medium at pH 10.0 and then to a high salt-neutral pH solution (standard medium). If the $^3$H-6 S component was added to undissociated native thyroglobulin and the mixture dialyzed against standard medium, again no change in the sedimentation pattern of the labeled material was observed (fig. 1A).

Table 1
Immunoprecipitation of $^3$H-L-leucine or $^3$H-D-mannose labeled 6 S component.

| Labeling conditions | Per cent radioactivity in precipitate* | | |
| --- | --- | --- | --- |
| | Ascending limb | Top fractions | Descending limb |
| 5 min *in vivo* leucine** | 30.0 | 39.1 | 72.8 |
| 10 min *in vitro* leucine | 43.7 | 68.6 | 77.3 |
| 10 min *in vitro* mannose | 46.8 | 73.8 | 81.3 |
| 30 min *in vitro* leucine | 54.1 | 78.6 | 83.3 |
| 30 min *in vitro* mannose | 55.2 | 85.5 | 93.1 |

\* The $^3$H-6 S component was obtained from pooled tubes 2–8 (ascending limb), 9–14 (top) and 15–20 (descending limb) of a sucrose gradient (see fig. 1A). To each fraction (10,000–100,000 dpm in 200 $\mu$l of standard buffer) were added 20 $\mu$l of anti-thyroglobulin serum, 100 $\mu$l of which precipitated 400 $\mu$g purified rat thyroglobulin at equivalence. The mixture was incubated 30 minutes at +37°C and 48 hr at +4°C. Almost complete inhibition of radioactivity precipitation was obtained by addition of 4 mg unlabeled rat thyroglobulin.

\*\* In this case a co-precipitation system was used (addition of 200 $\mu$l of immune goat serum against rabbit Ig, 50 $\mu$l of which precipitated approximately 100 $\mu$g of rabbit Ig at equivalence).

When dissociation and reassociation were carried out in the presence of unlabeled 19 S rat thyroglobulin, or when the $^3$H-6 S component was added to previously dissociated 19 S, and the mixture then dialyzed against "standard" medium, a significant proportion of the activity appeared under the 19 S absorbance peak. A minor, but clearly evident peak, sedimenting between the 12 S subunit and 19 S was also observed regularly (fig. 1B). The proportion of the $^3$H-6 S component converted to 19 S in the presence of dissociated rat thyroglobulin did not exceed 20% of the total radioactivity. Attempts to increase the conversion by varying the experimental conditions were unsuccessful.

### 3.2. *Immunological reactivity of the labeled 6 S component*

Since the sucrose gradient profile did not seem to indicate ultracentrifugal homogeneity of the purified 6 S, the labeled peak was arbitrarily divided in three fractions; the first comprising fractions from the ascending limb, the second from the top and the third from the descending limb of the peak. The three fractions were separately tested by the standard immunoprecipitation reaction. The results obtained, summarized in table 1, indicated that the proportion of the 6 S radioactivity which reacts with rat thyroglobulin increases from the slowest sedimenting fractions (ascending limb) toward the faster sedimenting ones

(top fractions and descending limb). The extent of precipitation was also higher at longer labeling intervals and slightly, but consistently, higher for the mannose than for the leucine label.

### 4. Discussion

Time-course studies on biosynthesis of thyroglobulin in tissue slices have failed to demonstrate a clear precursor-product relationship between the slow-sedimenting component (3–8 S) and 19 S thyroglobulin: in fact, the broad 3–8 S peak observed by sucrose gradient analysis was still present in considerable amounts even after 20 hr of incubation *in vitro* [1,2]. The results reported here strongly suggest that the 6 S component is a precursor of 19 S thyroglobulin: (1) the 6 S was able to associate *in vitro* with thyroglobulin 12 S subunits to form 19 S molecules; (2) it reacted to a large extent with antibodies against native thyroglobulin. Both these properties, however, were shared only by a variable fraction of the slow-sedimenting material (6 S) and it is possible that the purified 6 S component still contained newly formed proteins or peptide chains unrelated to thyroglobulin. Although this possibility cannot be excluded, it appears unlikely since, after purification by repeated salting out, complete disappearance with time of the slow-sedimenting component ($\sim$ 6 S) has been ob-

served in this laboratory (work in preparation, see also ref. [9]). This result was obtained either 48 hr after administration of the radioactive amino acid *in vivo* or after 10 min of slice labeling followed by 4 hr "chase" *in vitro* with unlabeled leucine.

The alternative hypothesis, therefore, of the intrinsic heterogeneity of the 6 S component seems to be more likely. Addition of carbohydrates, a stepwise process which follows the synthesis of the peptide chains [12,13], may be responsible for this heterogeneity. In fact the 6 S molecules which have incorporated labeled mannose have a higher degree of reactivity towards anti-thyroglobulin antibodies than those labeled with leucine. It has been recently reported that mannose and galactose are incorporated at different times during the formation of thyroglobulin [14]: since the acquisition by the 6 S component of the antigenic determinants typical of the 19 S molecules is also time-dependent, it is likely that the heterogeneity of the 6 S precursor of thyroglobulin is related to the different degree of completeness reached by the newly formed protein through the stepwise attachment of its carbohydrate moiety.

## Acknowledgements

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