Biosynthesis of Thyroglobulin: Incorporation of [^3H]Fucose into Proteins by Rat Thyroids *in vitro*

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(Received 26 January 1970)

Previous work (Herscovics, 1969) demonstrated that mannose and galactose are incorporated into the carbohydrate groups of thyroglobulin at different times in the elaboration of this glycoprotein from its subunit precursors. Mannose is incorporated on subunits of thyroglobulin at about the time of their synthesis, whereas galactose is incorporated over 30 min later near the time of aggregation of subunits to form non-iodinated thyroglobulin (17–18S protein). It was concluded that this difference in time is related to the positions of these two sugars in the oligosaccharides of thyroglobulin, mannose being found closer to the polypeptide backbone than galactose. Since fucose is located exclusively at the non-reducing terminal of some carbohydrate side chains of thyroglobulin (Spiro & Spiro, 1965a), it was decided to determine at what stage in the formation of thyroglobulin this sugar is incorporated.

Methods. Rat thyroid lobes were incubated in Krebs–Ringer bicarbonate medium containing both L-[^3H]fucose and L-[U- ^{14}C]leucine (New England Nuclear Corp., Boston, Mass., U.S.A.). Incubation was performed as described previously (Herscovics, 1969). After incubation the thyroid lobes were rinsed and homogenized with a Potter–Elvehjem homogenizer in 2 ml of saline (0.15 M-NaCl–0.01 M-sodium phosphate buffer, pH 6.8), and the homogenate was centrifuged in a Sorvall refrigerated centrifuge at 25000 g_{max} for 20 min. The pellet was homogenized again in 2 ml of saline, followed by centrifugation. The proteins in the supernatants (saline extract) were precipitated three times with 70%-saturated ammonium sulphate, pH 6.8, and separated by sucrose-density-gradient centrifugation as described previously (Herscovics, 1969). The pellet was washed by homogenization in 2 ml of saline and the wash, which was free of labelled proteins, was discarded after centrifugation. The pellet was then homogenized in 1 ml of 2% digitonin in saline and kept for 1 h at 4°C. The suspension was then centrifuged at 25000 g_{max} for 20 min, and the supernatant (digitonin extract) was dialysed overnight against saline and analysed by sucrose-density-gradient centrifugation. Extended periods of extraction with digitonin such as 18 h at 4°C caused some breakdown of 17–18S protein to 12S material.

Results. Radioactivity in thyroid proteins was determined after different times of incubation in the presence of [^3H]fucose and [^{14}C]leucine. Two fractions were examined by sucrose-density-gradient centrifugation: (1) a saline extract, which contains proteins derived from the colloid, from the cell sap and from fragile subcellular particles; (2) a digitonin extract of the resulting pellet, which contains proteins originally firmly bound to subcellular particles.

Within 10 min of incubation radioactivity from [^3H]fucose in both the saline extract and the digitonin extract was present mostly in the non-iodinated thyroglobulin fraction, which sediments with a peak at 17–18S (Fig. 1a). The 17–18S fraction was the only one labelled from [^3H]fucose in the digitonin extract, and accounted for most of the ^3H label in the saline extract. This pattern of incorporation of radioactivity from [^3H]fucose was the same after 5 min of incubation, and did not change with longer incubations up to 1 h, although the amount of ^3H incorporated into 17–18S protein increased markedly with time (Figs. 1b and 1c). The radioactivity from [^3H]fucose incorporated into thyroid proteins was recovered in a spot migrating as fucose after hydrolysis of the proteins in 0.05 M-sulphuric acid for 8 h at 100°C and paper chromatography of the hydrolysate by using the techniques described by Spiro & Spiro (1965b). All the radioactivity on the sucrose density gradients could be precipitated by cold 6% trichloroacetic acid–0.5% phosphotungstic acid.

As described previously (Herscovics, 1969, and references cited therein) radioactivity from labelled leucine appeared in the 17–18S protein only after a lag period greater than 30 min. During this lag period leucine label was observed only in 3–8S and 12S fractions containing subunit precursors of thyroglobulin (Figs. 1a and 1b). It may be noted that the $^{14}\text{C}/^3\text{H}$ ratio was much greater in the initial saline extract than in the subsequent digitonin extract, particularly at the earlier times of incubation. These observations indicate that leucine-labelled proteins were more easily extracted from the thyroid than were fucose-labelled proteins. It seems likely therefore that at these times a large part of the leucine-labelled proteins and the fucose-labelled proteins were not located within the same subcellular particles.

Fig. 1. Sucrose-density-gradient centrifugation of proteins from thyroids double-labelled with [^3H]fucose and [^{14}C]leucine *in vitro*. Whole thyroid lobes from ten rats (185 ± 10 mg wet wt.) were incubated in each flask at 37°C in 2 ml of Krebs-Ringer bicarbonate medium with a gas phase of $\text{O}_2 + \text{CO}_2$ (95:5). A preincubation of 15 min with ($\blacktriangle$ and $\bullet$) or without ($\triangle$ and $\circ$) cycloheximide ($70 \mu\text{g}/\text{ml}$) was first carried out before the addition of radioactive precursors. The lobes in the control and cycloheximide-treated samples were hemithyroids obtained from the same rats. For (a) $500 \mu\text{Ci}$ of L-[^3H]fucose (specific radioactivity $4300 \text{ mCi}/\text{mmol}$) and $5 \mu\text{Ci}$ of L-[^{14}C]leucine (specific radioactivity $278 \text{ mCi}/\text{mmol}$) were added to the flask, and for (b) and (c) $200 \mu\text{Ci}$ of L-[^3H]fucose (specific radioactivity $4300 \text{ mCi}/\text{mmol}$) and $5 \mu\text{Ci}$ of L-[^{14}C]leucine (specific radioactivity $263 \text{ mCi}/\text{mmol}$) were added to each flask. The thyroids were incubated with labelled precursors for: (a) 10 min; (b) 30 min; (c) 60 min. The thyroids were then extracted first with saline and then with digitonin, and the proteins in these extracts were separated by sucrose-density-gradient centrifugation as described in the text. ^{14}C ($\blacktriangle$ and $\triangle$) and ^3H ($\bullet$ and $\circ$) radioactivity and E_{280} (.....) of each fraction obtained from the gradients were measured. Fractions obtained from control and cycloheximide-treated thyroids were plotted on the same graph and showed identical E_{280} curves. The E_{280} of the digitonin extracts was negligible. The arrow at 19S in the digitonin extract of (a) indicates the position of a sample of purified rat thyroglobulin that was added to the extract before centrifugation. The direction of sedimentation is from right to left.

Cycloheximide at a concentration of 0.25 mM completely inhibited leucine incorporation into thyroid proteins within 30 min of incubation without any effect on fucose incorporation into 17–18S protein of either the saline extract or the digitonin extract (Fig. 1b). After 60 min of incubation cycloheximide inhibited fucose incorporation into saline-extracted 17–18S protein only slightly, but it

prevented this incorporation into digitonin-extracted 17–18S protein to a greater extent (Fig. 1c).

Discussion. These results demonstrate that, in rat thyroids *in vitro*, fucose is readily incorporated into the carbohydrate groups of the non-iodinated thyroglobulin fraction (17–18S protein). Fucose label is immediately associated with 17–18S protein, without the lag observed for the incorpora-

tion of leucine or mannose (Herscovics, 1969). Further, fucose incorporation can continue unaffected for at least 30 min in the absence of polypeptide-chain synthesis induced by cycloheximide, and therefore fucose is incorporated into preformed polypeptides. These results are very similar to those previously obtained with labelled galactose (Herscovics, 1969). Since there is no significant incorporation of fucose label into amino acids or other sugars, much less radioactivity is associated with the 3-8 S and 12 S fractions in the presence of [^3H]fucose than was observed with labelled galactose. The present results therefore confirm the idea that terminal sugars are incorporated into the carbohydrate groups of thyroglobulin much later in the synthesis of this protein than are sugars like mannose, which are closer to the polypeptide backbone. Fucose is incorporated either on subunits of thyroglobulin immediately before their aggregation to 17-18 S protein, or after aggregation directly on to 17-18 S protein.

With radioautography it has been demonstrated that mannose incorporation takes place within the rough endoplasmic reticulum of thyroid follicular cells, whereas galactose addition occurs within the Golgi apparatus after migration of thyroglobulin precursors there (Whur, Herscovics & Leblond, 1969). In view of the similarity of the kinetics of incorporation of fucose and galactose into 17-18 S protein, it seems likely that the intracellular site of incorporation of fucose likewise is within the Golgi apparatus.

The technical assistance of Miss C. Walker is gratefully acknowledged. This work was supported by The Medical Council of Canada.

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