BIOSYNTHESIS OF THYROGLOBULIN : ABSENCE OF ^{14}C-GLUCOSAMINE INCORPORATION ON THYROID POLYSOMES

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Thyroglobulin, the major protein synthesized in the thyroid gland is a glycoprotein containing 8,5 % of carbohydrate residues in the form of ca. 22 heteropolysaccharide units (Spiro and Spiro, 1963, 1965, Cheftel et al 1964). An investigation of the subcellular site of attachment of this glucidic moiety is described in the present Note.

Partly conflicting results have recently appeared concerning the site of incorporation of carbohydrates into serum glycoproteins synthesized in liver microsomes. Sarcione (1964) after hepatic perfusion with either ^{14}C-galactose or ^{14}C-glucosamine concluded that the endoplasmic membranes were the only site, and an attempt (Sinohara and Sky-Peck, 1965) to isolate a sRNA carrying an amino-acid linked glucosamine was negative. On the contrary other laboratories (Helgeland, 1965, Molnar et al, 1965) reported definite incorporation of ^{14}C-glucosamine on ribosomes ; an hypothesis was advanced that at least asparaginylglucosamine linkages could occur while the polypeptide chain was associated to polysomes.

After incubation of sheep thyroid slices with ^{3}H-leucine and ^{14}C-glucosamine (or mannose), we found no incorporation of ^{14}C-carbohydrate on polysomes, while ^{3}H-leucine is incorporated. A definite ^{14}C-labeling, associated with the ribosomal pellets, is partially removable by acids and is not related to the actively synthesizing polysomes as seen after sucrose density gradient centrifugation. An attempt to discriminate between rough and smooth membranes as a preferential site of carbohydrate incorporation has shown that proteins from the two kinds of membranes are equally labeled with regard to both ^{3}H-leucine and ^{14}C-mannose.

Incubations and cell fractionations. Sheep thyroid slices are incubated in Eagle's medium as described by Seed and Goldberg (1963) in the presence of labeled precursors. Streptomycin and leucine are omitted. After incubation, slices are washed with cold medium M (0.02M TRIS buffer pH 7.6, 0.1M KCl, 0.01M Mg acetate, 0.04M NaCl, 0.25M sucrose), containing 0.1 % leucine and carbohydrate, and homogenized in 5 vol of the same medium : Servall Omnimixer, 1 min at 2/5 max speed (cf. Cartouzou et al, in preparation), followed by 2 strokes at 800 rpm in a loosely fitting glass-teflon Potter homogenizer. Filtered homogenates contain, per g of thyroid, 140-185 mg of proteins (Lowry method), 1.0-1.25 mg RNA (Ceriotti's orcinol procedure after a modified Schmidt-Thannhauser fractionation, Begg et al, 1965) and 0.9-1.05 mg DNA (diphenylamine reaction after a Schneider's fractionation). Post-mitochondrial supernatants (after 10000 g or sometimes 14000 g centrifugation, 10 min) contain, per g of thyroid, about 9/10 of the abovementioned proteins, 0.6-0.9 mg RNA and no DNA. Microsomes are isolated from post-mitochondrial supernatants. Post-microsomal supernatants are free from particulate RNA. Microsomal subfractions are prepared as described by Chauveau et al (1962) and modified by Manganiello and Phillips (1965), but using the abovementioned saline concentrations. Ribosomes are prepared as described by Wettstein et al (1963), but with the medium M of Munro et al (1964) : post-mitochondrial supernatants are made up to 1 % Na deoxycholate, layered over 2 layers of sucrose and ultracentrifuged. The ribosomal pellets are suspended in medium M for assays ; supernatants of a short low speed centrifugation are analysed on sucrose density gradient. 1/ A 5h ultracentrifugation at 100000 g max (Spinco Rotor 30 or 40) with a 1M sucrose inferior layer yields 440-515 µg of ribosomal RNA per g of thyroid ($E^{1\%}_{1cm,260m\mu}$ = 290) a large amount of monomers being soluble whereas polysomes are largely agglutinated (they are susceptible to RNase). 2/ With a 2M sucrose inferior layer, the yield decreases to 147-165 µg of ribosomal RNA, mainly polysomal ; of this amount, 80-120 µg per g of thyroid, sometimes less after incubation, accounts for soluble material. Protein to RNA ratio was not inferior to 1.3. 3/ If no deoxycholate is added the polysomal RNA (free polysomes in these homogenizing conditions, ^{3}H-leucine-labeled after incubation) amounts to about 2/3 of the value with detergent.

Comparison of ^{3}H-leucine and ^{14}C-glucosamine incorporation in polysomes and microsomes. (Fig. 1 and Table 1). The ^{3}H-labeling on polysomes, prepared through 2M sucrose, is nearly identical after 30 or 120 min, whereas in total microsomes it increases with time, an observation in accordance with the polysomes being the site of polypeptidic chain synthesis. After 30 min there is no ^{14}C-radioactivity on polysomes and only a small amount in microsomal proteins. After 2h the ^{14}C-labeling in microsomes has increased and a definite ^{14}C-radioactivity is also present in the polysomal suspension : centrifugation on a density gradient shows this label not to be located in the polysomal region but in the ribosome dimer-trimer

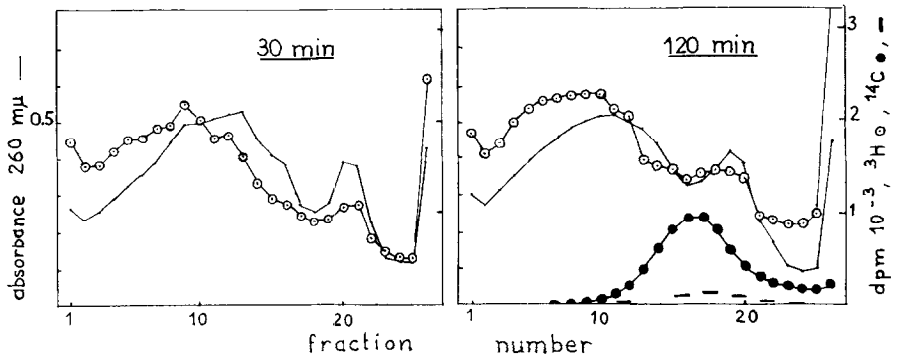

Fig. 1 - Radioactivity distribution after gradient centrifugation of polysomes (sucrose 30-10 % in buffered saline, linear, SW25 Spinco Rotor, 2h, 23 000 rpm).

Either 30 min (left) or 120 min (right) incubation, starting from 8 g thyroid in 50 ml (2 flasks) Eagle's medium + 170 μc [4,5-^{3}H] leucine (5C/mMole) + 42 μc [1-^{14}C] glucosamine (10mC/mMole). ○ and ● are respectively ^{3}H and ^{14}C dpm per 1.03 ml fraction (direct counting with 0.25 ml). — refers to the ^{14}C dpm after 5 % trichloracetic acid precipitation (0°) and washing (20°) on pooled fractions (2 mg serum albumin added) ; results for ^{3}H are essentially unmodified. Countings are done with a Packard Tri-Carb liquid scintillation spectrometer, model 314 EX, 500 D (discriminator settings : 10-50 with max gain, and 18-100 with gain = 20 or 25).

Values found for an always noticeable pellet at the bottom of the tube are not given here.

30 min :	214 000	^{3}H dpm	1 300	^{14}C dpm
120 min :	657 000	"	10 600	"

Table 1 - Incorporated radioactivities in microsomal proteins

(modified Schmidt-Thannhauser fractionation, starting from washed total microsomes). Incubation described in Fig. 1 legend. Values are for 1 g of thyroid.

zone and to be partially removed by 5 % trichloracetic acid. The same result is observed with ^{14}C-mannose.

A 10 min incubation at 37°, with 0.02 µg cryst. RNase, of the polysomal suspension before centrifugation does not modify the position of this ^{14}C-radioactivity peak, whereas the ^{3}H leucine labeling becomes entirely located with monomers and dimers. A similar incubation with RNase followed by a 3h delay at 5° before centrifugation, has been able once to entirely displace the unexplained ^{14}C-radioactivity to a lighter region than the ^{3}H-labeled monomers and dimers : this observation affords a new argument for the absence of correlation between ^{14}C-radioactivity and nascent polypeptide chains.

The observation of progressive removal by diluted acid washings (trichloracetic or perchloric acids) and of a displacement to lighter regions after incubations (37°, 30 min, has been tried successfully too, with an only slight decrease of the average S value of polysomes) would argue for an association to ribosomes, or to an unknown membrane, of a low MW labeled compound, perhaps a sugar nucleotide (Gregoire et al, 1961).

If thyroid slices are pre-incubated with puromycin ($2.5\ 10^{-4}$M), a 98 % inhibition of aminoacid incorporation is observed either on ribosomes or microsomes after 2h incubation, whereas there is only a 18 % inhibition of the ^{14}C-carbohydrate incorporation in microsomes (see also Spiro and Spiro, 1965). The ^{14}C radioactivity peak in the ribosome gradient is still present, which again argues for this radioactivity being unrelated to nascent polypeptidic material.

Incorporation of ^{3}H-leucine and ^{14}C-mannose in microsomal subfractions. Table II shows the results obtained after 1 or 2 h incubation. These preliminary data indicate the rough membranes to be a site of carbohydrate incorporation.

Thyroglobulin characterization and fate of the precursor glucosamine. After solubilization of washed total microsomes by 0,5 % digitonine (Nunez et al, 1965), a sucrose density gradient (linear 20-5 %) shows most of the radioactivity, ^{14}C-leucine or ^{14}C-glucosamine, in the case of a 2 h incubation, to be located with S$\sim$18 material, shoulders in S$\sim$12 and heterogeneous lighter regions being also present. Ratios of the specific activities of the particulate proteins and of the soluble cytoplasmic thyroglobulin are 16 for leucine and 34 for glucosamine. After pronase hydrolysis of

	rough membranes				smooth membranes			
	^{3}Hdpm	^{14}Cdpm	$\frac{^{14}C\text{dpm}}{\text{mg prot}}$	$\frac{^{3}H/^{14}C}{\text{dpm}}$	^{3}Hdpm	^{14}Cdpm	$\frac{^{14}C\text{dpm}}{\text{mg prot}}$	$\frac{^{3}H/^{14}C}{\text{dpm}}$
1h	32800	349	1230	94	60500	580	1030	104
2h	91000	1060	3180	86	108000	1150	2560	94

Table 2 - Incorporated radioactivities in the proteins of the two main submicrosomal fractions. - Incubation as in Fig. 1 but with 1-^{14}C mannose, 1 or 2 h. Samples for counting processed as in Table 1. Values are for 1 g of thyroid. Protein/RNA = 17-24 for rough membranes and 12-14 for smooth membranes.

the ^{14}C-particulate thyroglobulin, labeled glycopeptides are characterized by filtration through Sephadex G-10. After a 2 h 2N HCl hydrolysis, radioactivity was found in glucosamine as characterized by paper chromatography, only negligible amounts being in other carbohydrates. Sialic acid labeling has not been studied here.

In conclusion, these results suggest that the carbohydrate moiety of thyroglobulin is added to completed polypeptide chains, within the endoplasmic membranes, most probably in the rough reticulum. The early step of attachment is shown not to be on polysomes (which in the intact cell are expected to be largely associated to membranes). These conclusions are similar to those obtained by Cook et al (1965) in a study of the biosynthesis of glycoproteins of the Ehrlich ascites carcinoma cell membranes. Reports on specific glycosyltransferases in membranes have recently appeared ; in the case of thyroglobulin, a sequential addition of carbohydrates in microsomes has been proposed by Spiro and Spiro (1965). The question of recognition of the asparaginyl sites is an open one, and it would be interesting to know at what stage of completion of the protein the synthesis of carbohydrate units begins.

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