TENTATIVE IDENTIFICATION OF N-ACETYLGALACTOSAMINE INCORPORATING MEMBRANES FROM OVINE SUBMAXILLARY GLANDS (OSG).

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Received December 3, 1968

The subcellular sites of the metabolic events which cooperate in the synthesis of a complete glycoprotein have been, within recent years, the subject of a considerable amount of experiments and speculation.

Whereas it is now admitted that the biosynthesis of the protein moiety of this macromolecule occurs through the general mechanism of protein biosynthesis, as evidenced mainly by its sensitivity to classical inhibitors, the subcellular site of fixation of the individual sugars and primarily of the first sugar which is directly linked to the protein chain remains controversial (Molnar, J. et al. , 1965, Bouchilloux, S. et al., 1966, Neutra, J. et al., 1966, Lawford, R. G. , et al. , 1967, Simkin, J. L. et al. , 1968).

Ovine submaxillary mucoprotein (OSM) seems ideally suited for the study of this problem, as the polysaccharide chain of this glycoprotein consists exclusively of a disaccharide (α-N-acetylneuraminyl 2 ⟶ 6-N-acetylgalactosamine)(GalNAc-NANA) (Graham et al. , 1960) which is bound through an alkali labile glycosidic linkage (Bhavanandan et al. , 1964, Harbon et al. , 1964) to hydroxyl groups of seryl and threonyl residues of the peptide chain.

The present work aims to localize the subcellular incorporation sites of NAcgalactosaminyl residues by two different experimental procedures: incorporation of radioactive precursors into surviving gland slices with subsequent fractionation and incubation of subcellular fractions incorporating labelled aminoacids and sugars.

[*] This work, and a previously published one (Rossignol et al. , 1966) is part of a Doctoral Thesis (Doctorat ès Sciences) to be submitted by one of the authors (B. Rossignol).

Experimental.

Incubation of surviving gland slices is performed at 37° in the usual Krebs-Ringer bicarbonate medium (Umbreit et al., 1964) under 95 % O_2-5 % CO_2. The tissue is homogenized by the means of an Ultra-Turrax homogenizer in 0.35 M sucrose, 0.004 M $MgCl_2$, 0.001 M KCl, 0.075 M Tris, pH 7.8 and fractionated as described below. Incubations of subcellular fractions are performed in Medium 1 (aminoacid incorporation): 6 μM ATP, 2 μM PEP, 40 μM $MgCl_2$, 20 μM K-phosphate, 10 μg pyruvate-kinase, 150 μM sucrose, 4,000 U Penicilline; or in Medium 2 (sugar incorporation) 5 μM ATP, 3 μM PEP, 50 μM $MgCl_2$, 100 μM K phosphate, 10 μg pyruvate-kinase, 150 μM sucrose, 4,000 U penicilline; pH 7.0 (final volumes 1 ml; incubation time 60 min). OSM is purified on DEAE-Sephadex, NANA removed by mild acid hydrolysis and disaccharides cleaved by β-elimination (Harbon et al., 1968). Glycogen is isolated and estimated according to Palasi et al. (1960). Phospholipids are extracted by the method of Garbus et al. (1963).

Protein subfractions are precipitated with 15 % TCA - 0,5 % Phosphotungstic acid. All acid insoluble pellets are washed with TCA-phosphotungstic acid, dissolved in 0.5 NaOH and reprecipitated by adding TCA-phosphotungstic acid. The pellets are washed by ethanol-ether (1:1, v/v) and counted in a Nuclear Chicago counter, Model C 115; paper chromatograms are cut into small strips (1 cm/3 cm) and counted in a Packard Tricarb liquid scintillation counter.

Results.

It has not proven possible to apply the classical tissue fractionation procedures, yielding small microsomal particles, rich in RNA, to tissues like OSG. Under these circumstances, the greatest amount of RNA sediments at very low speeds (72 % at 5,000 g), and this situation which is also met with in the fractionation of oviduct (Hendler, 1956) and, more recently in the bovine submaxillary gland (Lawford et al., 1967) proved to be, up to the present time, resistant to all tentatives to improve the classical procedures.
Hence, to prepare RNA rich material, centrifugation had to be operated between 1000 g and 105,000 g which yielded a fraction devoid of cells and nuclei. Refractionation of this material on a discontinuous sucrose gradient (Schneider et al., 1953) yielded a sediment containing all of the RNA and three discrete zones of "smooth" membranes, the separation of which is not arti-

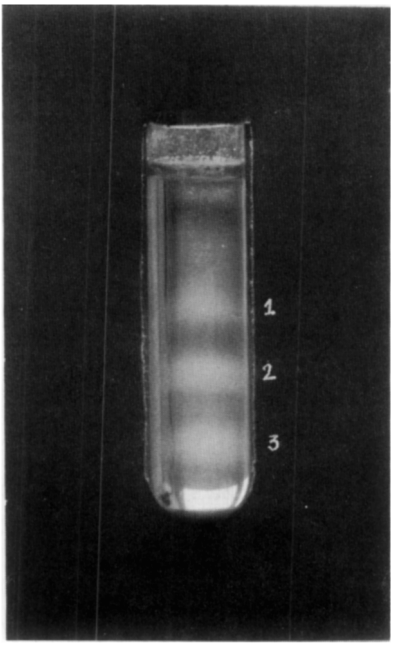

Fig. 1 : Centrifugal separation of microsomes from OSG on discontinuous sucrose gradient showing 3 described zones of smooth membrane material.

Table 1: Microsomal distribution of protein, RNA, cytochrome c oxydase, alkaline phosphatase and amino acid incorporation activities.

M sucrose		0,250	0,335	0,636	0,957	1,110	Sediment
Protein (mg)		6.3	2.3	1.8	3.7	4	16
RNA (mg)				0,064	0.111		0,990
^{14}C-leucine cpm/mg prot.			6	14	9		578
Phospholipid /RNA (w/w)				52			11
Total Cyto c oxydase activity*			0	0	0		650
Alkaline phosphatase activity**	(total	6	17.5	56	68	16.5	17.7
	(per mg/ prot.	0.95	7.7	30.7	18.5	4.15	1.07

* Expressed as mμmoles cytochrome c oxidized per minute.
** Expressed as mμmoles p-nitrophenol released in 30 minutes at 37° from p-nitrophenylphosphate.

factual, as they differ in many of their properties (fig. 1). Thus the highest specific activity of alkaline phosphatase (attributed to the Golgi bodies) appears in the middle zone of smooth membranes and other differences between the three zones will be outlined below. The sediment is still heavily contaminated with mitochondrial material (as evidenced by the high cytochrome oxydase content) and no reliable method was found, up to now, to purify the rough membranes, while preserving their biological activity (Table 1).

When OSG slices are incubated with ^{14}C-leucine, heavy labelling occurs in the sediment, where active protein synthesis is supposed to take place, labelling in zones 2 and 3 of smooth membranes are appreciable and there is some radioactivity in the soluble proteins, near the meniscus: zone 1, though being rich in proteins does not seem to incorporate aminoacids very actively, indicating membrane material of low turnover velocity (figure 2 a). When ^{14}C-glucose (figure 2 b) is used as a precursor, labelling occurs mainly in the sediment and in zone 2 (Golgi bodies) indicating that these are the loci where glycoprotein biosynthesis might take place. When ^{14}C-glucosamine, as good a precursor of OSM as glucose (Harbon et al., 1966), is used under the same experimental conditions (figure 2 c) there is a sharp breakdown of radioactivity in the smooth membrane fraction of zone 2 whereas labelling in the rough membranes sediment remains very high. Occurence of radioactive material at the meniscus on these figures is due to contamination of microsomes with various precursors and products and may be eliminated by previous washing of the insoluble fraction; this indicates that the labelling of the main peaks is not due to adsorption of proteins or sugar intermediates. The results suggest that incorporation of at least the first protein linked sugar, GalNAc, is an intrinsic property of rough, and not of smooth membranes. This view is confirmed by a preliminary analysis of labelled material (from ^{14}C-glucose) (Table 2): labelled OSM is found in a much higher percentage of the total labelled material in rough than in smooth membranes. Most of the radioactivity in the latter material seems to be due to glycogen and to phospholipids, a fact which provides a tentative explanation of high labelling of smooth membrane vesicles, including the Golgi bodies, which have been previously reported on the basis of histochemical and autoradiographic methods (Neutra et al., 1966). This interpretation is further supported by the very high turnover of ^{32}P labelled fractions in zone 2 of smooth membranes and in the sediment. Figure 2 d shows that under identical circumstances, the patterns of labelling

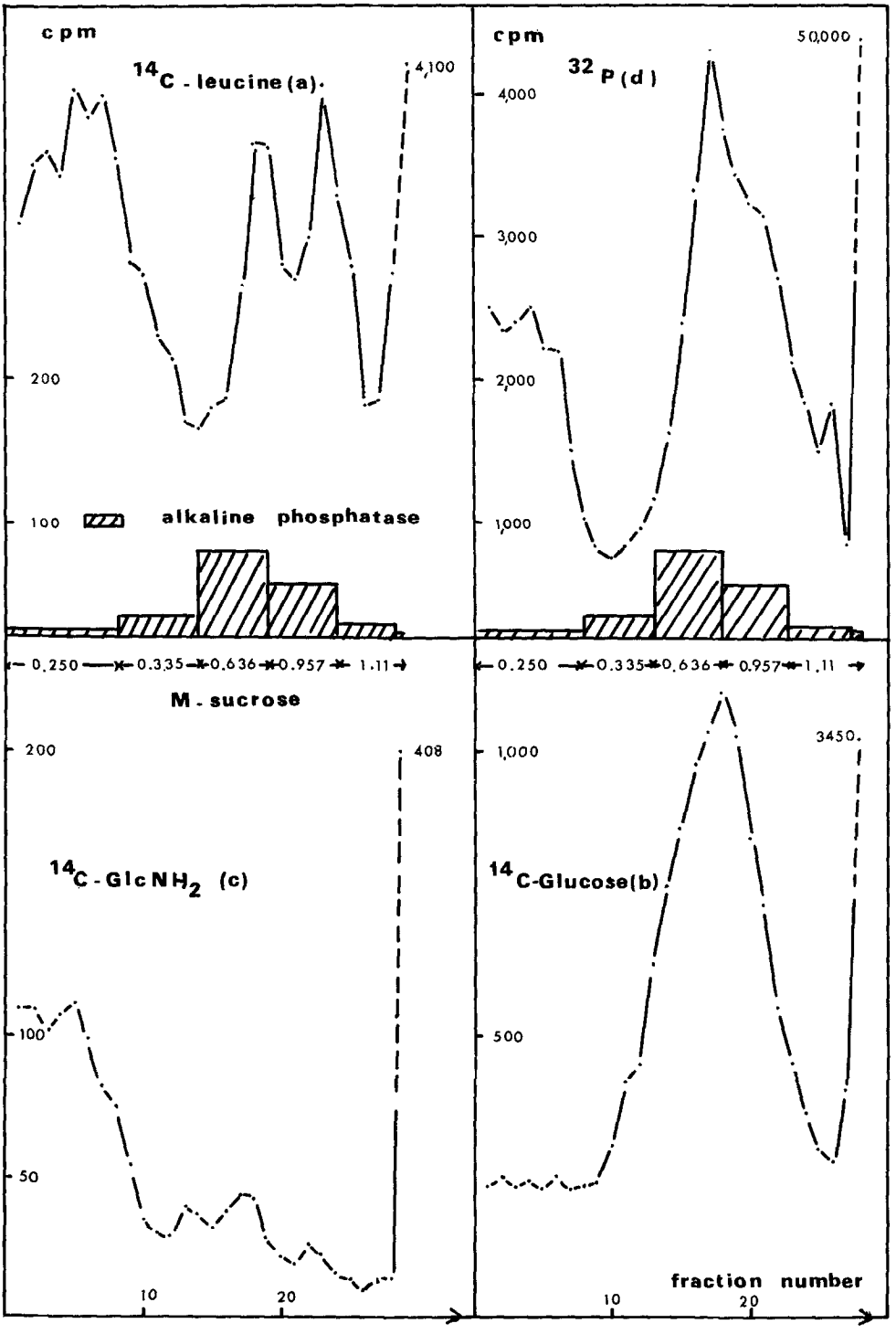

Fig.2 : Repartition of radioactivity on sucrose gradient

Table 2: Distribution of radioactivity of incubated OSG slices in smooth and rough membranes.(precursor: ^{14}C-glucose).

	"Smooth" membranes		"Rough" membranes	
	cpm	%	cpm	%
Total radioactivity	119,040		45,500	
Dialysable fraction*	50,040	42	4,500	10
Non dialysable fraction (OSM	2,800	2.3	7,500	17
Non dialysable fraction (Glycogen	28,200	23	33,500	74
Non dialysable fraction (unidentified**	35,800	30		

* After treatment with 1 % laurylsulfate.

** Not precipitated by TCA-phosphotungstic acid.

Table 3: Radioactivity incorporated into rough membranes of a cell-free system (OSG) from ^{14}C-leucine under varying conditions.

Experiment	System	cpm	%inhibition
A	Control	578	
	+ RNAse (500 µg)	242	58
B	Control	3,240	
	+ puromycine (500 µg)	955	70
	+ streptomycine (20 µg)	3,029	7

Supernatant protein: 2 mg, "rough" protein: 1,5 mg.

Table 4: Incorporation of radioactivity into total microsomes and rough membranes of a cell-free system (OSG) from ^{14}C-glucosamine.

	Supernatant	Total microsomes	Rough membranes
cpm/mg protein	16	68	32
% eliminated after 0.1 N KOH	0	84	68
% eliminated after 0.01 N HCl	0	62	35

of the various fractions with ^{14}C-glucose and ^{32}P-phosphate coincide almost completely.

A further step to identify the locus of glycoprotein biosynthesis consisted in fractionating the microsomes by the abovementioned method and incubating the

main fractions separately with the suitable precursor systems. Under these conditions, none of the smooth membrane fractions incorporates any radioactive aminoacid precursor, whereas the sediment displays a noticeable ability of biosynthesizing protein material. The extent of this synthesis shows a linear relationship with the amount of microsomes present and the biosynthesis is sensitive to the usual inhibitors of acellular protein biosynthesis in mammalian systems, RNAse and puromycine, whereas it is insensitive to streptomycine (Table 3). This latter fact and the sensitivity to RNAse suggest that the system may be devoid of bacterial contaminants which are one of the pitfalls one has to expect in such experiments. It has been previously shown (B. Rossignol et al., 1966) that the supernatant from OSG homogenates contains all the enzymes necessary to convert glucosamine to UDP-GalNAc, a direct precursor of the protein bound GalNAc (Mc Guire et al., 1967). Labelling of microsomal and rough membrane fractions with ^{14}C-glucosamine as precursor is appreciable. The radioactivity is firmly bound to the insoluble protein material, but may be almost totally split by moderate alkaline treatment (Table 4) under conditions where it has been shown that the diosides linked to the protein chain are cleaved through a β-elimination mechanism. Mild acid treatment removes part of this radioactive material, under conditions where the labelled neuraminic acid, accounting for approximately 50 % of the disaccharide material should be split.

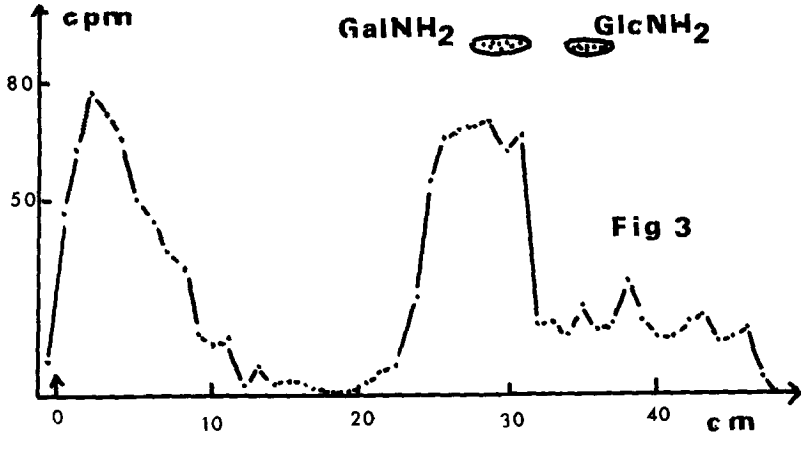

Fig. 3 : Chromatography of labelled sugars after hydrolysis of rough membranes incubated with ^{14}C-GluNH$_2$ as a precursor.

This result suggests that the radioactive compounds incorporated into rough membrane proteins with ^{14}C-glucosamine as precursor behave at first sight, as the authentic disaccharides of OSM. Finally it is shown that extensive hydrolysis (HCl 3 N 12 hours 100°) of the proteic material of rough membranes and identification through paper chromatography of the labelled compounds arising from ^{14}C-glucosamine, yielded a main radioactive peak which migrates like galactosamine (Figure 3).

Discussion.

The conclusions which may be drawn from the results outlined above are twofold: the first set concerns the site of incorporation of the first sugar linked to the protein chain viz. GalNAc. In the experiment performed with tissue slices, incorporationsof radioactivity from both labelled glucose and glucosamine are equally high in the sediment (rough membranes) whereas radioactivity incorporation from ^{14}C-glucosamine into the smooth membrane of zone 2 (Golgi bodies ?) is very low indeed. This would localize the incorporation site of GalNAc in the rough membranes and is strongly supported by the data observed with subcellular fraction incubation which show appreciable fixation of GalNAc from ^{14}C-glucosamine into the rough membranes. It may be added that the chromatographic identification of galactosamine as the major constituent of the incorporation rules out any aspecific contamination of the material with glucosamine, which normally is present in great excess in the incubation medium; it also rules out any definite bacterial growth which, if present, may result in incorporation of GluNAc into the bacterial cell wall.

The second set of conclusions concerns the biological specificity of the smooth membrane fraction isolated according to Schneider et al. (1953). It is demonstrated that this fractionation is not artifactual, as evidenced by the differential content of alkaline phosphatase and the differential incorporation rate of ^{14}C-glucose and ^{32}P. The very high rates observed with zone 2 (Golgi bodies) indicates a fraction of high turnover velocity and might contribute to explain earlier results obtained with histoautoradiographic methods and interpreted as showing specific incorporation of sugars into glycoproteins within the Golgi apparatus. Our results seem to indicate an alternative possibility: rapid renewal of phospholipids and other, hitherto unidentified, constituents of membrane material.

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