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Biosynthesis of Ovine Submaxillary Gland Mucoprotein in Surviving Submaxillary Gland Slices in the Presence of Various Substrates and Inhibitors*

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ABSTRACT: Surviving ovine submaxillary gland slices have been incubated in a Krebs–Ringer medium (1 mg of glucose/ml) in the presence of [U-^{14}C]glucose and [U-^{14}C]valine or [U-^{14}C]serine. A method has been devised for the isolation of the labeled mucoprotein "OSM." Radioactive glucose has been found to be incorporated in the glycosidic part of OSM (*N*-acetylgalactosamine and *N*-acetylneuraminic acid) and in the glycogen which has also been isolated from the gland tissue. Specific radioactivities of individual sugars have been determined. Labeled amino acids are incorporated into various protein fractions of the gland extract as well as in the protein moiety of OSM. It has been possible to estimate the quantity of the glycoprotein synthesized *de novo* during 3-hr incubation under physiological conditions; this amounts to an average of 1 mg/g of tissue and represents 5% of the actual amount of OSM found in the gland. In the absence of glucose in the incubation medium, degradation of glycogen takes place and its metabolic products may replace exogeneous glucose for the synthesis of the glycoprotein. Puromycin inhibits both the incorporation of sugars and amino acids into OSM but has little effect on the incorporation of glucose into glycogen. 2-Diazo-5-oxonorleucine (DON) which inhibits the glutamine fructose 6-phosphate transamidase reaction prevents the labeling of the hexosamine moiety of OSM from [^{14}C]glucose, and indirectly inhibits by 90% the synthesis of OSM as well from [^{14}C]glucose as from [^{14}C]valine. Addition of glucosamine to the incubation medium restores a normal glycoprotein synthesis. Despite the fact that in the presence of DON and in the absence of glucosamine, only 10% of OSM is synthesized from [^{14}C]serine, an increase in the labeling of other protein fractions is observed. It is suggested that this labeled material may be a nonglycosylated precursor of OSM. Incorporation into glycogen and OSM of [^{14}C]glucosamine and [^{14}C]galactose has also been studied and the results of a series of mutual dilution experiments with glucosamine, galactose, galactosamine, and *N*-acetylneuraminic acid are discussed.

Extensive studies on the structure of glycoprotein within recent years, resulted in the elucidation of the links between sugars and amino acids in several of these macromolecules as being either a β-aspartylglycosylamine linkage as in ovalbumin (Marshall and Neuberger, 1964; Bogdanov *et al.*, 1964) or an *O*-serylglycosidic linkage as in chondroitin sulfate (Anderson *et al.*, 1963) and in heparin (Lindahl and Roden, 1964) and *O*-seryl- and *O*-threonylglycosidic linkages as in submaxillary gland mucoprotein (Tanaka *et al.*, 1964; Harbon *et al.*, 1964; Carubelli *et al.*, 1965) and other mucins (Adams, 1965). These studies have been closely followed by investigations concerning the biosynthesis of these macromolecules either from amino acid precursors or from sugar precursors or from both (Richmond, 1963; Sarcione, 1963; Athineos *et al.*, 1964). Biosynthetic pathways for the formation of individual sugars and their activation to various nucleotide sugar compounds are well established and have been recently reviewed (Ginsburg, 1964). Their incorporation into some glycoproteins has been studied: this includes incorporation of [^{14}C]glucosamine into serum glycoproteins (Robinson *et al.*, 1964; Shetlar *et al.*, 1964) and submicrosomal fractions of the liver (Sarcione *et al.*, 1964; Molnar *et al.*, 1965; Helgeland, 1965) and of [^{14}C]galactose into serum glycoproteins (Sarcione, 1964; McGuire *et al.*, 1965). The occurrence of an enzyme catalyzing the transfer of *N*-acetylneuraminic acid[1] (NANA) residues from cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA) into glycoproteins has been described in bovine submaxillary gland extract (Carlson *et al.*, 1964) and in liver subcellular fractions (O'Brien *et al.*, 1966). Recently, studies on the biosynthesis of chondroitin sulfate–protein complex (Telser *et al.*, 1965) and of thyroglobulin (Spiro and Spiro, 1966) raise several interesting problems concerning the stepwise addition of sugars to polypeptide chains and the possible spatial separation of the loci

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[1] Abbreviations used: OSM, ovine submaxillary gland mucoprotein; NANA, *N*-acetylneuraminic acid; GalNac, *N*-acetylgalactosamine; CMP-NANA, cytidine monophospho-*N*-acetylneuraminic acid; UDP-G, uridine diphosphoglucose; DON, 2-diazo-5-oxonorleucine.

where biosynthesis of either the peptidic moiety or the glycosidic moiety of the glycoprotein takes place. However the precise mechanism whereby the first sugar of the polysaccharidic chain is directly linked to the peptide chain remains presently unknown.

Submaxillary glands seem to provide an interesting material for the study of glycoprotein biosynthesis. The reasons for this are: first, that the links between the sugar and polypeptidic parts of the submaxillary mucoprotein are well established (Tanaka *et al.*, 1964; Harbon *et al.*, 1964; Carubelli *et al.*, 1965); second, that the level of the glycoprotein present in the gland is unusually high, suggesting the occurrence of a very active biosynthesis which has been recently spotted by the demonstration that extracts of the gland actively catalyze the conversion of glucosamine to UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine (Rossignol *et al.*, 1966); third, that the osidic moiety of the submaxillary glycoprotein consists of very small chains of simple structure (Graham and Gottschalk, 1960); and fourth, that the considerable acidity of this glycoprotein due to the presence of more than 25% of NANA facilitates the isolation and separation of the specific submaxillary mucoprotein from the remaining protein material. The present work which has been performed on ovine submaxillary glands, aims to study several aspects of the biosynthesis of OSM (ovine submaxillary mucoprotein) in sliced tissue, in other words, in cells as intact as possible. The first part of the experiments described below concerns the determination of the rate of synthesis of OSM under conditions as close to physiological requirements as possible, and the effect of specific inhibitors on protein or hexosamine biosynthesis. The second part concerns the mutual interferences of possible precursors of the sugar moiety and aims to investigate the enzymatic equipment which enables the submaxillary gland to synthesize the specific glycosidic moiety of OSM from glucose.

Experimental Procedure

Material. The following labeled substrates[2] were used: D-[U-^{14}C]glucose (40 mc/mM), L-[U-^{14}C]valine (60 mc/mM), L-[U-^{14}C]serine (36.6 mc/mM) (C. E. A., Saclay, France); D-[1-^{14}C]galactose (4.0 mc/mM), D-[1-^{14}C]glucosamine–HCl (3.75 mc/mM) (The Radiochemical Center, Amersham, England). Appropriate chromatographic controls of purity were performed on these substrates. The corresponding unlabeled substrates were: D-glucose, D-glucosamine–HCl, D-galactose (Hoffman-LaRoche and Cie., Basel, Switzerland); D-galactosamine–HCl, *N*-acetylneuraminic acid (Calbiochem, Lucerne, Switzerland). Inhibitors used were: puromycin dihydrochloride (Nutritional Biochemicals Corp., Cleveland); actinomycin-D (Merck Sharp and Dohme, West Point, Pa.); 2-diazo-5-oxonorleucine (DON), through the courtesy of Dr. J. R. Dice, (Park Davis and Co., Ann Arbor, Mich.). DEAE-Sephadex was purchased from Pharmacia Uppsala, Sweden. All chemical reagents used throughout this work were of analytical grade. Glass-bidistilled water has been used for the incubation procedures. Radioactivity was determined with a Nuclear-Chicago gas-flow detector; measurements reported are not corrected for efficiency and are expressed as counts per minute (cpm).

Analytical Methods. The following analytical methods have been used: glucose through glucose oxidase (Huggett and Nixon, 1957), hexosamines by the procedure of Elson and Morgan as modified by Boas (1953), free and bound sialic acid by the procedure of Warren as modified by Aminoff (1961), and Bial's orcinol reaction according to Böhm and Baumeister (1955). Proteins were determined by the Folin reaction according to Lowry *et al.* (1951); chromatographic separation of amino acids was occasionally performed by the automatic procedure of Spackman *et al.* (1958). Chromatographic separations of sugars in order to determine their specific radioactivities have been performed in the following way: glucose, after acid hydrolysis of glycogen (HCl, 2 N, 2 hr at 100°), on Schleicher and Schüll 2043b paper; solvent, isoamyl alcohol–pyridine–0.1 N HCl (2:2:1); elution from paper is carried out with H_2O and the specific activity of glucose is determined on the eluate; galactosamine, after hydrolysis of the glycoprotein fraction (HCl, 6 N, 16 hr at 100°) by the same procedure; NANA, after hydrolysis of the glycoprotein by 0.1 N HCl, 50 min at 80°, following adsorption on a Dowex 2-X8 resin (formate form) and elution with 0.3 M formic acid. It has been checked by subsequent paper chromatography with the above mentioned system as well as with the butanol–acetic acid–H_2O (4:1:5) system that the radioactivity of the formic acid eluate of the Dowex column was due to NANA only.

Animals. Submaxillary glands[3] were obtained from sheep of either sex and an age ranging from 5 to 18 months. The glands were kept in a chilled vessel (0–4°) without any buffer added, sliced at 0° with a razor blade between 20 and 60 min after the animals have been killed, and immediately transferred into the incubation medium. The average thickness of the slices was 200–300 μ.

Incubation Procedures. The buffer used was the usual Krebs–Henseleit bicarbonate medium (gas phase 95% O_2–5% CO_2; Umbreit *et al.*, 1964). Incubations were performed for the varying periods indicated below. A single assay consists usually of 700–1000 mg of slices (wet weight) in 8 ml of buffer. At the end of the incubation the slices are withdrawn from the medium, blotted, weighed, immediately homogenized with a 10-fold amount of cold water (0°) in a Virtis 45 homogenizer, and centrifuged (30 min at 13,800*g*).

[2] Purchase of labeled substrates has been supported in part by C. E. A.-Saclay, France (partial subvention N° 05952, October 6, 1965).

[3] We are indebted to Mr. Jame (C. N. R. Z., Jouy en Josas, France) for the glands used in this work.

Extraction of OSM is complete under these conditions. As far as glycogen is concerned the yields obtained with this method average 50–75% of the quantities extracted by the usual hot KOH method. Therefore when a quantitative assay of the glycogen contents of the glands was estimated necessary, the latter method has been employed.

Fractionation of the Extracts. The extracts are dialyzed against a 100-fold amount of distilled water which is changed four to five times. After lyophilization, the material is extracted with 8 ml of pyridine–acetic acid buffer, 0.075 M at pH 5.0, and centrifuged. With this procedure, extractions of OSM and glycogen are nearly complete whereas 50% of the protein material remains insoluble. Finally the soluble material, adjusted to pH 6.5 with aqueous pyridine, is fractionated on a DEAE-Sephadex A-50 column (1 × 16 cm). DEAE-Sephadex has been previously prepared according to Schmidt (1962) and equilibrated with the pyridine–acetic acid buffer 0.075 M at pH 6.5. The column is washed with 10–15 ml of the same buffer and elution proceeds by increasing stepwise the ionic strength of the pyridine–acetic acid buffer by the addition of NaCl up to a final concentration of 0.1–0.2 and 0.3 M. Four peaks are thus obtained (Figure 1), the first of which emerging with the holdup volume of the column contains polysaccharide and some protein material. When incubation is performed with [^{14}C]glucose, the sole radioactive material found in peak I seems to be due to a polyglycan which on hydrolysis yields radioactive glucose quantitatively. Moreover, the specific activity of the radioactive glucose from this material determined as described above is identical with the specific radioactivity of glycogen after extraction by the classical alcaline extraction method. Hence it seems reasonable to conclude that the polysaccharidic material present in peak I is due to glycogen only. Peaks II and III seem to contain protein material devoid of any carbohydrate. Protein-bound NANA and *N*-acetylgalactosamine (GalNac) are detected in significant amount only in peak IV (eluted with 0.3 M NaCl) indicating that OSM is present in this fraction only. It must be noted that whereas the recovery of glycogen from the column is usually quantitative, some of the OSM seems to be irreversibly adsorbed as evidenced by the loss of an identical percentage of NANA, GalNac, and radioactive material. Usually the yields of OSM obtained varied between 50 and 75%. This value is currently corrected by estimating the total loss of NANA during the chromatographic procedure. Peak IV is dialyzed, lyophilized, and sometimes submitted to additional electrophoresis on pevikon as described in a previous publication (Harbon *et al.*, 1963). By this electrophoretic procedure, as well as by the estimation of amino acid and sugar contents, it appeared that peak IV consists of 80–90% pure ovine submaxillary mucoprotein.

Estimation of Specific Radioactivities. The specific activities of glycogen have first been estimated on the purified glucose which has been separated from the acid hydrolysate of peak I by paper chromatography

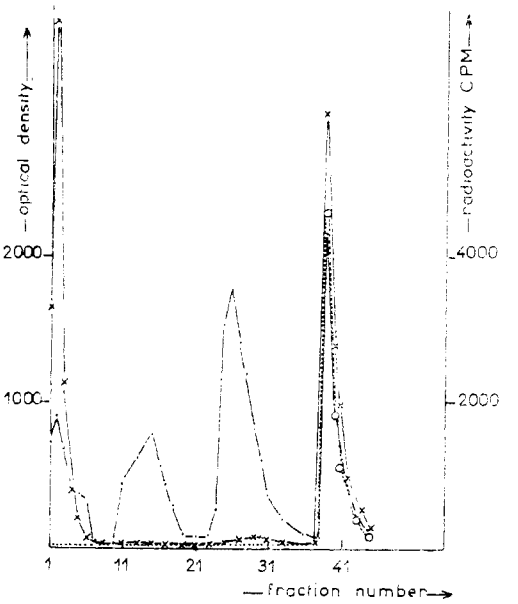

FIGURE 1: Elution pattern of labeled submaxillary gland extract from DEAE-Sephadex column. The submaxillary gland slices have been incubated during 3 hr at 37° in the Krebs–Ringer bicarbonate buffer containing [U-^{14}C]glucose (1 mg/ml). For details, see Experimental Procedure. ●——●, folin reaction, o.d. 640 mμ. O-----O, Bial's reaction, o.d. 570 mμ. ×——×, radioactivity (counts per minute).

as outlined above. However, the specific activities determined by this method proved to be identical with the specific activities calculated by dividing the total radioactivity of material from peak I by the amount of glucose as estimated enzymatically after acid hydrolysis. Thus in subsequent experiments chromatographic purification of glucose has frequently been omitted. It has already been mentioned that the specific activity of glycogen as calculated from the material present in peak I is identical with the specific activity obtained when glycogen is extracted by the hot KOH method.

The specific radioactivities of NANA and GalNac are determined on the material from peak IV after the selective hydrolysis and purification procedures mentioned above. In the case where the radioactive precursors are glucose, galactose, or glucosamine, the labeling of the protein moiety of OSM is negligible and the contribution of radioactivity from the small protein contaminant of this peak, which contains no sugar, may be neglected. On the contrary, when the radioactive precursor is an amino acid ([^{14}C]valine or [^{14}C]serine), the glycosidic moiety of OSM is unlabeled, but the protein contaminant contributes to the total radioactivity of peak IV in a way which cannot be neglected: in this case an electrophoretic purification of this material must be performed prior to the determination of specific radioactivities.

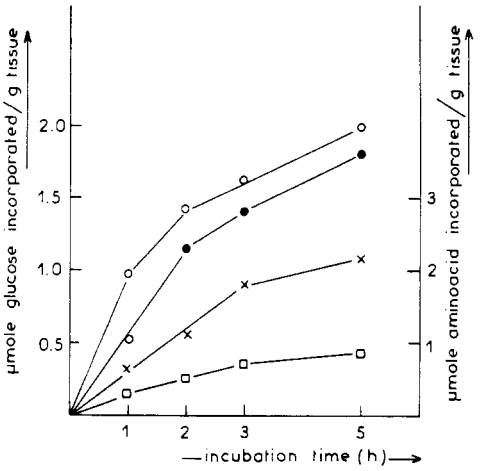

FIGURE 2: Kinetics of incorporation of [^{14}C]glucose into glycogen and OSM, and of [^{14}C]valine and [^{14}C]serine into OSM. O——O, micromoles of glucose incorporated into glycogen; ●——●, micromoles of glucose incorporated into OSM; □——□, micromoles of valine incorporated into OSM; ×——×, micromoles of serine incorporated into OSM.

It has been assumed throughout this work that the specific activities of *N*-acetylgalactosamine and of the *N*-acetylmannosamine moiety of NANA are identical. This assumption seems justified by the fact that in the presence of trace amount of labeled glucosamine and substrate quantity of unlabeled glucose (the latter compound diluting the radioactivity of the pyruvate moiety of NANA, which becomes negligible), the molar specific activities of GalNac and of NANA are identical. Conversely, in the presence of trace amount of labeled glucose and substrate quantity of unlabeled glucosamine, it will be shown that solely NANA is labeled, which seems to indicate that the labeling in this case derives from the pyruvate moiety. In addition, the relative labeling of pyruvate and *N*-acetylmannosamine, calculated according to this assumption, coincides exactly with the relative values determined by Eichberg and Karnovsky (1963) through the enzymatic cleavage of NANA by NANA-aldolase. Thus, it may be estimated that the separate determination of specific radioactivities of glucose, GalNac, and NANA provides a mean for the separate calculations of the specific radioactivities of the pools of glucose, pyruvate, and *N*-acetylhexosamines in the cell.

Results

Kinetics of Incorporation of Glucose and Amino Acids into OSM and of Glucose into Glycogen. The total amount of OSM present in the gland at the beginning of the incubation time may be estimated from the amount of nondialyzable bound *N*-acetylneuraminic acid to be 30 mg/g of fresh gland; during incubation this quantity drops to a value of 25 mg/g of fresh tissue, indicating excretion of the glycoprotein into the incubation medium (Eichberg and Karnovsky, 1963). The corresponding average values for glycogen are 720 μg/g of fresh gland at the beginning of the incubation and 1000 μg/g of fresh tissue at the end of the incubation. These glycogen values are about four to five times lower than the contents found in striated muscle and 10–12 times lower than the contents in hepatic tissue. Figure 2 shows that the incorporation of [^{14}C]glucose, [^{14}C]serine, and [^{14}C]valine into OSM follows an almost linear course between 0- and 2-hr incubation time and slows down between 3- and 5-hr incubation. The incorporation of [^{14}C]glucose into glycogen slows down very markedly after 1-hr incubation. These experiments essentially show that the surviving glands withold their ability of synthesizing glycoprotein during a reasonable amount of time, when incubated.

Influence of the Concentration of Glucose in the Incubation Medium on the Biosynthesis of OSM. The total amount of OSM synthesized by sliced submaxillary glands after 3-hr incubation in the presence of physiological concentrations of glucose (1 mg/ml) has been calculated in two different ways: either in the presence of radioactive glucose, considering that the phosphoenolpyruvate incorporated into sialic acid is not diluted by metabolites derived from glycogen (the total amount of which seems to remain stable under these conditions) and that the specific radioactivity of the pyruvate is thus in equilibrium with the specific radioactivity of the added glucose. The quantity of OSM synthesized has been calculated from 10 different experiments and amounts to 1030 ± 92 μg/g of fresh gland, which corresponds to 4% of the quantity of OSM found in the gland; or by estimating the amount of incorporated [^{14}C]valine into the protein moiety of OSM, the intracellular pool of this amino acid in the gland being taken as 0.5 μmole/g of fresh tissue (B. Rossignol and C. Gros, 1966, personal communication). In this case, the value found is 450 μg of OSM/g of fresh tissue, almost two times less than the amount calculated from the incorporation of [^{14}C]glucose. It must however be emphasized that this value is certainly underestimated, no account being taken for the kinetics of penetration of valine into the intracellular space which have not yet been determined. Hence, it seems reasonable to consider the basic assumption of isotopic equilibrium between phosphoenolpyruvate and extracellular glucose as a valid working hypothesis.

Table I shows the amount of glycoprotein synthesized when the extracellular glucose concentration is varied. In this table the total amount of OSM synthesized in the presence of 1 mg of glucose/ml (calculated from incorporation of [^{14}C]glucose) has been arbitrarily taken as 100% OSM biosynthesized. Under these conditions, the amounts of glycoprotein synthesized as estimated either from glucose incorporated into the sugar moiety or from valine incorporated into the protein moiety seem to be roughly equal if the extracellular glucose concentration ranges between 100 and 2000 μg/ml, whereas increasing discrepancies appear when

TABLE I: Influence of the Concentration of Glucose in the Incubation Medium on the Biosynthesis of OSM (after 3-hr Incubation).

Glucose Present in the Incubn Medium (μg/ml)	Total Incorp from [^{14}C]Glucose into OSM Diosides (cpm/g of tissue)	OSM Synthesized from Incorp of [^{14}C] Glucose (μg/g of tissue)	%	Total Incorp from [^{14}C]-Valine into the Protein Moiety of OSM (cpm/g of tissue)	%	OSM Synthesized from Incorp of [^{14}C]-Valine[a] (μg/g of tissue)	Glycogen Present at the End of Incubn (μg/g of tissue)
1	258,000	68	6	78,000	59	610	180
50	172,000	228	22	61,500	46.5	482	
100	215,000	632	61	66,000	50	510	
500	52,000	755	73	113,000	86	890	
1,000	35,500	1,030	100	132,000	100	1,030	800
2,000	15,700	935	90	145,000	110	1,130	

[a] The quantity of OSM synthesized under physiological conditions (1 mg of glucose/ml) has been calculated from [^{14}C]glucose data and it was estimated that this quantity (1030 μg) corresponds to 100% incorporation of [^{14}C]glucose and [^{14}C]valine.

TABLE II: Effect of Puromycin on the Incorporation of [^{14}C]Glucose into Glycogen and OSM and on the Incorporation of [^{14}C]Valine into OSM.[a]

Labeled Substrate	[^{14}C]Glucose (150,000 cpm/ml)		[^{14}C]Valine (135,000 cpm/ml)	
Puromycin	−	+	−	+
Total Incorp into Glycogen (cpm/g of tissue)	40,000	28,000	−	−
Total Incorp into OSM (cpm/g of tissue)	40,000	1,000	120,000	2,000

[a] Each flask contained about 800 mg of submaxillary gland slices in 8 ml of Krebs–Ringer bicarbonate buffer (1 mg of glucose/ml). Puromycin was added to a final concentration of 500 μg/ml. After 30-min preincubation with or without the inhibitor, [^{14}C]glucose and [^{14}C]-valine were added to the appropriate flask, and incubation was continued for 3 hr.

TABLE III: Total Radioactivity Incorporated into OSM (cpm/g of Gland) from [^{14}C]Serine, for Varying Periods of Incubation in the Presence or Absence of Actinomycin D.[a]

Incubn Time (hr)	1	2	3	5
− Actinomycin	68,000	116,000	165,000	216,000
+ Actinomycin	51,500	95,500	187,000	160,000

[a] Each flask contained about 800 mg of submaxillary gland slices in 8 ml of Krebs–Ringer bicarbonate buffer (1 mg of glucose/ml). Preincubation with or without the inhibitor (100 μg/ml) has been performed for 20 min before the addition of [^{14}C]serine (135,000 cpm/ml) followed by varied incubation periods.

the glucose concentration decreases below 100 μg/ml. In the latter case, the amount calculated from the data with [^{14}C]valine is considerably higher than the amount calculated from [^{14}C]glucose. This discrepancy seems to be due to an increasing dilution of exogenous glucose by endogenous substrates derived from glycogen, an interpretation which is confirmed by the fact that glycogen is degraded under these conditions. It is concluded that below an extracellular concentration of 100 μg of glucose/ml, the quantities of glycoprotein synthesized must be taken from valine data, the glucose data being systematically too low. It appears that even in the almost complete absence of extracellular glucose, the surviving gland still synthesizes about 50% of the OSM which is synthesized at optimal concentrations of glucose, and that the synthesis proceeds at the expanse of intracellular glycogen.

Inhibition by Puromycin and Actinomycin D. PUROMYCIN. Table II shows that puromycin inhibits completely the incorporation of either [^{14}C]valine or [^{14}C]glucose

TABLE IV: Effect of DON on the Incorporation of Different Labeled Precursors into OSM and Glycogen.[a]

Addn to the Incubn Medium Krebs–Ringer Bicarbonate	Radioactivity in Glycogen (cpm/g of tissue)		Radioactivity in OSM (cpm/g of tissue)		Sp Radioactivity of Hexosamine (cpm/μmole)		Sp Radioactivity of Pyruvate (cpm/μmole)	
DON (20 μg/ml)	−	+	−	+	−	+	−	+
Glucose (1 mg/ml) + [^{14}C]Glucose	26,000	30,000	40,000	1,140	700	0	740	65
Glucose (1 mg/ml) + [^{14}C]Valine			112,000	15,000				
Glucosamine (1 mg/ml) + [^{14}C]-Glucose	41,600	40,000	48,600	42,000	200	40	2,030	1,990
Glucosamine (1 mg/ml) + [^{14}C]-Glucosamine	8,100	11,000	48,500	55,000	1,070	1,200	280	320
Glucosamine (1 mg/ml) + [^{14}C]-Valine			112,000	140,000				

[a] About 800 mg of sliced submaxillary gland were suspended in 8 ml of Krebs–Ringer bicarbonate buffer containing glucose (1 mg/ml) or glucosamine (1 mg/ml). DON was added to a final concentration of 20 μg/ml. After 15-min preincubation with the inhibitor, radioactive precursors were added to the appropriate flask and incubation was continued for 3 hr at 37° (gas O_2 95%-CO_2 5%).

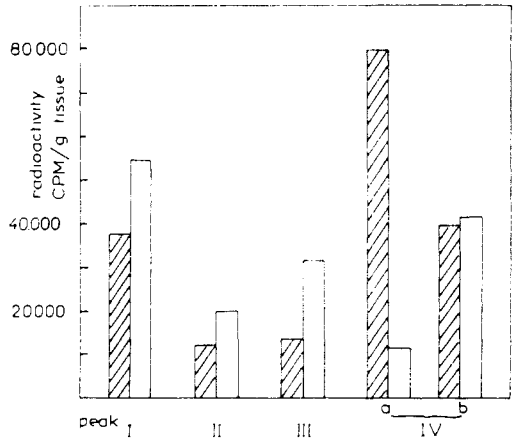

FIGURE 3: Relative labeling of various protein fractions of submaxillary gland extract after incubation with [^{14}C]serine in the presence or absence of DON. Diagonal -line bars, without DON; open bars, with DON. a, OSM; b. OSM contaminant.

into the OSM fraction of surviving submaxillary glands. As could be expected, the inhibition of incorporation of [^{14}C]valine is also complete as far as other protein fractions synthesized by the gland are concerned. With [^{14}C]glucose, however the incorporation into material from peak I, mainly consisting of glycogen, is only decreased by about 30%, which confirms that this material is of polysaccharidic nature and that its synthesis does not depend on the preliminary biosynthesis of any protein material.

ACTINOMYCIN D. Table III shows the total radioactivity incorporated during various incubation times from [^{14}C]serine into the glycoprotein fraction, in the presence or absence of actinomycin D. Although there seems to be in some cases a small inhibition of incorporation by actinomycin, this inhibition does not increase with the incubation time, a phenomenon which has in certain cases been used in order to calculate the half-life of messenger ribonucleic acid (m-RNA). It is possible that the incubation time used in the present work has not been extended enough. In any case, the m-RNA of the secreted glycoprotein does not seem to be characterized by a peculiar instability, a hypothesis which has been favoured by Davidson *et al.* (1962) concerning RNA of acid mucopolysaccharide material (AMPS) secreted by cultured ARE 2-60 cells.

Inhibition of Hexosamine Biosynthesis by 2-Diazo-5-oxonorleucine (DON). DON is known to inhibit specifically the L-glutamine D-fructose 6-phosphate transamidase (Ghosh *et al.*, 1960); thus under our experimental conditions, this inhibition may be expected to counteract the biosynthesis of both GalNac and NANA either from extracellular glucose or from intracellular substrate like glycogen. Table IV shows this to be indeed the case, the amount of protein synthesized in the presence of DON, either from [^{14}C]valine or from [^{14}C]glucose, dropping to values around 10% of the quantity of glycoprotein synthesized in the absence of the inhibitor. It must be added that the radioactivity incorporated from labeled glucose into galactosamine is naught, the only radioactivity incorporated into the glycosidic part of the glycoprotein being present in sialic acid. However the total radioactivity incorporated into the extract from amino acid precursors is by no means diminished in the presence of the inhibitor, but an increase in the radioactive

TABLE V: Uptake of Glucose, Glucosamine, and Galactose by Surviving Submaxillary Gland Slices.[a]

Unlabeled Substrate Present	Labeled Substrate Present (1,000,000 cpm)		
	[^{14}C]Glucose (1 μg/ml)	[^{14}C]Glucosamine (20 μg/ml)	[^{14}C]Galactose (30 μg/ml)
None	390,000	166,000	355,000
Glucose (1 mg/ml)	200,000	185,000	286,000
Glucosamine (1 mg/ml)	302,000	190,000	
Galactose (1 mg/ml)	370,000		165,000

[a] Estimated by the total incorporation into submaxillary gland extract after 3-hr incubation (counts per minute per gram of tissue).

material in other peaks which do not correspond to glycoprotein material seems to compensate the striking decrease of radioactivity in peak IV (Figure 3). When glucosamine (1 mg/ml) is added to the incubation medium, normal rates of synthesis of the glycoprotein are restored as evidenced by the normal incorporation rate of labeled valine into OSM under these conditions. Conversely, if the biosynthesis of glycogen from [^{14}C]glucosamine is estimated under the same experimental conditions in the presence of DON, no inhibition of incorporation of the radioactivity into the polysaccharide is observed. Despite the fact that some labeled glucosamine is found in the glycogen fraction after hydrolysis, which agrees with recent findings of Maley *et al.* (1966), at least 80% of the radioactivity incorporated into glycogen is recovered as glucosyl residues. This indicates that conversion of glucosamine to glucose actually takes place in the gland through a desaminating reaction (Comb and Roseman, 1958) which is not inhibited by DON.

Sequential Inhibition of OSM Biosynthesis by DON and Puromycin. As will be discussed below, accumulation of labeled material from radioactive amino acid precursors in peaks I and III in the presence of DON may suggest a precursor–product relationship between the proteins from these peaks and the final glycoprotein material. To test this possibility, about 800 mg of submaxillary gland slices have been suspended in 8 ml of Krebs–Ringer bicarbonate buffer (glucose, 1 mg/ml). A preincubation in the presence or absence of DON (20 μg/ml) has been performed for 2 hr at 37°; the slices were then withdrawn from each flask, gently blotted, and transferred to a corresponding flask containing 8 ml of Krebs–Ringer bicarbonate buffer and glucosamine in a final concentration of 1 mg/ml. After a second preincubation with or without puromycin (500 μg/ml), [^{14}C]glucosamine has been added and incubation was continued for 3 hr. Under these conditions, the amounts of radioactivity incorporated into the glycoprotein material were as follows: preincubation without DON and puromycin, 8100 cpm/g of tissue; with DON and without puromycin, 10,000 cpm; without DON and with puromycin, 260 cpm; and with both inhibitors, 320 cpm. The results of these experiments essentially show that the protein material which accumulates in the presence of the transamidation inhibitor cannot quantitatively serve as a precursor for glycosylation once the inhibition has been bypassed by adding hexosamine. Implications of these results will be discussed below.

Mutual Dilution Experiments of Radioactive Precursors of the Disaccharides. GLUCOSE–GLUCOSAMINE. Table V shows the uptake of glucose and glucosamine present either separately or together in the incubation medium; while glucose uptake exhibits a saturation phenomenon which indicates active transport, the penetration of glucosamine is proportional to the amount of substrate added to the incubation medium. This seems to indicate either passive transport or saturation at glucosamine levels above the concentration used. In any case, the two penetrations seem to be mutually independent and competition at this level may be neglected in the further evaluation of the results obtained. Table VI shows the results of dilution experiments with labeled and unlabeled glucose as well as labeled and unlabeled glucosamine. It has already been mentioned that in the presence of labeled glucose (1 mg/ml) the radioactivities of the hexosamine and pyruvate moieties of the disaccharides seem to derive from the radioactive precursor without much dilution from endogeneous substrate. The same seems to be the case when the experiments are performed in the presence of labeled glucosamine (1 mg/ml) as the sole substrate. As may be expected, incubation in the presence of trace amount of labeled glucose and large quantities of unlabeled glucosamine (column 5) results in a preferential labeling of the pyruvate moiety of NANA whereas the hexosamine moiety of NANA and GalNac are only slightly radioactive. The opposite is true when large quantities of unlabeled glucose dilute small quantities of labeled glucosamine (column 4). Beside these expected results, the main conclusions which can be drawn from these experiments, concern a regulatory phenomenon at the level of the biosynthesis of hexosamines from glucose, as evidenced by the difference in the dilution factor of a trace amount of [^{14}C]-glucose by either 1 mg of unlabeled glucose or 1 mg of unlabeled glucosamine (columns 3–5). In the first

TABLE VI: Mutual Dilution of Glucose and Glucosamine in Surviving Slices of Ovine Submaxillary Gland.[a]

Unlabeled Substrate Present (1 mg/ml)	None (1)	None (2)	Glucose (3)	Glucose (4)	Glucosamine (5)	Glucosamine (6)
Labeled Substrate Present (135,000 cpm/ml)	[^{14}C]Glucose (1 μg/ml)	[^{14}C]-Glucosamine (20 μg/ml)	[^{14}C]Glucose	[^{14}C]Glucosamine	[^{14}C]-Glucose	[^{14}C]Glucosamine
Amt of Glycogen (Peak I) (μmoles of glucose/g of tissue)	0.93	0.58	3.0	3.2	2.85	3.02
Total Incorp into Glycogen (cpm/g of tissue)	19,000	6,600	20,500	4,660	41,600	6,400
Total Incorp into OSM (cpm/g of tissue)	320,000	213,000	42,000	57,000	48,600	48,500
Hexosamine, Sp Act. (cpm/μmoles)	6,200	3,900	700	1,240	200	1,070
Pyruvate, Sp Act. (cpm/μmoles)	3,600	2,820	740	350	2,030	280

[a] Mean values calculated from three different experiments.

case, incorporation of labeled glucose into hexosamines is considerably higher than in the second case, which suggests an active inhibition of the conversion of glucose to glucosamine in the presence of high quantities of amino sugars. Such an inhibition may be caused by the accumulation of UDP-*N*-acetylhexosamines which inhibit the L-glutamine D-fructose 6-phosphate transamidase (Kornfeld *et al.*, 1964). The labeling of glycogen in the presence of glucose, glucosamine or both, indicates the existence of several precursor–products relationships which are irrelevant to the purpose of the present work; it can be seen (columns 3–6) that in the presence of either [^{14}C]glucose or [^{14}C]glucosamine of the same specific activities, approximately three-times less glycogen is synthesized from glucosamine than from glucose, and if this factor is applied to column 5 where only a trace amount of labeled glucose is present with large quantities of unlabeled glucosamine, it appears that glucosamine dilutes glucose for the biosynthesis of glycogen 8–10 times less than does unlabeled glucose. This result can only be explained by the fact that in the presence of large amounts of glucosamine and in the virtual absence of glucose, the precursor pool for the biosynthesis of glycogen (*viz.*, UDP-glucose) is 8–10 times smaller than in the presence of physiological quantities of glucose. However, the presence of glucosamine definitely counteracts glycogen breakdown which normally occurs in the absence of glucose; this is evidenced by the fact that the glycogen level in the gland remains constant when incubation is performed in the presence of glucosamine. The fact that conversion of glucose to glucosamine and conversion of glucosamine to glucose do not proceed through the same enzymatic mechanism has already been mentioned.

GLUCOSE–GALACTOSE. The initial aim of these experiments was to investigate if no direct conversion of galactose to galactosamine could occur, although such a pathway has to our knowledge never been described. It can be seen from Table V that the transport of glucose and galactose exhibits identical saturation kinetics which seem to be mutually independent. This result coincides with experiments of Battaglia and Randle (1960) who found two separate systems for galactose and glucose transports in the muscle. Both systems being independent, no account has to be taken for competition between the two substrates at the transport level. Table VII shows the results of dilution experiments with glucose and galactose. It appears that the presence of galactose prevents the degradation of glycogen (columns 1–5); the radioactivity incorporated into the glycogen fraction from [^{14}C]galactose as evidenced by hydrolysis and chromatographic analyses, consists only of radioactive glucose. As far as the pattern of labeling itself is concerned, the following comments can be made. The total biosynthesis of glycogen from galactose is three times slower than from glucose (columns 3–6). When this correction factor is applied to column 5, it appears that the synthesis of glycogen in the presence of trace amount of glucose and substrate quantities of galactose proceeds from a pool of UDP-glucose (UDP-G) which is 10 times more labeled than the pool in the presence of substrate quantities of labeled glucose. This indicates that the enzymatic system which synthesizes UDP-G from galactose has an over-all maximum activity which is 10 times lower than that which synthesizes UDP-G from glucose. When this type of calculation is applied to OSM, it also appears that galactose dilutes trace amount of labeled glucose 10 times less than does the same amount of unlabeled glucose; thus there is no discrepancy between the dilution factors in glycogen and OSM when the experiment is performed in the presence of large quantities of galactose and in the virtual absence of glucose. On the contrary, if the experiments are performed in the

TABLE VII: Mutual Dilution of Glucose and Galactose in Surviving Slices of Ovine Submaxillary Gland.[a]

Unlabeled Substrate Present (1 mg/ml)	None (1)	None (2)	Glucose (3)	Glucose (4)	Galactose (5)	Galactose (6)
Labeled Substrate Present (135,000 cpm/ml)	[^{14}C]-Glucose (1 μg/ml)	[^{14}C]-Galactose (30 μg/ml)	[^{14}C]-Glucose	[^{14}C]-Galactose	[^{14}C]-Glucose	[^{14}C]-Galactose
Amt of Glycogen (Peak I) (μmoles of glucose)	1.7	1.44	4.4	3.3	4.56	4.0
Total Incorp into Glycogen (cpm/g of tissue)	44,000	51,000	40,000	76,000	115,000	14,000
Total Incorp into OSM (cpm/g of tissue)	260,000	91,000	25,000	10,500	120,000	12,500
Hexosamine, Sp Act. (cpm/μmole)	4,700	1,700	420	170	1,980	215
Pyruvate, Sp Act. (cpm/μmole)	3,600	1,300	390	205	2,190	205

[a] Mean values calculated from three different experiments.

presence of trace amount of labeled galactose and large quantities of unlabeled glucose (column 4), galactose is preferentially used for the biosynthesis of glycogen, and its incorporation into the glycoprotein occurs to a small extent only. The discrepancy between both series of results cannot to our knowledge, be explained solely by a compartmentation on the cellular or subcellular level. It seems that a regulation phenomenon must be involved, a model of which may be found in the well known activation of glycogen synthetase by glucose 6-phosphate (Traut and Lipmann, 1963). Such a hypothesis would account for the fact that in the first case (large amounts of galactose and the virtual absence of glucose), incorporation of radioactivity into glycogen does not seem to proceed preferentially to its incorporation into OSM, whereas in the second case (large amounts of glucose and trace amount of galactose), biosynthesis of glycogen seems to act as a galactose trap, preventing the radioactivity from galactose to be incorporated into OSM. However, it must be emphasized that this explanation, at the present stage, is only a tentative one.

DILUTION BY GALACTOSAMINE. No influence whatsoever of added galactosamine on the patterns of glycogen or OSM labeling with [^{14}C]glucose could be detected. It has not been investigated if this lack of interference is due to an absence of penetration, an absence of galactosamine phosphorylation or an absence of interference between the metabolism of galactosamine and the biosynthetic pathways studied. It is noteworthy that galactosamine does not prevent the breakdown of glycogen as do glucosamine and galactose.

DILUTION BY NANA. Dilution experiments performed with unlabeled NANA (1 mg/ml) in the presence of labeled glucose show a 40% decrease in the specific radioactivity of NANA in OSM with no influence on the specific activity of GalNac in the glycoprotein. It is concluded that NANA penetrates readily into the cell and dilutes the pool of sialic acid present, but that its catabolism is not fast enough as to interfere with the radioactivity of other metabolic pools than its own.

Discussion

The estimation of the actual amount of OSM synthesized in the gland under conditions as close to the physiological requirements as possible offers a suitable means for the study of the biosynthesis of the protein and osidic moieties of a glycoprotein like OSM and their mutual interactions. The total inhibition of the biosynthetic process under the influence of puromycin is not unexpected. The possibility of an incorporation of sugars into small peptidic fragments which could escape detection by the present methods is not excluded. However present evidence obtained with similar inhibition experiments on α_1-acid glycoprotein biosynthesis in the liver (Molnar *et al.*, 1964), on the biosynthesis of blood group substances in hog gastric mucosa (Kornfeld *et al.*, 1965), on thyroglobulin biosynthesis in the thyroid gland (Spiro and Spiro, 1966), and on the biosynthesis of chondroitin sulfate–protein complex in isolated epiphysis tissue (Telser *et al.*, 1965) seem to indicate that small peptides or amino acids cannot substitute for the protein as acceptor molecules during the first enzymatic transglycosylation reaction which results in the attachment of the first sugar of the glycosidic chain to the polypeptide precursor. Moreover, the specificity of the acceptor molecule for the transfer of terminal sialyl residues to a desialoglycoprotein is also very narrow (Carlson *et al.*, 1964; O'Brien *et al.*, 1966). Hence the conclusion that puromycin completely inhibits the biosynthesis of OSM throughout the experiments reported above, seems presently reasonable.

Whereas inhibition of the biosynthesis of the protein moiety of OSM results in a complete absence of any glycoprotein synthesized, impairment of the biosynthesis of the sugar moiety does not completely inhibit the glycoprotein synthesis. Attempts to starve the gland by incubations in a glucose-free medium results in only about 50% inhibition of the biosynthesis of the glycoprotein, glycogen substituting under these conditions for the endogenous substrate. When the conversion of fructose 6-phosphate to glucosamine 6-phosphate is inhibited by the addition of DON, the biosynthesis of the glycoprotein drops to about 10% of the control in the presence of glucose, normal biosynthesis of the glycoprotein being restored by the addition of glucosamine. Similar results have been obtained by Telser *et al.* (1965), on chondroitin sulfate protein complex. The main question which arises from the inhibition experiments with DON, concerns the possibility of an actual biosynthesis of the OSM protein moiety alone. Figure 3 shows that the total incorporation of [^{14}C]serine into the protein fractions of the submaxillary gland extract is by no means decreased in the presence of DON. On the contrary, the labeling of the protein material appears to be much more abundant in peaks I and III (which do not correspond to the glycoprotein) in the presence of DON than in its absence. Hence, the assumption that this material could at least partly consist of the protein moiety of OSM seemed a reasonable one; however, it has not been possible to demonstrate its quantitative glycosylation by the sequential inhibition experiment with DON and puromycin. Despite this negative result, it does not seem possible to dismiss the hypothesis mentioned above without any further critical consideration. Quantitative glycosylation of the precursor may be difficult because the protein has accumulated at the apical region of the cell and cannot flow back to the locus where glycosylation occurs (*e.g.*, the Golgi apparatus), or on the contrary, because this protein accumulates in the ribosomal region and does not proceed to the Golgi apparatus in the presence of puromycin which stops the biosynthesis of further amounts of protein. In the absence of any definite conclusion, it is postulated that the protein moiety of OSM may be synthesized without any interference or control by glycosylation reactions, thus adding some circumstantial evidence to the experimental results recently published, indicating that the biosynthesis of the protein and glycosidic part of a glycoprotein do not take place in one and the same subcellular structure.

A few comments may be added concerning the experiments of mutual dilutions between glucose, glucosamine, galactose, galactosamine, and *N*-acetylneuraminic acid, although the main conclusions which may be drawn from these experiments have been already discussed; the interest in performing this kind of experiments is threefold; first, the specific dilutions which have been obtained, though not unexpected, provide a useful mean of checking the accuracy of the techniques used to study metabolic correlations; second, it has been possible to deduce from these results quantitative data concerning the relative efficiency of metabolic pathways deriving from glucose, glucosamine, and galactose, which agree with the precursor role of glucose for the biosynthesis of the disaccharide chains under physiological conditions; third, it appears that some of the results which have been discussed with the relevant experiments can only be explained by regulatory mechanisms. Although these mechanisms have not been systematically studied in the present work, preliminary evidence for their occurrence in intact cells, demonstrates that the techniques and experimental conditions used, are suitable for the study of such regulatory phenomena.

The occurrence of biosynthesis of glycogen or OSM from glucosamine and galactose needs some explanation. It is of course unlikely that these metabolic pathways operate to an appreciable extent *in vivo*, but it is well established that most hexokinases of animal tissues exhibit a pronounced lack of specificity (Crane and Sols, 1955) which enables them to utilize glucosamine as substrate in a very efficient way. It may be postulated that the same is true for the submaxillary gland. The possibility to convert glucosamine to UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine by enzymatic extracts of submaxillary glands has already been mentioned (Rossignol *et al.*, 1966). The metabolic significance of an active galactokinasic system within the submaxillary gland is not immediately evident. There is no reason to believe that galactose plays a quantitatively important role as substrate under physiological conditions. It may however be suggested that the galactokinase plays a role when actual degradation of the glycoprotein takes place, by participating in the catabolism of galactosamine. However, it must be noted that no evidence for an active galactosamine metabolism has been obtained throughout this work.

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