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# ROLE OF GALACTOSYL-TRANSFERASES IN RAT GASTRIC EPITHELIAL GLYCOPROTEIN SYNTHESIS

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## Summary

Two galactosyl-transferases have been found in the Golgi-enriched subcellular fractions derived from rat gastric mucosa. One incorporates galactose into ovomucoid at optimal pH 6.8. The reaction can be completely inhibited by acetylglucosamine. The apparent $K_m$ for UDPgalactose is 0.024 mM. The other galactosyl-transferase incorporates galactose into desialated ovine submaxillary mucin at optimal pH 7.5 and the transfer cannot be inhibited by acetylglucosamine. The apparent $K_m$ for UDPgalactose is 0.191 mM. Both enzymes require $Mn^{2+}$ and Triton X-100 for optimal galactose incorporation. The enzymes could be separated by polyacrylamide gel electrophoresis. Incorporation into endogenous glycoprotein was studied in conditions optimal for the two galactosyl-transferases: (1) at pH 6.8, using Mes as buffer system, and (2) at pH 7.5, using Tris-HCl in the presence of an inhibitory excess of acetylglucosamine. In both cases, most radioactive galactose is incorporated into macromolecules, which could be identified as epithelial glycoprotein. Endogenous incorporation in the presence of excess acetylglucosamine results in the formation of a substantial amount of a disaccharide (probably galactose-β-(1—3)acetylgalactosamine), whereas upon incorporation at pH 6.8 almost no disaccharide is formed. Quantitative immunoprecipitation experiments with specific antibodies to the endogenous product, labelled by [$^3$H]galactose in the presence of varying amounts of desialated ovine submaxillary mucin and/or acetylglucosamine, indicated that other galactosyl-transferases are involved in the biosynthesis of epithelial glycoprotein.

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Abbreviations: GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Mes, 2-(*N*-morpholino)-ethanesulfonic acid. All glycoses in this paper are in the pyranose form and are of D configuration, except fucose, which is of the L configuration.

## Introduction

Macromolecules composing the mucus layer of the inner surface of the mammalian stomach are glycoproteins. Many purified epithelial glycoproteins consist of 15% amino acids, about 80% carbohydrate, and 3% ester sulphate [1]. Porcine gastric mucus glycoprotein contains over 150 carbohydrate side chains per molecule of $M_r$ $5 \cdot 10^5$, and each side chain consists of 14—20 sugar residues joined by ten different glycosidic linkages. The ester sulphate residues occur as *N*-acetylglucosamine-6-sulphate [2]. The oligosaccharides are glycosidically-linked to the polypeptide backbone via GalNAc-serine/threonine bonds. Also other, non-mucin glycoproteins like human chorionic gonadotropin [3], fetuin [4] and the erythrocyte membrane glycoprotein glycophorin [5] contain glycosidic linkages between sugar and amino acid.

In gastric glycopeptides with blood group A activity about six galactose residues occur, possibly in five different glycosidic linkages [2,6]. Whether each of these various bonds requires a different galactosyl-transferase is unknown. In porcine submaxillary gland the occurrence of three galactosyl-transferases has been described [7]: one transferring galactose to GalNAc residues of desialated ovine submaxillary mucin, a second transferring galactose to GlcNAc in $\alpha_1$-acid glycoprotein or to free GlcNAc, and a third transferring galactose to Tay-Sachs ganglioside. Only the first enzyme seems to be involved in the attachment of the galactose residue of porcine submaxillary mucus.

Previous studies from our laboratory have been concerned with the attachment of the first sugar (GalNAc) to the peptide backbone [8] and with the intracellular site of galactose incorporation in the gastric surface mucous cells [9,10]. Initial GalNAc attachment occurs at peptidyl-tRNA level on membrane-bound polysomes [8], while the majority of galactose is bound in the trans-Golgi cisternea [10]. The present study deals with the characteristics of two galactosyl-transferases from gastric surface epithelium and their role in epithelial glycoprotein synthesis.

## Materials and Methods

*Materials.* UDP-6-[$^3$H]galactose, spec. act. 10.4 Ci/mmol, was obtained from the Radiochemical Centre, Amersham; unlabelled UDPgalactose and ovomucoid type III from Sigma Chemical Co. Ovine submaxillary mucin was prepared as described by Tettamanti and Pigman [11]. Ovine submaxillary asialo-mucin was prepared by treatment of the mucin (50 mg/ml) with neuraminidase from *Clostridium perfringens*, 0.1 U/ml (type IX, Sigma Chem. Co.). After incubation in 50 mM sodium acetate, pH 5.0, at 37°C for 24 h, the asialo-mucin was heated at 85°C for 10 min to destroy neuraminidase and then dialyzed against deionized water. Quantitation of the sialic acid [12] showed that more than 95% of the sialic acid was removed from the glycoprotein. The GalNAc content was determined by the Morgan-Elson procedure [13] as modified by Ressig [14] with *N*-acetylgalactosamine as standard. Rabbit antiserum against bovine serum albumin and goat IgG fraction against rabbit IgG were obtained from Miles Laboratories.

*Enzyme preparation.* Microsomes were prepared from postmitochondrial

supernatant of homogenated scrapings of the fundic part of the rat stomach as described previously [15]. The 100 000 $\times g$ pellet was resuspended in 0.25 M sucrose and layered over a discontinuous gradient consisting of 1.5 ml 1.5 M sucrose, 1.5 ml of 1.1 M sucrose, 3 ml 0.9 M sucrose and 3.5 ml 0.4 M sucrose. The tube was centrifuged at 40 000 rev./min in a Spinco SW-41 rotor for 3 h. The gradient was displaced by a heavy sucrose solution, pumped under the gradient through a needle. The absorbance was monitored with a Gilford spectrophotometer. Fractions of 1.0 ml were collected and used for enzyme analyses. The interphase between 0.4 and 0.9 and between 0.9 and 1.1 M sucrose contained nearly all galactosyl-transferase activities towards both ovomucoid and asialo-mucin. These fractions were pooled and used for the experiments. By this procedure 20—80-fold purification of the galactosyl-transferase activities was achieved. Protein concentration was estimated by the method of Lowry et al. [16].

*Enzyme assays.* Unless indicated otherwise the galactosyl-transferases were assayed in incubation mixtures containing the following components: 1 mM dithiothreitol, 5 mM $MnCl_2$, 2 mg/ml Triton X-100, 0.7 mM UDPgalactose, 15 $\mu$Ci/ml UDP[$^3$H]galactose, and acceptor protein (3 mg/ml asialo-mucin or 10 mg/ml ovomucoid). If the transfer of galactose to asialo-mucin was determined, 0.05 M Tris-HCl (pH 7.5) was used; for the transfer reaction to ovomucoid 0.05 M Mes, pH 6.8, was used as buffer system. The total volume of the assay was 0.05 ml. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 1 ml ice-cold 1% phosphotungstic acid in 0.5 M HCl. The precipitate was collected on glass fibre filters and washed with 5% trichloroacetic acid. Then the filters were dried and the radioactivity was counted in a liquid-scintillation counter. At the specific radioactivity of UDPgalactose used in the experiments, the incorporation into endogenous acceptors was negligible. For galactose incorporation into endogenous glycoprotein the same incubation mixture was used except that unlabelled UDPgalactose was omitted. Each assay was conducted under optimal conditions, in which product formation was proportional to incubation time and to the quantity of enzyme protein.

*Polyacrylamide gel electrophoresis.* Cylindrical gels of polyacrylamide (7%, v/v) were prepared according to Davis [17] in 0.375 M Tris-HCl buffer, pH 8.9. After electrophoresis for 180 min in the presence of 0.005 M Tris-glycine, pH 8.3, at 2 mA/gel, the gels were cut into 3-mm slices, which were incubated in the enzyme incubation mixture containing asialo-mucin or ovomucoid.

Analysis of endogenous products was performed by electrophoresis as described by Peacock and Dingman [18]. The gels contained 2% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, and 0.5% (w/v) agarose. Gels and buffer contained 0.1% (w/v) sodium dodecyl sulphate. Prior to electrophoresis the samples were reduced for 30 min at 37°C in 0.01 M 2-mercaptoethanol. Electrophoresis was performed at 10 mA per gel for about 120 min. After the run the gels were stained with Coomassie Brilliant blue or periodic acid Schiff reagent. The gels were cut into 2 mm slices, which were solubilized with 0.2 ml Lumasolve at 60°C for 16 h. After addition of a toluene-based scintillator, the radioactivity was counted in a liquid scintillation counter.

*Immunoprecipitation.* For immunoprecipitation, 0.05 ml aliquots of galactosyl-transferase assay mixtures were used. At room temperature, 0.9 ml of a

solution of 0.15 M NaCl, 0.01 M Tris-HCl (pH 8.3), 0.01 M EDTA, 1% Triton X-100, 100 units of trasylol per ml as well as 3 μl of a rabbit antiserum raised against rat gastric epithelial glycoprotein was added. In a pilot experiment, the amount of rabbit anti-rat epithelial glycoprotein serum was determined to obtain quantitative immunoprecipitation of the glycoprotein. Following incubation for 60 min at 37°C and 16 h at 4°C, goat anti-rabbit-immunoglobulin IgG was added and the incubation was continued for 2 h at 4°C. As a blank, 3 μl of rabbit anti-bovine serum albumin-serum was added to 0.05 ml incubation mixtures. The precipitates were collected by centrifugation for 5 min at 4000 × $g$, washed three times with immunoprecipitation buffer, dissolved in 0.4 ml Lumasolve and the radioactivity was counted in a liquid-scintillation counter. The epithelial glycoprotein, used for immunization, was purified on CsCl-density gradients and preparative polyacrylamide gel electrophoresis on polyacrylamide-agarose gels and administered subcutaneously to a rabbit. The specificity of the serum was tested by immunoprecipitation of [$^3$H]galactose-labelled epithelial glycoprotein and subsequently by analysis of the precipitate by polyacrylamide gel electrophoresis.

## Results

### *Optimization of the galactosyl-transferase assays*

To characterize and measure the galactosyl-transferase activities in gastric epithelial scrapings, we have used ovine submaxillary asialo-mucin and ovomucoid as exogenous acceptors. The lattter glycoprotein contains terminal GlcNAc residues and has therefore the advantage of accepting galactose without any previous chemical or enzymatic modification. Asialo-mucin is used as acceptor for the galactosyl-transferase reaction that requires terminal GalNAc residues.

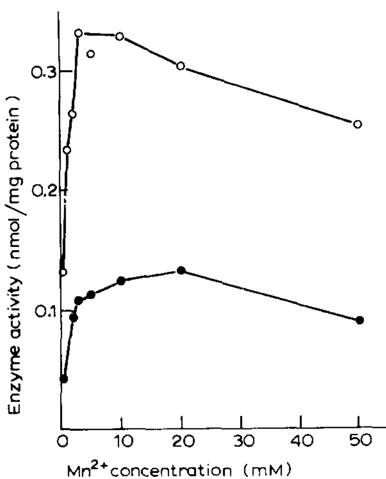



Fig. 1. Effect of the concentration of $Mn^{2+}$ on the galactosyl-transferase activity. Standard incubations were performed using sialic acid-free ovine submaxillary mucin (○——○) and ovomucoid (●——●) as acceptors.

Both enzyme activities showed a strong requirement for $Mn^{2+}$ (Fig. 1) with an optimal concentration of 5 mM when asialo-mucin and 20 mM when ovomucoid was used as acceptor. All further assays were performed at a $Mn^{2+}$ concentration of 5 mM. The rate of incorporation was constant for more than 30 min of incubation, indicating an excess of the exogenous acceptors. Under these conditions, the rate of the reaction was proportional to the quantity of enzyme protein added to the standard assay mixture, up to 0.15 mg. The optimal pH values are different for the two exogenous acceptors: 7.5 for asialo-mucin and 6.8 for ovomucoid. Moreover, Tris ions turned out to be inhibitory for the galactose transfer to ovomucoid (Fig. 2). Fig. 3 shows the effect of UDPgalactose concentration on the enzyme activities towards asialo-mucin and ovomucoid. The apparent $K_m$ for UDPgalactose is 0.024 mM with ovomucoid and 0.191 mM with asialo-mucin as acceptor.

To ascertain the presence of at least two galactosyl-transferases we electrophoresed Triton X-100 treated microsomes on a polyacrylamide gel in the presence of the detergent and the transferase activities were determined with asialo-mucin and ovomucoid. Fig. 4A shows the profile of galactosyl-transferase activity to ovomucoid. Two main peaks of galactosyl-transferase activity were present. The transferase activity to asialo-mucin has a different electrophoretic

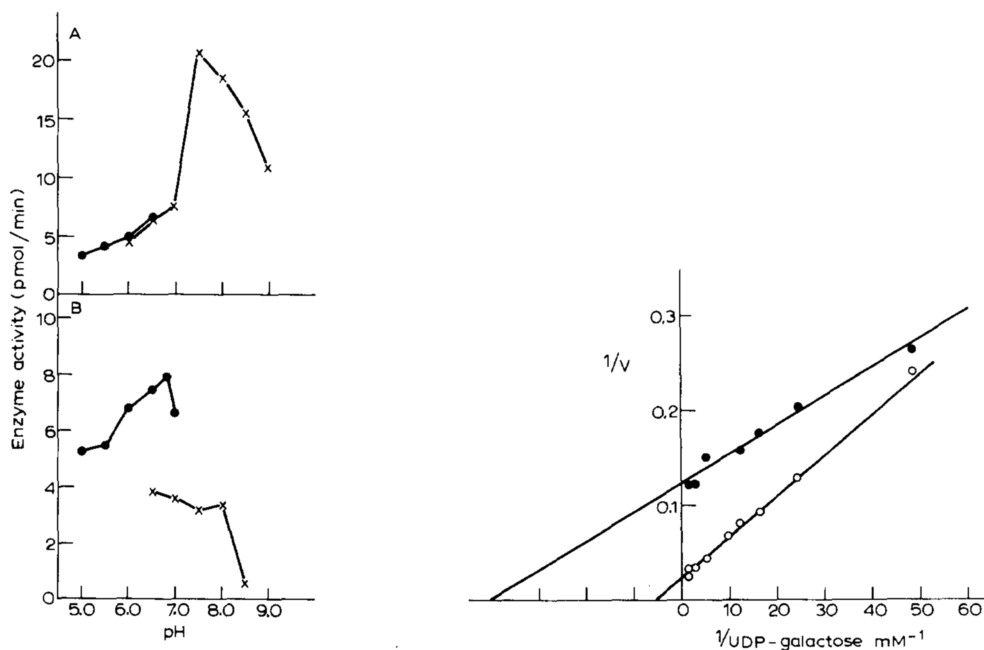
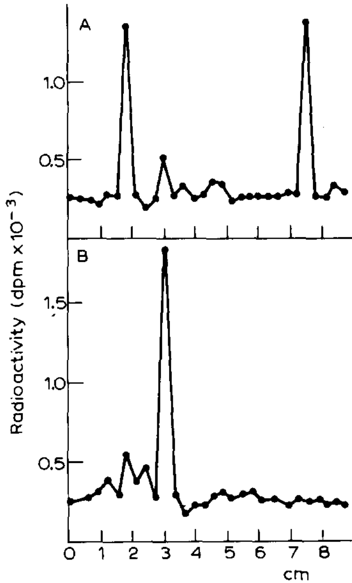



Fig. 2. Effect of pH on the galactosyl-transferase activity. Standard incubations were conducted with Mes buffer (●——●) and Tris-HCl buffer (X——X) A, sialic acid-free ovine submaxillary mucin; B, ovomucoid.

Fig. 3. Effect of UDPgalactose concentration on galactosyl-transferase activity. Standard incubations were conducted in the presence of UPD[$^3$H]galactose and varying amounts of unlabelled UDPgalactose. Acceptors were sialic acid-free ovine submaxillary mucin (○——○) and ovomucoid (●——●). The data were plotted by the method of Lineweaver and Burk.



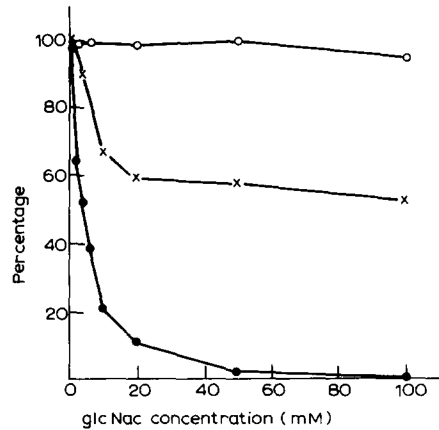




Fig. 4. Polyacrylamide gel electrophoresis of a purified Golgi fraction. Galactosyl-transferase activities were determined with ovomucoid (A) and sialic acid-free ovine sumaxillary mucin (B) as acceptors. Slices (3 mm) of the gels were incubated following standard incubation conditions except that the incubation time was 3 h.

Fig. 5. The effect of GlcNAc concentration on the galactosyl-transferase activities towards exogenous acceptors. Standard incubations with sialic acid-free ovine submaxillary mucin (○———○), desialo-degalacto-fetuin (10 mg/ml) (X———X) and ovomucoid (●———●) as acceptors.

mobility (Fig. 4B). Apparently, some overlap between substrate specificities of the two enzymes occurs.

*Competition studies*

As shown for liver (Fraser and Mookerjea [19], Schachter et al. [20]), mammary gland (Smith and Brew [21]), and spleen cells (Martin et al. [22]), Golgi membranes contain a galactosyl-transferase activity towards modified high molecular weight acceptors like fetuin and $\alpha_1$-acid glycoprotein as well as towards free GlcNAc. In all cases, the enzyme catalyzes the formation of galactosyl-$\beta$(1-4)acetylglucosaminyl linkages. In order to get more insight in the specificities of the two galactosyl-transferases in the gastric epithelium, the enzyme reactions were conducted in the presence of free GlcNAc. If ovomucoid was used as acceptor, total inhibition of the transfer reaction to that macromolecule could be achieved (Fig. 5). The transfer of galactose to asialomucin was not influenced even by high GlcNAc concentrations. Incorporation into asialo-agalacto-fetuin, which contains terminal GalNAc residues besides terminal GlcNAc [4], could only be inhibited to 50%.

*Cell-free endogenous incorporation of galactose*

In order to examine whether the two galactose-transferases are involved in the synthesis of gastric glycoprotein, we have incubated post-mitochondrial

supernatant with UDP[$^3$H]galactose, its specific radioactivity not decreased by the addition of unlabelled UDPgalactose. To discriminate between the results of the two enzyme activities, the incubation was carried out in the presence of different buffer systems, namely Mes at pH 6.8 and Tris-HCl at pH 7.5. Moreover, to the incubation mixture containing Tris-HCl, 50 mM GlcNAc was added to prevent endogenous incorporation by the enzyme with pH optimum 6.8. At this GlcNAc concentration, complete inhibition of the transfer reaction to ovomucoid is achieved (Fig. 5).

The labelled endogenous products were analyzed by electrophoresis of the post-mitochondrial supernatant in agarose-acrylamide gels in the presence of sodium dodecyl sulphate (Fig. 6). If the incubation was carried out in the presence of Tris-HCl, pH 7.5, and GlcNAc (Fig. 6B), [$^3$H]galactose was almost exclusively incorporated in material having the same electrophoretic mobility as native epithelial glycoprotein. If Mes at pH 6.8 was used, a substantial

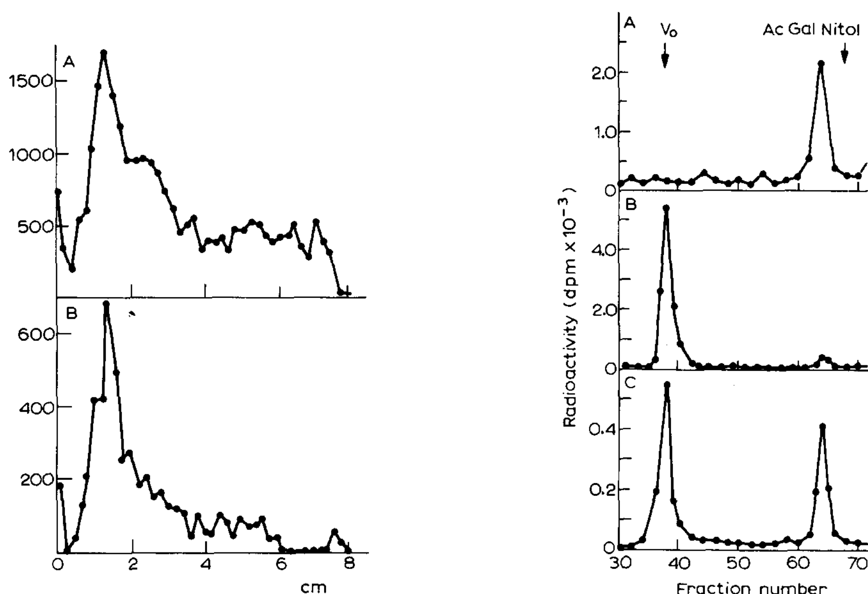



Fig. 6. Electrophoretic analysis on polyacrylamide-agarose gels of endogenous [$^3$H]galactose-labelled glycoproteins. Endogenous incubations in a total volume of 0.25 ml were done. A, incubation in the presence of Mes buffer, pH 6.8; B, incubation in the presence of Tris-HCl, pH 7.5, and 50 mM GlcNAc. Ordinate: radioactivity (dpm $\times$ $10^{-3}$).

Fig. 7. Gel filtration profiles of [$^3$H]galactose-labelled components on a Biogel P-2 column. Endogenous incubations in a total volume of 0.25 ml were done. After incubation the reaction was terminated with phosphotungstic acid/HCl, the precipitate washed three times and subjected to reductive alkaline treatment (0.1 M NaOH/1 M $NaBH_4$ for 24 h at 45°C). The $\beta$-eliminated material was acidified and then deionized on Dowex-50 ($H^+$). The remaining borate was removed as methyl borate. The labelled material was applied on a calibrated Biogel P-2 column (90 $\times$ 1 cm) and eluted with 0.1 M ammonium acetate. 2-ml fractions were collected. For the determination of radioactivity, aliquots of 0.2 ml were mixed with 10 ml Lumagel and counted. B, incubation in the presence of Mes, pH 6.8; C, incubation in the presence of Tris-HCl, pH 7.5, and 50 mM GlcNAc. As a reference, $\beta$-eliminated material derived from an exogenous incubation as described in Materials and Methods section with sialic acid-free ovine submaxillary mucin as acceptor was used (A). Unlabelled acetylgalactosaminitol (AcGalNitol) was cochromatographed as a reference.

amount of radioactivity comigrated with other (glyco)proteins, a great part of it just ahead of the main compoment (shoulder) Fig. 6A).

In order to find out whether the two transferases catalyze different linkages in endogenous glycoprotein, we have hydrolyzed the peptide sugar bonds of the two acid-insoluble reaction products by $\beta$-elimination in the presence of 1 M $NaBH_4$. The hydrolysates were treated with Dowes 50 ($H^+$) to remove peptides, and the [$^3$H]galactose-labelled oligosaccharides were analyzed on a Biogel P-2 column. Fig. 7B shows the distribution of radioactivity after incubation in the presence of Mes, pH 6.8. Since nearly all radioactive material eluted in the void volume, the [$^3$H]galactose-bearing saccharides formed by this enzyme activity have molecular weights of more than 800, and almost no disaccharide was formed. Saccharides derived from the incubation mixture containing Tris-HCl, pH 7.5, and GlcNAc, partly eluted in the void volume of the column and partly in a retarded volume (Fig. 7C). The latter position was shared by the $\beta$-elimination product obtained after incubation of asialo-mucin with UDP[$^3$H]galactose (Fig. 7A). The identity of the retarded radioactive material, derived from the incubation in Tris-HCl buffer and GlcNAc, was further established by paper chromatography. It had the same $R_F$ value as the $\beta$-elimination product from [$^3$H]galactose-labelled asialo-mucin. Van den Eijnden et al. [23] showed that the anomeric configuration of the latter oligosaccharides is Gal$\beta$(1—3)GalNAc.

Part of the $\beta$-elimination products of the incubation with GlcNAc in Tris-HCl, pH 7.5, eluted in the void volume of the Biogel P-2 column (Fig. 7C). The molecular weights of these products must be higher than 800. The presence of incompletely hydrolyzed glycoprotein is unlikely, as it should be absorbed to Dowex-50 resin. Therefore, this material consists of oligosaccharides with a rather high molecular weight. As the radioactive saccharides, synthesized in the presence of GlcNAc and Tris, probably do not contain [$^3$H]-

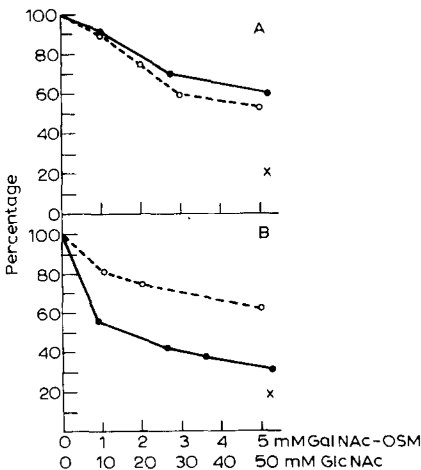



Fig. 8. The effect of sialic acid-free ovine submaxillary mucin and GlcNAc on the incorporation of [$^3$H]-galactose into endogenous epithelial glycoprotein. Standard incubations were conducted as described for endogenous incorporation with Mes, pH 6.8 (A) and Tris-HCl, pH 7.5 (B), except that the incubation time was 5 min. ○······○, GlcNAc; ●———●, sialic acid-free ovine submaxillary mucin; X, sialic acid-free ovine sumaxillary mucin plus GlcNAc.

Gal$\beta$(1—4)GlcNAc linkages, the radioactive oligosaccharides present in the void volume of the Biogel P-2 volumn in Fig. 7C differ from those in the void volume of Fig. 7B. Whether the former [$^3$H]galactose-labelled material is synthesized by a third galactosyl-transferase, different from the enzyme which catalyzes the formation of the disaccharide present in fraction 64, is not clear from this experiment.

The possibility of other galactosyl-transferases involved in the synthesis of epithelial glycoprotein was examined by competition experiments with GlcNAc and asialo-mucin. Endogenous incorporation of [$^3$H]galactose during incubation with various concentrations of the two competitive acceptors was measured by quantitative immunoprecipitation with specific antibodies raised against gastric epithelial glycoprotein. The incorporation into endogenous glycoprotein was linear for at least 5 min at the highest concentration of competitors used. At pH 6.8 (Mes buffer) the incorporation of [$^3$H]galactose could be inhibited by 40% with asialo-mucin and by 50% with GlcNAc, whereas at pH 7.5 (Tris-HCl) inhibition with asialo-mucin was 70% and with GlcNAc 40% (Fig. 8). When GlcNAc and asialo-mucin were used together at high concentrations, the maximal inhibition was about 80% at both pH values.

These results indicate that 80% of the incorporation into endogenous glycoprotein can be ascribed to two galactosyl-transferases. The results do not exclude the possibility of other galactosyl-transferases.

## Discussion

Rat gastric surface mucous cells contain at least two galactosyl-transferases, involved in mucous glycoprotein biosynthesis. Epithelial glycoproteins, as produced by the gastric mucosa, might contain five different types of galactose linkages: Gal$\beta$(1—3)GalNAc, Gal$\beta$(1—3)GlcNAc, Gal$\beta$(1—4)GlcNAc, Gal$\beta$(1—3)-Gal and Gal$\beta$(1—6)Gal [2,8]. In case of blood group B activity, present in only negligible amounts in our rats, Gal$\alpha$(1—3)Gal also occurs.

As different galactosyl-transferases are distinguished by their specificities towards different acceptors, we have used two macromolecular acceptors, namely asialo-mucin and ovomucoid. The different behaviour of galactose transfer towards these two acceptors in the presence of a competitor molecule, GlcNAc, demonstrates the presence of at least two galactosyl-transferases. One of them directs the transfer of galactose to GlcNAc, either free or at the non-reducing end of ovomucoid oligosaccharides. The apparent $K_m$ value of this galactosyl-transferase (24 $\mu$M) is identical to the value obtained for human serum galactosyl-transferase with $\alpha_1$-acid glycoprotein devoid of sialic acid and galactose [24]. A comparable enzyme is also isolated from fetal calf serum [25] and from rat liver ($K_m$ 10.8 $\mu$M) and serum ($K_m$ 12.5 $\mu$M); both $K_m$ values were determined with asialo-agalacto-fetuin [19]. The molecular structure of the acceptor can significantly influence the apparent $K_m$ for UDPgalactose [26]. Berger et al. have found a high specificity for the human serum enzyme that catalyzes only Gal$\beta$(1—4)GlcNAc bond formation [27]. The enzyme present in the gastric epithelial cells resembles the galactosyl-transferase of serum, liver, mammary gland and spleen [19,27], and might therefore be involved in galactose transfer towards the oligosaccharide side-chain of the

Asn-GlcNAc type glycopeptides as well as towards those of the Ser(Thr)-GalNAc type.

Schachter et al. described a UDPgalactose:mucin galactosyl-transferase from porcine submaxillary gland, which catalyzes a Galβ(1—3) linkage to GalNAc residues of ovine submaxillary asialo-mucin [7]. Since the same linkage occurs in gastric mucin [2,6], one might expect a similar enzyme to be present in the gastric mucus cells. However, the optimal pH (7.5) for the reaction as well as the apparent $K_m$ for UDPgalactose (191 $\mu$M) differ considerably from those of the porcine submaxillary gland enzyme (pH 5.5—6.5; $K_m$ 500 $\mu$M).

The two galactosyl-transferases can be separated on a polyacrylamide gel (Fig. 4). The enzyme with activity towards ovomucoid is resolved into two main bands, the other in only one. Some overlap in specificity between the two enzymes occurs. There are two possibilities to explain this phenomenon: trace amounts of acceptor places for galactose, different from the main specificities, might be present in the two acceptor preparations, or the specificity of the galactosyl-transferases for their acceptors is not absolute. In the latter case, the enzyme which catalyzes the linkage Galβ(1—4)GlcNAc might for example exhibit a low affinity for GalNAc. Both possibilities can explain that, upon quantitative immunoprecipitation of reaction mixtures incubated with increasing amounts of the competitors asialo-mucin and GlcNAc (Fig. 8), the addition of each of the inhibitory effects on the endogenous incorporation of [$^3$H]galactose amounted to more than 100% at pH 7.5.

If in a Golgi preparation the different endogenous acceptor sites should be present in a distribution reflecting the ratio of the different types of Gal-sugar bonds, other enzymes must take part in the endogenous galactose incorporation. The data of Fig. 8 suggest their part to be at least 20%. This might be an underestimate for the reasons mentioned before.

## Acknowledgements

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