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CHARACTERIZATION OF UDP-GALACTOSYL:ASIALO-MUCIN TRANSFERASE ACTIVITY IN THE GOLGI SYSTEM OF RAT LIVER

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Summary

UDPgalactosyltransferase activity (UDPgalactose:mucopolysaccharide galactosyltransferase, EC 2.4.1.74) was measured in a well-characterized fraction of Golgi membranes in the presence of UDPgalactose and exogenous acceptor sites. Substrate saturation for 0.05 mg Golgi protein was achieved at a concentration of 4.6 mM UDPgalactose. Desialylated mucin proved to be the most suitable acceptor protein. Access to galactose acceptor sites was not rate limiting for the reaction when 20 mg of asialo-mucin/ml of incubation mixture was used. With these concentrations of substrates the use of nucleotides to inhibit pyrophosphatases and of detergents to perturb the membrane structure was not necessary and proved, in fact, to be inhibitory to galactose transfer. UDPgalactosyl:asialo-mucin transferase activity in Golgi membranes was 230 nmol galactose transferred/mg Golgi protein per 30 min.

Introduction

Galactosyltransferase activities towards protein and lipid acceptors have been measured in preparations of endoplasmic reticulum and Golgi membranes [1—4] and in plasma membranes [5]. In addition exo-galactosyltransferases have been described in the serum by Ip and Dao [6]. With different cytoplasmic membranes there are large variations in galactosyltransferase activity using endogenous membrane protein acceptors [4]. Preliminary investigations in our laboratory have revealed differences in the properties of the transferase activity in different cell components when exogenous glycoproteins are used as galactose acceptors (Andersson, G.N. and Eriksson, L.C., unpublished data). In investigations where the transfer of galactose to added glycoprotein has been

studied, various quantities of substrate, detergents and acceptor proteins have been used to effect enzyme activity. In order to study changes in the properties of this activity in different cytoplasmic membrane fractions under both normal and certain pathological conditions, it is important to optimize the assay and to use membrane fractions which are as pure as possible. In this study we have optimized the galactosyltransferase (UDPgalactose:mucopolysaccharide galactosyltransferase, EC 2.4.1.74) assay for a well-characterized Golgi membrane fraction. To obtain maximal enzyme activity, the transfer of radioactively labeled galactose to an excess of an exogenously added glycoprotein was studied.

Materials and Methods

In all experiments male adult albino Sprague-Dawley rats starved for 20 h were used for the preparation of Golgi membranes. This was achieved by a sequential procedure recently developed in our laboratory [7], where the Golgi fractions are floated on the 1.10 M-0.25 M sucrose interface. In order to reduce interference by non-membraneous galactosyltransferases, galactosidases, and pyrophosphatases, the membranes were washed once with 0.15 M Tris-HCl, pH 8.0. This washing procedure did not release galactosyltransferase activity from the Golgi membrane (Andersson, G.N. and Eriksson, L.C., unpublished data), a result also obtained by Fleischer and Smigel [8]. The acceptor protein used was bovine submaxillary mucin type I (Sigma Co., St. Louis, U.S.A.), which was desialylated by dissolving 1 g of mucin in 20 ml 0.05 M H_2SO_4 and heating to 80°C for 1 h under gentle stirring. Following the acid hydrolysis, the pH was adjusted to 8.0 with NaOH to avoid aggregation. The hydrolysate was dialysed against 5 mM Tris (pH 8.0) and 0.9% NaCl overnight and concentrated in a Diaflo cell equipped with a PM-10 filter to a final concentration of 100 mg/ml.

The incubations were performed at 37°C for 30 min, during which time period no deviation from linearity was found. The optimal assay medium in a final volume of 0.4 ml contained 30 mM Tris/maleate buffer, pH 7.5, 5 mM $MnCl_2$, 30 mM β-mercaptoethanol, 8 mg asialo-mucin and UDP[^{14}C]galactose (Radiochemical Centre, Amersham, U.K.) diluted with non-radioactive UDP-galactose (Sigma Chemicals Co, St. Louis, U.S.A.) to a final concentration of 4.62 mM and with a specific activity of 26.7 μCi/mmol. In some experiments Triton X-100 (Kebo-Grave, Stockholm, Sweden) was used. The assay mixture contained about 0.05 mg of Golgi protein. All incubations were performed with freshly prepared Golgi membranes. The enzyme reaction was stopped by adding 5 ml of cold 8% trichloroacetic acid and the precipitate was spun down at 2500 × *g* for 20 min. The pellet was resuspended and recentrifuged in the trichloroacetic acid solution twice. The pellet was then dissolved in 2 ml of 2% sodium dodecyl sulfate (Kebo-Grave, Stockholm, Sweden) and counted in 10 ml Aquasol-2 (New England Nuclear Chemicals, F.R.G.) with a Beckman LS-100 Liquid Scintillation Counter.

Measurements of hydrolysis of β-glycoside bonds were performed as previously described [4] using *p*-nitrophenyl-β-D-galactopyranoside (Sigma Co., St. Louis, U.S.A.) as substrate. Protein was measured according to Lowry et al. [9] using bovine serum albumin as a standard.

In order to identify radioactive sugar moiety incorporated in asialo-mucin, the product was hydrolysed and radioactivity was determined in amino and neutral sugars in the following fashion. The transferase assay mixture was scaled up ten-fold and incubated for 1 h at 37°C. Golgi membranes were removed by centrifugation at 105 000 × *g* for 90 min. The supernatant was then gelfiltrated on a Sephadex G-25 column preequilibrated with 50 mM Tris-HCl, pH 8.0. Labeled mucin, eluated with the void volume, was clearly separated from free UDPgalactose. 5 mg of the radioactive acceptor protein was subjected to acid hydrolysis in 2 M HCl for 16 h at 100°C. Amino sugars were purified by the method of Boas [10] where the hydrolysate, diluted to a final concentration of 0.3 M HCl, was applied to a Dowex 50W-2X 200—400 mesh cation-exchange column (1 × 3 cm). Radioactivity was measured in the fraction passing through the column, and in the fraction obtained after elution with 2 M HCl.

The labeled mucin preparation was also subjected to β-galactosidase treatment (from bovine liver, Sigma Chemicals Co, U.S.A.) in an assay medium consisting of 20 mM citrate/phosphate buffer, pH 3.5, 8 mg of the labeled mucin and 0.02 U β-galactosidase, for 1 h at 37°C. The precipitable radioactivity after two washes with 8% trichloroacetic acid was measured and the amount of hydrolysed sugar was calculated by subtracting the value obtained when β-galactosidase was present in the assay from the value of the control incubation without enzyme.

Lipids were not extracted before the determination of radioactivity. Lipid acceptors for galactose have been detected in plasma membranes and Golgi membranes [4,5,11], but the amount of galactose incorporated into the lipid phase of the membranes is negligible in comparison with the amount incorporated into exogenous acceptor glycoproteins.

Results and Discussion

The importance of divalent cations to the optimal function of the galactosyltransferases is well-documented in the literature [2,12]. Mn^{2+} is the most effective activator. Fig. 1a illustrates the effect of different concentrations of Mn^{2+} in our incubation medium. Maximal activation occurs with 5 mM $MnCl_2$. If higher concentrations of the divalent cation are used, the enzyme activity decreases, a result recently obtained for Golgi membranes by Freilich et al. [13]. Beaufay et al. [1] and Bergeron et al. [14] used EDTA in order to inhibit Mg^{2+}-dependent nucleoside pyrophosphatases, which would degrade UDPgalactose. EDTA has no stimulatory effect on the incorporation of galactose into exogenously added acceptor protein. Instead, EDTA inhibits the reaction, probably due to complex binding of Mn^{2+} (not shown in the figure). Furthermore, in our assay system the action of pyrophosphatases is of minor significance due to the large excess of UDPgalactose used; this probably explains why EDTA fails to increase the galactose transfer.

Various buffer systems have been used in the assay of galactosyltransferase in the literature, with pH values in the interval of 5.7—7.4 [1,3,6,15,16]. For pure Golgi membranes the optimal pH in our assay turns out to be 7.5 (30 mM Tris/maleate, 37°C), as shown in Fig. 1b.

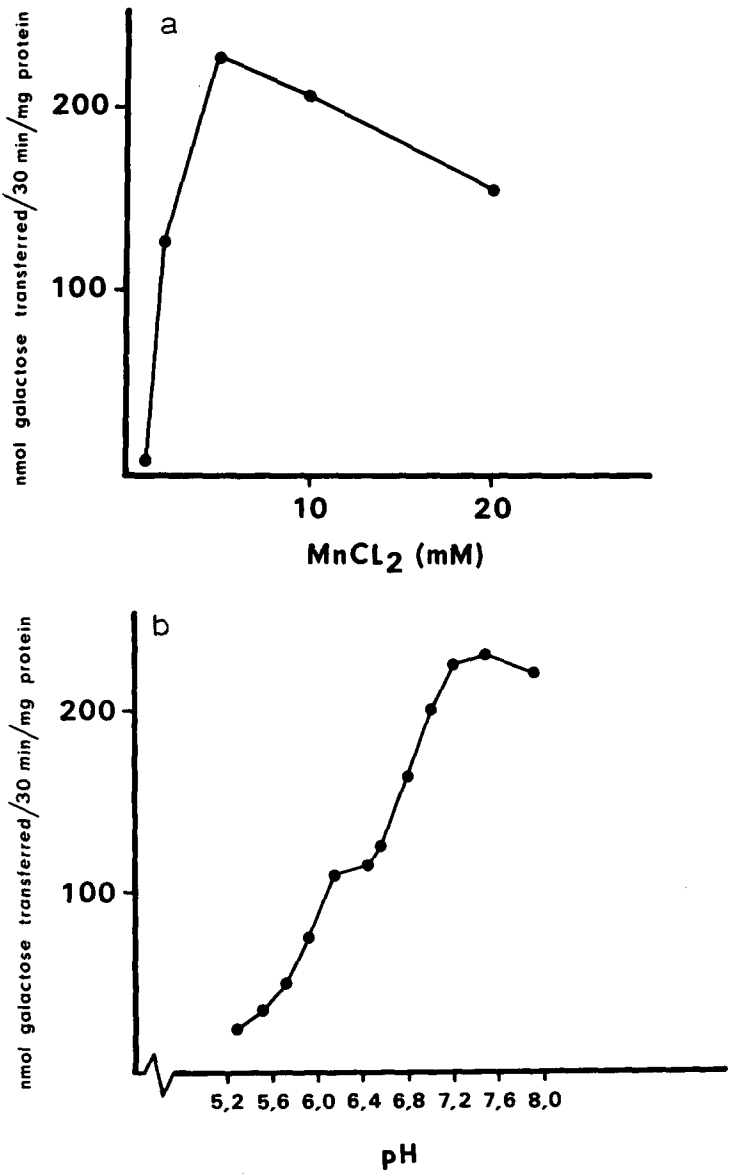

Fig. 1. UDPgalactosyl:asialo-mucin transferase activity in Golgi membranes as a function of $MnCl_2$, and pH. The Golgi fraction was prepared as described earlier [7]. Incubation mixture contained 30 mM Tris/maleate buffer, pH 7.5, 5 mM $MnCl_2$, 30 mM β-mercaptoethanol, 4.62 mM UDPgalactose, desialidated mucin 20 mg/ml and around 0.05 mg of Golgi protein in a final volume of 0.4 ml. In (a) the $MnCl_2$ concentration was varied as indicated, in (b) the incubations were performed at different pH, using the same buffer. The incubations were carried out at 37°C for 30 min and the activity is expressed as nmol [^{14}C]galactose transferred/30 min per mg Golgi protein. The values are the means of five experiments.

Several well-known glycoproteins with terminal mannosyl or *N*-acetylglycosaminyl residues function as acceptors for galactose. In Fig. 2 the acceptor properties of three different glycoproteins are shown. In this study asialomucin proved to be the most practical galactose acceptor. Ovomucoid and

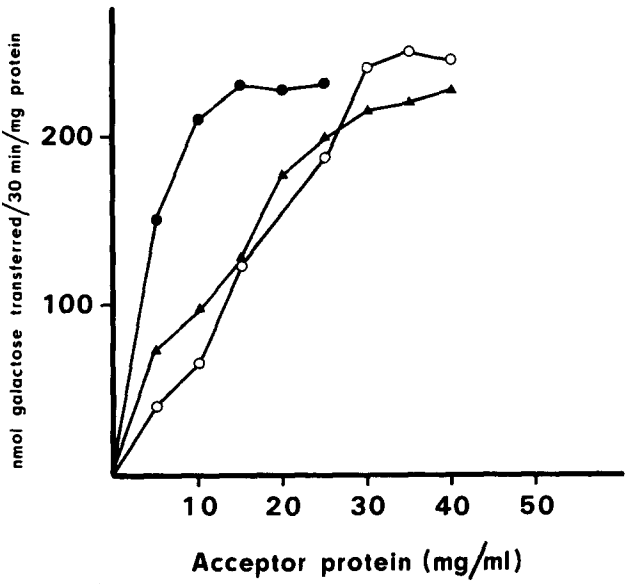

Fig. 2. [^{14}C]Galactose transfer to different exogenous galactose acceptors. Asialo-mucin was prepared by acid hydrolysis followed by neutralization and dialysis as described in Results and Discussion. Ovalbumin and ovomucoid were dissolved in water without pretreatment. After incubation (37°C, 30 min), the amount of radioactively labeled galactose was measured in the trichloroacetic acid-insoluble protein precipitate. Mucin could be quantitatively precipitated by trichloroacetic acid and spun down at 2500 × *g* for 20 min, while ovalbumin and ovomucoid had to be centrifuged at 20 000 × *g* for 60 min to be fully recovered. The concentrations of acceptor proteins are expressed as mg protein/ml incubation mixture. ●———●, asialo-mucin; ▲———▲, ovalbumin; ○———○, ovomucoid. Incubation as in Fig. 1. The values are the means of three experiments.

ovalbumin must be present in a much higher molar concentration to accept as much galactose. In addition these proteins required ultracentrifugation to be precipitated. Furthermore, bovine submaxillary mucin is easily desialidated enzymatically by neuraminidase or by mild acid hydrolysis in 0.05 M H_2SO_4. In most assays described in the literature β-mercaptoethanol is added to the incubation mixture. At the optimal concentration of this reducing agent, 30 mM, galactose transfer was stimulated by 60% (not shown in the figure). A possible explanation is that β-mercaptoethanol breaks up the disulfide bridges of the acceptor protein, which may lead to exposure of more galactose-accepting sites.

In Fig. 3 the activity of UDPgalactosyltransferase with increasing amounts of UDPgalactose is depicted. Enzyme saturation occurred at a substrate concentration of 5 mM using 0.05 mg of Golgi protein.

To determine if the added UDPgalactose actually was incorporated in the desialylated mucin as a galactose residue and not further metabolized to nucleotide derivates of amino sugars and/or other neutral sugars, ^{14}C-labeled mucin was prepared as described in Materials and Methods. After incubation with bovine liver β-galactosidase at pH 3.5 around 85% of the incorporated radioactivity was detached from the trichloroacetic acid-precipitated pellet (not shown in the figure). The labeled mucin was also non-enzymatically hydrolysed in 2 M HCl for 16 h at 100°C, a procedure sufficiently mild not to damage the

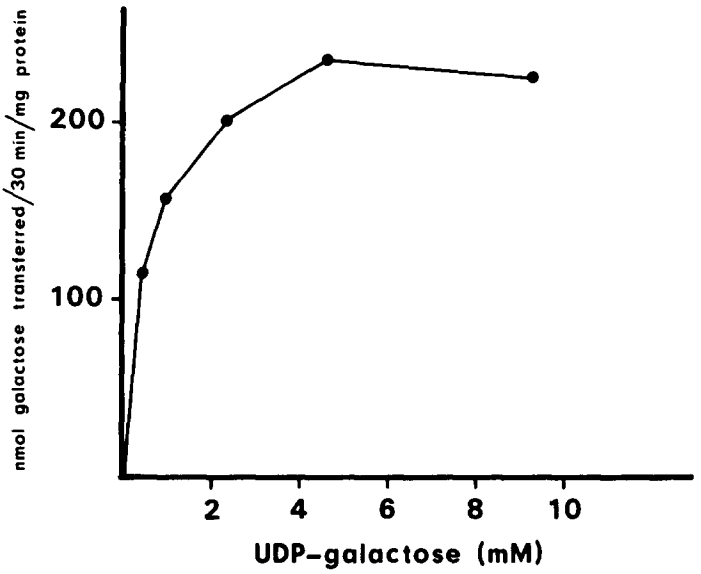

Fig. 3. Incorporation of [^{14}C]galactose into asialo-mucin at varying concentrations of UDP[^{14}C]galactose. Incubation mixture and assay as in Fig. 1. The values are the means of six experiments.

chemical structure of hexosamines and to completely release protein bound sugars [10]. As can be seen in Table II, after passage of the hydrolysate on a Dowex 50W-2X ion-exchange column, a 93% recovery of radioactivity was obtained in eluate I containing neutral sugars, and only insignificant amounts recovered after elution with 2 M HCl. The results of these two experiments support the conclusion that the protein-bound radioactivity under our assay conditions can be considered as [^{14}C]galactose.

When assaying enzyme activities in membrane preparations, it is important to bear in mind the influence of the membrane structure on the reaction, and the possible interference by other enzymes which affect the concentrations of substrate and product.

In our assay we have almost completely eliminated interference by pyrophosphatases acting on UDPgalactose and by β-galactosidases. Several authors have studied different nucleotides as inhibitors of the pyrophosphatases [2,16]. In Table I is illustrated the effect of adenosinetriphosphate on galactose

TABLE I

THE INFLUENCE OF ATP ON THE ACTIVITY OF UDP-GALACTOSYL:ASIALO-MUCIN TRANSFERASE IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF UDP-GALACTOSE

Incubation and assay as in Materials and Methods. Results expressed in nmol galactose transferred/30 min per mg Golgi protein. Values are the means of three experiments.

UDPgalactose (mM)	ATP (mM)		
	0	2	4
0.048	0.71	3.35	3.0
0.925	121.7	114.3	97.7
4.60	230.0	191.0	159.0

TABLE II

IDENTIFICATION OF [^{14}C]HEXOSAMINE IN HYDROLYSATE FROM ^{14}C-LABELED ACCEPTOR PROTEIN

2.5 mg of ^{14}C-labeled mucin prepared as described in Materials and Methods was subjected to hydrolysis in 2 M HCl at 100°C for 16 h. The hydrolysate, diluted to a final concentration of 0.3 M HCl, was applied to a Dowex 50W-2X ion-exchange resin, and the fraction passing through the column, eluate I, was collected. After elution of bound hexosamines with 2 M HCl, eluate II was recovered and radioactivity in the two fractions was measured after addition of 10 ml Aquasol-II scintillation liquid.

	cpm	Radioactivity (%)
Control	1220	100
Eluate I	1135	93.0
Eluate II	53	4.5

transfer in the presence of increasing amounts of UDPgalactose. At low substrate concentrations, adenosinetriphosphate strongly increases the incorporation of galactose; while with excess UDPgalactose adenosinetriphosphate inhibits galactose transfer. The results point out the danger of using nucleotides as a substitute for high amounts of substrate.

Fig. 4 illustrates the activity of β-galactosidase at different pH values using *p*-nitrophenyl-β-galactopyranoside as substrate. At pH 7.5 the β-galactosidase activity is very low and insignificant in comparison with the activity of the transferase.

The necessity of using detergents for the analysis of galactosyltransferase to several exogenous glycoprotein acceptors has been reported. Fleischer and

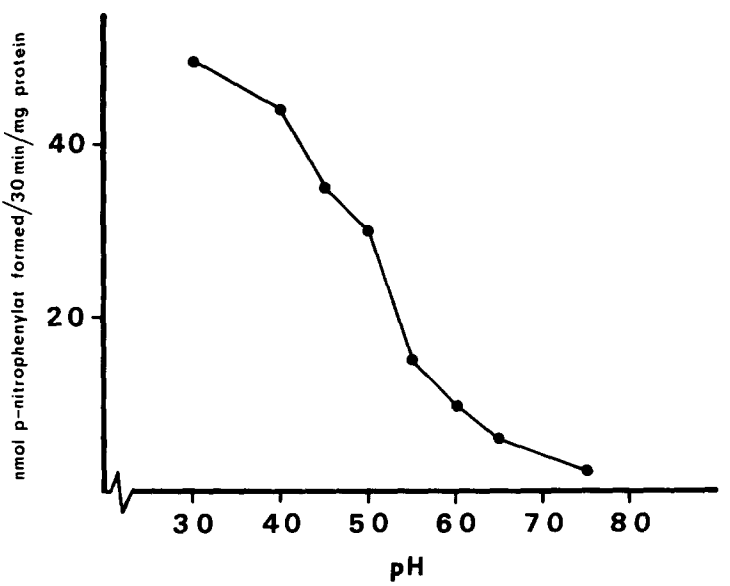

Fig. 4. β-Galactosidase activity at different pH. The incubation medium contained 0.02 M citrate/phosphate buffer, 0.04% Triton X-100, 4 mM *p*-nitrophenyl-β-galactopyranoside and sample 0.1 mg in a final volume of 0.5 ml.

Smigel [8] have shown that more than 90% of the transferase activity can be found in the 100 000 × *g* supernatant after treating the Golgi membrane with 2 mg Triton/mg protein. Beaufay et al. [1] showed a 30-fold stimulation of the enzyme activity by Triton in microsomes using ovalbumin as acceptor. For postnuclear supernatant and Golgi membranes Bretz and Stäubli [3] described a three-fold increase in galactose transfer to ovomucoid. In Golgi membranes we observed a slight decrease in galactose transfer to asialo-mucin using up to 4 mg of Triton X-100/mg of protein (Fig. 5). This decrease amounted to at most 30% of the total activity using 1 mg Triton/mg Golgi protein. With higher quantities of Triton/protein, no further inhibition could be detected. These results indicate that Triton X-100 is not required to measure maximal activity with our acceptor. The specific activity of UDPgalactosyl:asialo-mucin transferase in our system was 230 nmol galactose transferred/30 min per mg protein, which equals the activity reported by Bretz and Stäubli [3] with maximal Triton stimulation. For the galactosyltransferase activity in microsomal fractions a 50—100% increase in enzyme activity could be detected using 2 mg Triton X-100/mg membrane protein (Andersson, G.N. and Eriksson, L.C., unpublished data). Thus, there is a difference between membrane fractions which makes it important to study the properties of the enzyme in as pure and well characterized membrane fractions as possible. In a recently published paper [17] it has been reported that the latency of UDPgalactosyltransferase activity in the Golgi system drastically decreases with aging of the fractions, indicating either cessation of the membrane permeability barrier or alterations in the properties of the enzyme molecule itself. In this study, Golgi membranes were assayed for galactosyltransferase activity the same day as the preparation,

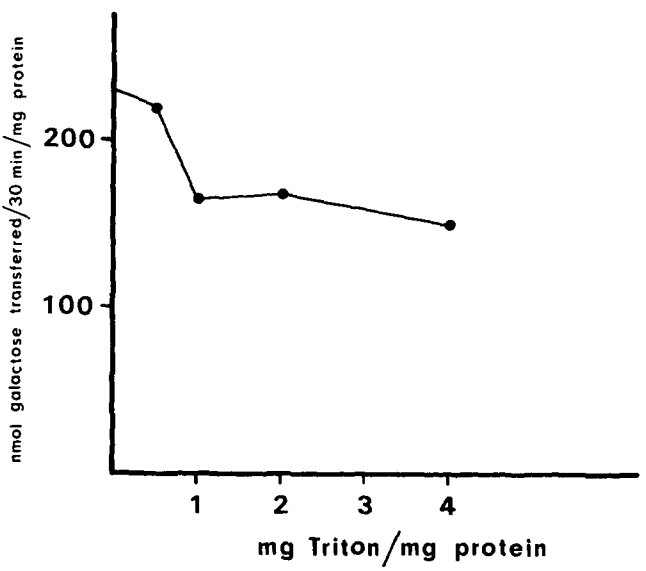

Fig. 5. The effect of Triton X-100 on the incorporation of [^{14}C]galactose into asialo-mucin. Triton was added to the incubation mixture to give the indicated final concentrations. The incubation and assay were carried out as described in Fig. 1 except the addition of Triton X-100 to the incubation medium. The values are the means of five experiments.

indicating that the aging factor cannot be responsible for the inhibitory effect of Triton X-100 on the enzyme activity.

In this study we have developed a procedure for the optimal determination of UDPgalactosyltransferase in the Golgi system of rat liver. Using high concentrations of UDPgalactose we could avoid the addition of nucleotides which seems to inhibit galactose incorporation. In contrast to other reports we could not find any stimulatory effect of Triton in Golgi membranes. This fact cannot yet be explained by current knowledge.

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