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EFFECTS OF URIDINE NUCLEOTIDES AND NUCLEOTIDE PYROPHOSPHATASE ON GLYCOLIPID α- AND β-*N*-ACETYLGALACTOSAMINYLTRANSFERASE ACTIVITIES IN GUINEA PIG MICROSOMES

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Summary

Membrane-bound α- and β-*N*-acetylgalactosaminyltransferases (EC 2.4.1.0) which catalyze formation of non-reducing terminal linkages of Forssman hapten and globoside, respectively, could be differentiated with respect to the different effects of UDP on the two enzyme activities. UDP markedly inhibited the α-transferase activity, in contrast to its stimulatory action on the β-transferase. These effects of UDP were similar to those of UDPglucose, which was demonstrated to be a competitive inhibitor (K_i, $3.3 \cdot 10^{-5}$ M for UDP-*N*-acetylgalactosamine) for the α-transferase reaction. Other uridine derivatives tested suppressed both the transferase activities, being more inhibitory for the α-transferase than for the β-transferase.

Under the synthetic conditions of these aminoglycolipids, UDP-*N*-acetylgalactosamine as a donor was simultaneously degraded into *N*-acetylgalactosamine-1-phosphate and finally into *N*-acetylgalactosamine by UDP-*N*-acetylgalactosamine pyrophosphatase, which is part of the membrane system. UDPglucose was confirmed as being able to prevent the enzymatic hydrolysis of UDP-*N*-acetylgalactosamine. UDPglucose, therefore, acts to suppress both the α-*N*-acetylgalactosaminyltransferase (but not the β-transferase) and the pyrophosphatase activities. The inhibitory effect of UDPglucose on the α-transferase activity was most probably due to its direct action on the transferase rather than its function in protecting UDP-*N*-acetylgalactosamine donor from pyrophosphatase action.

Introduction

Previous studies from this laboratory have shown that microsomal preparations of guinea pig tissues can catalyze the transfer of *N*-acetylgalactosamine

from UDP-*N*-acetylgalactosamine to trihexosylceramide (α-Gal-Gal-Glc-ceramide) and globoside (β-GalNAc-Gal-Gal-Glc-ceramide), synthesizing globoside [1] and Forssman hapten (α-GalNAc-GalNAc-Gal-Gal-Glc-ceramide) [2], respectively. Although the properties of UDPGalNAc: trihexosylceramide β-*N*-acetylgalactosaminyltransferase (EC 2.4.1.0) and UDPGalNAc: globoside α-*N*-acetylgalactosaminyltransferase of microsomes were found to be similar to each other, the effects of UDPglucose on the two enzyme reactions were quite different. Among the compounds tested, such as NaF, NaN_3, *p*-chloromercuribenzoic acid, reduced glutathione, *N*-acetylgalactosamine, *N*-acetylglucosamine and ATP, UDPglucose slightly stimulated the activity of the β-transferase [1], whereas it strongly suppressed that of the α-transferase [2]. On the other hand, an extensive degradation of a donor, UDP-*N*-acetylgalactosamine, was observed in the conditions of synthetic reactions of these aminoglycolipids owing to hydrolase(s), which was in the enzyme preparation [1]. A high level of nucleotide pyrophosphatase (dinucleotide nucleotidehydrolase, EC 3.6.1.9) activity has been demonstrated in microsomes [3–5]. Since membrane-bound glycosyltransferases have not been purified, the membrane systems used for their assay contained variable amounts of nucleotide pyrophosphatase, and caused hydrolysis of sugar nucleotides even under the synthetic conditions of glycolipids [1,6] and glycoproteins [5,7]. It is possible, therefore, that a concerted action of glycosyltransferases and sugar nucleotide pyrophosphatase may exert a control on glycolipid biosynthesis.

The present paper describes the study of the different effects of uridine and its derivatives on glycolipid α- and β-*N*-acetylgalactosaminyltransferase reactions, and the relationship between the transferase and the pyrophosphatase under the conditions of the glycolipid synthesis. A preliminary communication of this study has been reported [8].

Materials and Methods

Sources of the following chemicals were as indicated. UDP-*N*-acetyl-[^{14}C]-galactosamine (47.2 Ci/mol) from New England Nuclear; uridine, UMP and UDPglucose from Kyowa Hakko Co.; and UDP, UDPgalactose and UDP-*N*-acetylglucosamine from Sigma Chemical Co. Acid phosphatase (from horseradish) was obtained from Miles Servac Ltd. The other chemicals were analytical grade.

Trihexosylceramide and globoside from human kidney [9], and Forssman hapten from equine spleen [10] were prepared as described previously.

The microsomal fraction was prepared from newborn guinea pig kidney [1], and used as enzyme preparation. Protein was measured by the method of Lowry et al. [11].

The α- and β-*N*-acetylgalactosaminyltransferase activities for biosynthesis of Forssman hapten [2] and globoside [1], respectively, were assayed according to the previous methods. Each incubation mixture (total volume, 100 μl) contained 50 μg of glycolipid acceptor (trihexosylceramide and globoside, for globoside and Forssman hapten synthesis, respectively), 300 μg of Triton X-100, 0.1 M of sodium cacodylate-HCl buffer (pH 6.9), 10 mM of $MnCl_2$, 500 μg of microsomal protein and 50 nCi of UDP-*N*-acetylgalactosamine (1.06

nmol). This mixture was employed throughout the experiments in this study unless otherwise indicated. After the reaction mixture was incubated at 37°C for 30 min, the reaction was stopped by adding 10 μl each of 0.5 M KCl and 0.25 M EDTA and 0.6 ml of chloroform/methanol (2 : 1, v/v). After shaking and centrifugation, the lower phase was used for the determination of *N*-acetylgalactosaminyltransferase activities, and the upper phase for that of sugar nucleotide hydrolases. The lower phase was applied to thin-layer chromatography with a solvent system of chloroform/methanol/water (60 : 35 : 8, v/v). The radioactivity corresponding to the spots of Forssman hapten and globoside was counted by the liquid scintillation technique.

UDP-*N*-acetylgalactosamine pyrophosphatase activity was determined on Folch's upper phase [12] obtained from an identical incubation mixture as described for the assay of glycolipid glycosyltransferase activity. An aliquot of the upper phase was subjected to descending paper chromatography at 22°C on filter paper (Toyo-Roshi No. 51), with a solvent system of ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, by vol.). The area corresponding to each radioactive peak (Peak 2 in Fig. 4a, Peaks 3 and 4 in Fig. 4b) on the paper was cut out and counted for radioactivity of *N*-acetylgalactosamine, UDP-*N*-acetylgalactosamine and *N*-acetylgalactosamine 1-phosphate, respectively, in a toluene scintillation liquid by a liquid scintillation spectrometer.

A high-voltage electrophoresis for separation of *N*-acetylhexosamines and of their phosphate esters was carried out using the Whatman 3 MM paper in 0.05 M sodium tetraborate buffer (pH 9.3) at 60 V/cm for 30 min.

Results

Effects of uridine derivatives on glycolipid α- and β-N-acetylgalactosaminyltransferase

UDPglucose had been found to exert reverse effects on glycolipid α- and β-*N*-acetylgalactosaminyltransferase activities [1,2]. In this relation, uridine and its derivatives at a fixed concentration (2 mM) were examined for their action on both the transferase activities (Table I). UDP showed an inhibitory action

TABLE I

EFFECT OF URIDINE AND ITS DERIVATIVES ON THE INCORPORATION OF *N*-ACETYLGALACTOSAMINE INTO GLOBOSIDE AND FORSSMAN HAPTEN

Chemicals added (2 mM)	% of incorporation to the control experiments	
	Globoside	Forssman hapten
None	100	100
Uridine	60	66
UMP	86	58
UDP	139	34
UDP glucose	104	16
UDP galactose	56	9
UDP-*N*-acetylglucosamine	98	12

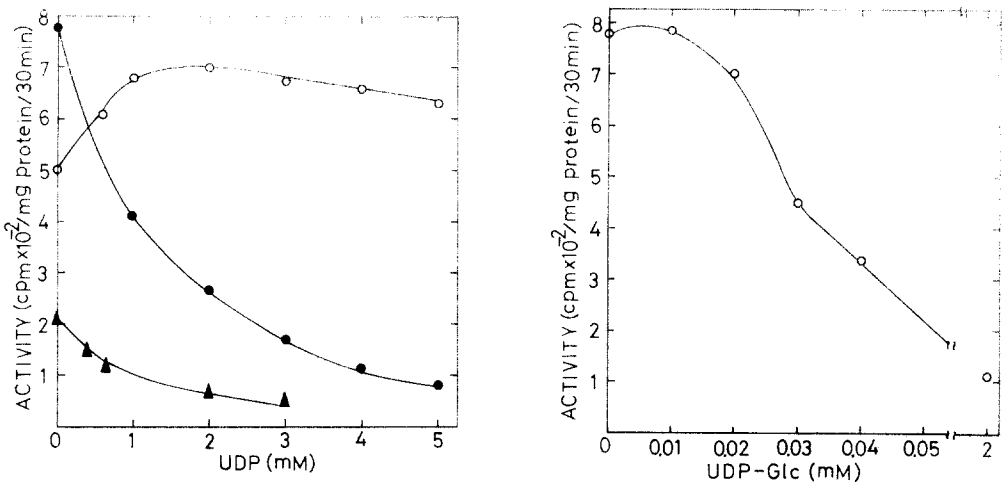


Fig. 1. Effects of UDP on the glycolipid α- and β-*N*-acetylgalactosaminyltransferase activities. The assay method was the same as described in Materials and Methods, with the exception that UDP of the indicated concentration was added to the complete mixture. The incorporation of *N*-[^{14}C]acetylgalactosamine into globoside (○) and Forssman hapten (● and ▲, using globoside and trihexosylceramide as acceptor, respectively) are shown.

Fig. 2. Effects of UDPglucose on the incorporation of *N*-acetylgalactosamine into Forssman hapten. The assay was performed as described in Materials and Methods, except that UDPglucose of the various concentrations was added to the complete incubation mixture.

on the α-transferase, but a stimulatory action on the β-transferase, similar to those of UDPglucose. UDP-*N*-acetylglucosamine had strong inhibitory effect on the α-transferase, but no effect on the β-transferase. The other compounds tested were more or less inhibitory toward both the enzymes, though the degree of inhibition was greater for the α-transferase than for the β-transferase. Sugar nucleotides generally had stronger effects compared to those of uridine and its phosphates. The markedly different effects of UDP on both the enzyme activities were also confirmed by varying its concentration, as illustrated in Fig. 1. The biosynthesis of Forssman hapten from trihexosylceramide by the β- and α-*N*-acetylgalactosaminyltransferases was also markedly inhibited by UDP. The synthesis of Forssman hapten catalyzed by the α-*N*-acetylgalactosaminyltransferase was suppressed by UDPglucose at higher concentrations than 20 μM, of which concentrations were much lower than the effective concentrations of UDP (Fig. 2). The effect of UDPglucose on the α-transferase was similar to that of UDP. Fig. 3 illustrates that 40 μM UDPglucose is a competitive inhibitor of α-*N*-acetylgalactosaminyltransferase for UDP-*N*-acetylgalactosamine with a K_i of $3.3 \cdot 10^{-5}$ M.

The enzymatic hydrolysis of UDP-N-acetylgalactosamine by microsomes and the effects of UDPglucose

In order to identify the degradation products, radioactive materials were examined using Folch's upper phase from the incubation mixture for the biosynthesis of Forssman hapten. As shown in Fig. 4a and 4b, three radioactive materials (Peaks 2, 3 and 4) were separated by means of paper chromatog-

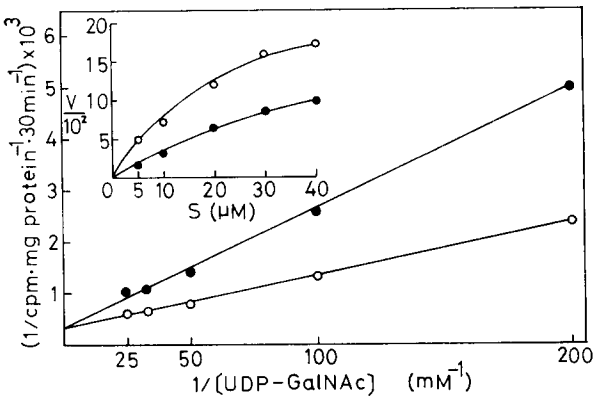


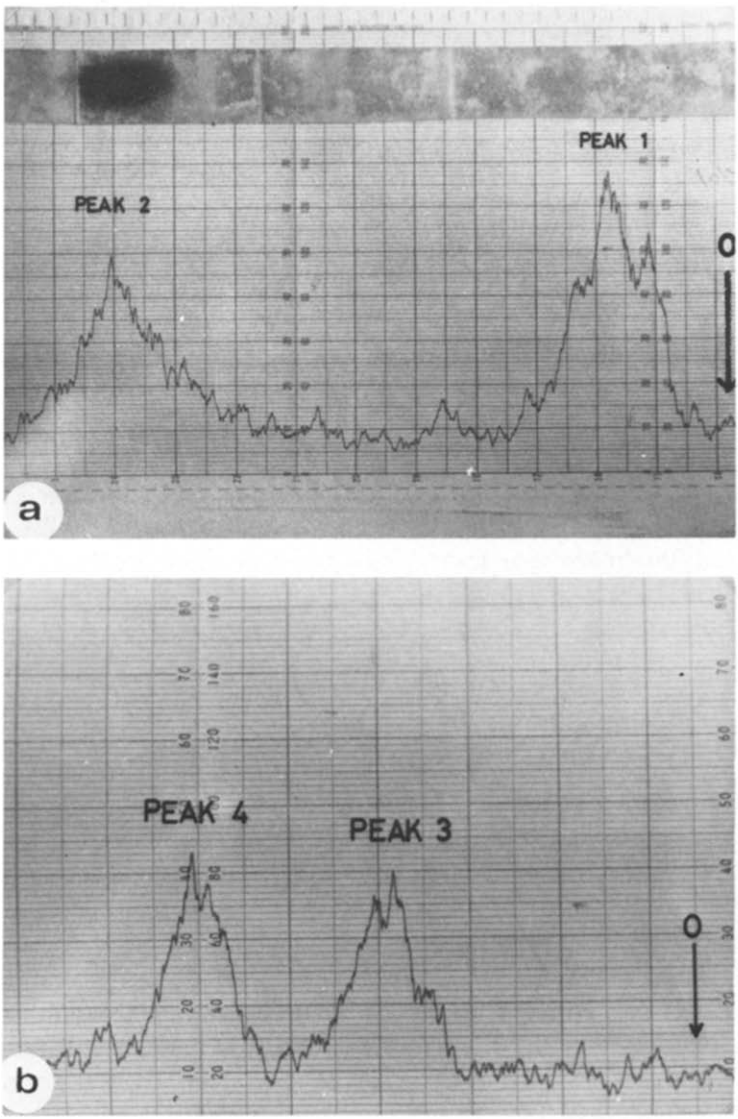
Fig. 3. Effects of UDP-*N*-acetylgalactosamine concentration on the velocity of Forssman hapten biosynthesis in the absence (○) and presence (●) of UDPglucose. The assay was carried out as described in Materials and Methods, except that concentration of UDP-*N*-acetylgalactosamine was varied as indicated.

raphy. Peak 3 material migrated together with UDP-*N*-acetylgalactosamine by a paper chromatography as well as by an electrophoresis. After the area corresponding to Peak 2 and 4 was cut out separately, the materials were eluted with water and then lyophilized. When Peak 2 material was subjected to high-voltage paper electrophoresis, it had the same mobility as an authentic *N*-acetylgalactosamine (Fig. 4c). Peak 4 material was identified as *N*-acetylgalactosamine 1-phosphate, as follows. After hydrolysis of the material with mild acid (0.01 M HCl for 7 min at 100°C) or with acid phosphatase from horseradish (1 mg, for 3 h at 37°C in 0.1 M acetate buffer, pH 5.0), followed by paper electrophoresis, the radioactive Peak 4 completely disappeared, and moved to the position of *N*-acetylgalactosamine (Fig. 5). Non-lipid radioactive products recovered from the incubation mixture for globoside synthesis were also identified as *N*-acetylgalactosamine, *N*-acetylgalactosamine 1-phosphate and intact UDP-*N*-acetylgalactosamine. Under the assay conditions described here, any other *N*-acetylhexosamines, their phosphates or their nucleotides were not detected, indicating that UDP-*N*-acetylhexosamine epimerases and phospho-*N*-acetylhexosamine mutase are absent in the membrane system used or not operative.

To examine the effects of nucleotide pyrophosphatase on the *N*-acetylgalactosaminyltransferase activities, the hydrolysis of UDP-*N*-acetylgalactosamine by guinea pig microsomal enzyme was determined in the presence or absence of UDPglucose under the biosynthetic conditions of Forssman hapten. The formation of labeled *N*-acetylgalactosamine 1-phosphate and *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine was proportional to the incubation time (data not shown), and with the various concentrations of the sugar nucleotide in absence of UDPglucose (Fig. 6). The relative rate of UDP-*N*-acetylgalactosamine (50—55%) left in the mixture, *N*-acetylgalactosamine (30%) and *N*-acetylgalactosamine 1-phosphate (15—20%) produced were almost constant irrespective of the concentrations of UDP-*N*-acetylgalactosamine added, as shown in the inserted diagram in Fig. 6. On the other hand, in the presence of UDPglucose the hydrolysis of UDP-*N*-acetylgalactosamine was significantly inhibited, as shown in Fig. 7.

Discussion

In the present study, UDPGalNAc : globoside α-*N*-acetylgalactosaminyltransferase and UDPGalNAc : trihexosylceramide β-*N*-acetylgalactosaminyltransferase activities of guinea pig tissue were examined for the influence of uridine nucleotides. The two transferases could be clearly distinguished with respect to the effects of UDP on the enzyme activities (Fig. 1). UDP enhanced the activity of the β-*N*-acetylgalactosaminyltransferase activity to a small degree, whereas it strongly inhibited the α-transferase. These effects were similar to those of UDPglucose. It was found that the inhibition of the α-*N*-acetylgalactosaminyltransferase activity by UDPglucose, and also probably



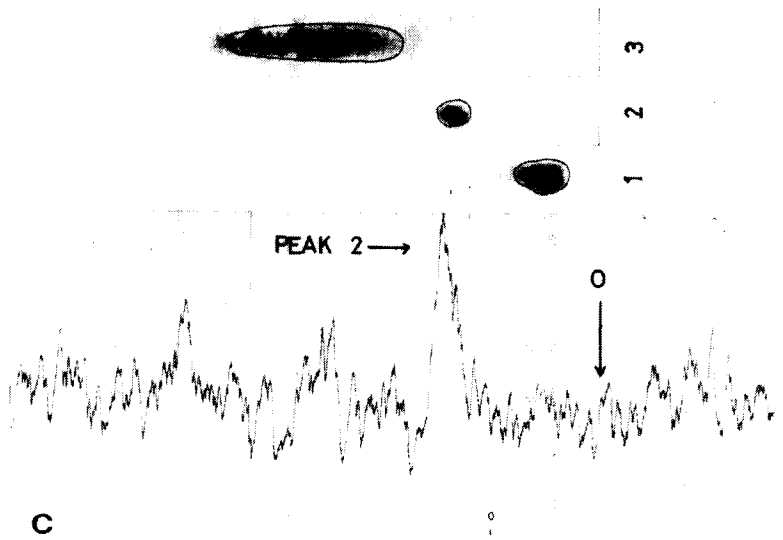


Fig. 4. Radiochromatographic scans of degradation products from UDP-*N*-acetylgalactosamine under the conditions of Forssman hapten biosynthesis. An aliquot of the upper phase obtained from the incubation mixture as described in Materials and Methods was subjected to paper chromatography, developing with ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3, by vol.) for (a) 10 h and (b) 40 h at 22°C. Then the paper was scanned for radioactivity. By chromatography for 40 h, Peak 1 was separated into two further peaks (Peaks 3 and 4), though Peak 2 moved off the paper. (c) Scan of Peak 2 material after high-voltage paper electrophoresis. The spot above Peak 2 on the paper chromatogram in (a) indicates standard *N*-acetylgalactosamine. Lanes 1, 2 and 3 are authentic *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylmannosamine, respectively, colored by spraying with alkaline $AgNO_3$ reagent. Arrows indicate the origin. For details see Materials and Methods.

by UDP, was of the competitive type (Fig. 3). In studies on the biosynthesis of glycoproteins [13,14], inhibition of glycosyltransferases activities by nucleotides and their glycosyl derivatives has been demonstrated. Baker et al.,

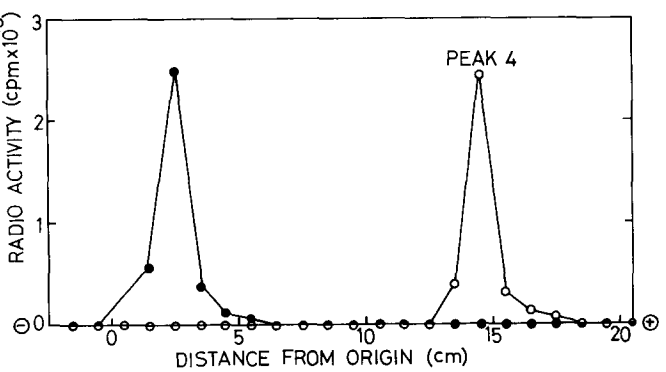
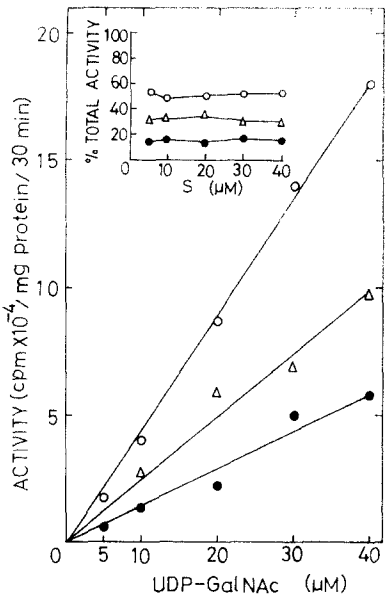


Fig. 5. Electrophoretogram of Peak 4 material before and after mild acid hydrolysis. The material from Peak 4 (Fig. 4b) was subjected to high-voltage paper electrophoresis before (○) and after (●) acid hydrolysis with 0.01 M HCl at 100°C for 7 min. After electrophoresis, 1-cm pieces of the paper were cut out and counted for radioactivity by a liquid scintillation technique.



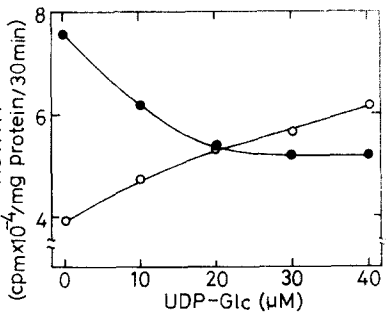


Fig. 6. Enzymatic hydrolysis of UDP-*N*-acetylgalactosamine occurred during the Forssman hapten biosynthesis by guinea pig microsomes. The hydrolytic activities of microsomal sugar-nucleotide hydrolases were assayed as described in Materials and Methods, using the upper phase from the incubation mixture for the α-*N*-acetylgalactosaminyltransferase assay. ○, UDP-*N*-acetylgalactosamine remained in the reaction mixture; △, *N*-acetylgalactosamine produced; ●, *N*-acetylgalactosamine 1-phosphate produced.

Fig. 7. UDP-*N*-acetylgalactosamine pyrophosphatase activities of guinea pig microsomes in presence of UDPglucose. The pyrophosphatase activities were assayed as described in Materials and Methods, using the upper phase from the incubation mixture for the α-*N*-acetylgalactosaminyltransferase reaction, except that UDPglucose at various concentrations was added. The enzymatic activities are expressed as the sum of *N*-acetylgalactosamine 1-phosphate and *N*-acetylgalactosamine produced. ○, UDP-*N*-acetylgalactosamine remaining; ●, *N*-acetylgalactosamine 1-phosphate plus *N*-acetylgalactosamine produced.

using tracheal microsomes, reported that some uridine nucleotides inhibited to varying extents the transfer of *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine into a glycoprotein acceptor; for example, 24% inhibition at 1 mM of UDP and 72% at 10 mM [13]. On the other hand, various nucleotides and their derivatives have been demonstrated to stimulate the transfer of sugars from sugar nucleotides into glycoprotein acceptors [5,15]. Mookerjea and Yung [5] showed that the activity of UDPGal : glycoprotein galactosyltransferase of liver microsomes was activated 2- to 4-fold by addition of UDP or its glycosyl derivatives. It remains to be determined whether the type of effect obtained by nucleotides on glycosyltransferases depends on the type of transferase or on the sugar nucleotide donor. Compounds exhibiting opposite effects on the activities of anomeric glycosyltransferases, which use the same sugar nucleotide as donor, have not up till now been found.

As observed before [1], in addition to the glycosyltransferase activities, guinea pig microsomes showed very high UDP-*N*-acetylgalactosamine pyrophosphatase activity even under the synthetic conditions of aminoglycolipids. Besides, *N*-acetylgalactosamine of the degraded product can not be derived from the labeled glycolipid by the action of *N*-acetylhexosaminidase, since

enzymatic hydrolysis of the labeled glycolipid products by glycosidases was negligible under the conditions of the transferase assay [1]. An appreciable amount of sugar nucleotide pyrophosphatase activity was detected in a particulate fraction from normal and transformed cell lines in tissue culture under the biosynthetic conditions of gangliosides [6]. Nucleotide pyrophosphatase purified from liver particulates [3,4] or plasma membranes [16—18], possessed low specificity for substrates such as ATP, UDP-sugars, pyridine nucleotides and acyl-CoA. A number of these nucleotides have been demonstrated to be competitive inhibitors for the pyrophosphatase [17,19]. In this communication, UDPglucose was confirmed to prevent the hydrolysis of UDP-*N*-acetylgalactosamine even under the synthetic conditions of aminoglycolipids. Although UDPglucose itself will undergo hydrolysis to give UMP and uridine by a coupled function of pyrophosphatase and 5′nucleotidase, both of which are operative in the membrane system, the inhibitory action on the α-transferase will mainly be due to UDPglucose left in the reaction mixture, because UMP and uridine are much less effective for the transferase enzyme compared to that of UDPglucose (Table I). The powerful competitive inhibition of UDPglucose on the α-transferase must be superior to its protective effect against hydrolysis of the donor nucleotide by pyrophosphatase. Some enhancement of the β-*N*-acetylgalactosaminyltransferase activity observed in presence of UDP and UDPglucose could be related to their protective effect from pyrophosphatase and to their blocking action on the formation of Forssman hapten which will be synthesized from trihexosylceramide by two-step reactions (see Fig. 1).

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