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THE BIOSYNTHESIS OF A N,N'-DIACETYLCHITOBIOSE CONTAINING LIPID BY LIVER MICROSOMES. A PROBABLE DOLICHOL PYROPHOSPHATE DERIVATIVE.

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SUMMARY: Incubation of liver microsomes with dolichol monophosphate, Mg^{++} and UDP-[^{14}C]-N-acetylglucosamine leads to the appearance of radioactivity in the lipid fraction. Mild acid treatment results in the formation of N-acetylglucosamine and N,N'-diacetylchitobiose (2-acetamido-2-deoxy-O-β-D-glucopyranosyl-(1⟶4)-2-acetamido-2-deoxy-D-glucose). The formation of the disaccharide containing lipid was increased by incubation with crude liver lipids or by reincubation with unlabelled UDP-N-acetylglucosamine. The labelling in the latter compound varied according to whether one of the N-acetylglucosamyl residues arose from a crude lipid or from unlabelled UDP-N-acetylglucosamine. Evidence is presented indicating that the compounds are dolichol pyrophosphate derivatives.

It has been reported (1-3) that liver and brain microsomes catalyze the formation from UDP-N-acetylglucosamine of a substance soluble in organic solvents. Since dolichol monophosphate greatly enhanced the formation of the compound it was assumed to be a dolichol derivative (3). It has now been found that under appropriate conditions a N,N'-diacetylchitobiose containing compound is also formed.

Methods. The substrates and methods were described in previous papers (4,5). N,N'-diacetylchitobiose was prepared by partial acid hydrolysis of chitin followed by paper chromatography (6). The standard incubation mixture was as follows: a dolichol monophosphate concentrate purified up to the DEAE-cellulose step (4), containing 30 ug of total phosphate, was dried with 0.5 μmoles of Mg-ethylendiamine tetracetate and 0.5 μmoles of $MgCl_2$. Rat liver microsomes (about 1.5 mg protein) were suspended in 0.1 M mercaptoethanol, 0.1 M tris maleate buffer pH 7.8 and 0.5 % deoxycholate in a final volume of 50 μl. Where indicated UDP-[^{14}C]-N-acetylglucosamine (New England 45 Ci/M, 60,000 cpm)

was included. After 30 min at 30°, chloroform, methanol and 4mM $MgCl_2$ 3:2:1 were added and the organic phase was separated and washed with theoretical upper phase (7).

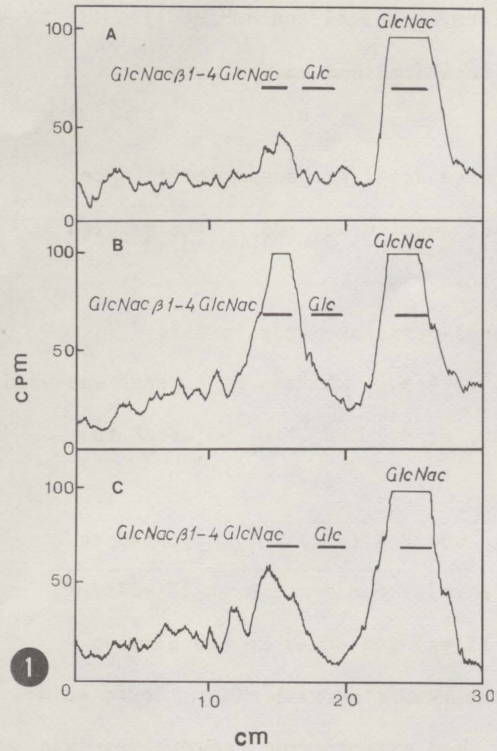
Mild acid hydrolysis. The labelled lipids were dried and suspended in water. Hydrogen chloride was added until thymol blue just turned red. The samples were then heated 10 min at 100°.

Paper chromatography. Solvent A was 1-butanol-pyridine-water, 6:4:3. Solvent B was 2-propanol-acetic acid-water, 27:4:9. Whatman N° 1 paper was used as such or previously dipped into 0.1 M $ZnSO_4$ or 0.2 M $Na_2B_4O_7$ adjusted to pH 8.5 with H_2SO_4 and dried.

Paper electrophoresis. The electrolyte used was 2 % $(NH_4)_2MoO_4$ adjusted to pH 5 with H_2SO_4 (8). Electrophoresis was carried out for 1 h at 25 volt/cm.

Borohydride reduction. Sodium borohydride (5 mg) was added to the samples in 0.3 ml water. After 18 h at 5° they were acidified with HCl. Boric acid was removed by repeated addition of methanol and evaporation. Hydrolysis was carried out in 2 N HCl at 100° for 4 h. The samples were then spotted on a small paper (2.5 x 7 cm) and chromatographed several times with pyridine. The salts remained at the origin and the radioactive substances could be eluted from the rest of the paper for electrophoresis.

Results - The formation of a slow moving substance. Incubation of liver microsomes with dolichol monophosphate and UDP-$[^{14}C]$-N-acetylglucosamine followed by extraction with chloroform-methanol, mild acid hydrolysis and paper chromatography, showed that besides a "fast moving" substance running like N-acetylglucosamine a trace amount of a "slow moving" substance was formed (Fig. 1 A). If the chloroform-methanol extract of an identical incubation mixture was dried and incubated with fresh enzyme and non-labelled UDP-N-acetylglucosamine, the amount of slow moving substance was greatly increased (Fig. 1 B). An experiment in which the order of addition was inverted (Fig. 1 C), that is, a first incubation was carried out with non-labelled and a second with labelled UDP-N-acetylglucosamine, showed that slightly more slow-



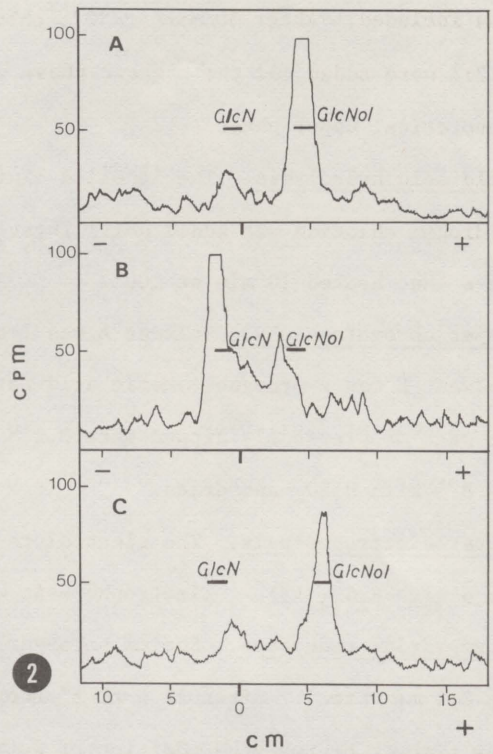


Figure 1. Paper chromatography (solvent A) of the products of mild acid hydrolysis of the lipid fraction obtained by incubation with the standard mixture (see Methods) under the following conditions:
A incubation with UDP- [^{14}C] -acetylglucosamine.
B incubation with UDP- [^{14}C] -acetylglucosamine. The lipid fraction was extracted, dried and reincubated with 0.25 µmoles of unlabelled UDP-N-acetylglucosamine and the standard mixture.
C Same as B but incubated first with unlabelled and then with labelled UDP-N-acetylglucosamine.

Figure 2. Electrophoresis in molibdate was carried out with the substances obtained as follows: the labelled lipids from standard incubation mixture were hydrolyzed with mild acid, and the sugars were separated by paper chromatography (solvent A). The radioactive zones were eluted from the paper, reduced with borohydride and hydrolyzed in 2 N HCl for 4 h at 100° and then submitted to electrophoresis.
A corresponds to the fast peak of an incubation with crude lipid extract and UDP- [^{14}C] -acetylglucosamine.
B corresponds to the slow peak of same.
C corresponds to the slow peak of a double incubation with labelled and then unlabelled UDP-N-acetylglucosamine similar to that shown in Fig. 1 B.
GlNol stands for glucosaminitol and GlcN for glucosamine.

moving substance than in the control was formed.

An increased formation of slow moving substance was also obtained if crude fractions of liver lipids were included in the standard incubation mixture.

TABLE I. Paper chromatography of the products of mild acid hydrolysis.

Solvent	A	A	A	A	B
Paper	untreated	borate	zinc	untreated	untreated
Substance	Mobility relative to N-acetylglucosamine				
Fast moving	0.98	0.99	1.01	0.64*	1.03
Slow moving	0.70	0.45	0.70	0.63*	0.76
N,N'-diacetylchitobiose	0.70	0.44	0.73	----	0.76
Glucose	0.73	0.03	0.77	0.79	0.76
Glucosamine	----	----	0.13	0.65	0.69

* after heating 3 h at 100° in 3 N HCl

The mobility of the slow moving substance was the same as that of N,N'-diacetylchitobiose, with two solvents and with borate or zinc treated paper (Table 1). Under these latter conditions the relative mobility of other sugars is considerably modified. Acid hydrolysis of the slow moving substance lead to the formation of a product having the mobility of glucosamine.

Borohydride reduction. The distribution of the radioactivity within the N,N'-diacetylchitobiose was measured after reducing with borohydride, hydrolyzing and separating the products by electrophoresis. In one experiment UDP-$[^{14}C]$-N-acetylglucosamine and a crude lipid extract of liver were added to the standard reaction mixture. After the incubation the lipid fraction was subjected to mild acid hydrolysis and the products were separated by paper chromatography. The substances corresponding to the fast and slow peaks (Fig. 1) were eluted and treated with borohydride, hydrolyzed and submitted to electrophoresis. As shown in Fig. 2 the substance from the fast peak gave mainly glucosaminitol as would be expected for free N-acetylglucosamine. The substance from the slow peak gave mainly glucosamine showing that the radioactivity in the disaccharide resided mainly in the non-reducing moiety (Fig. 2 B).

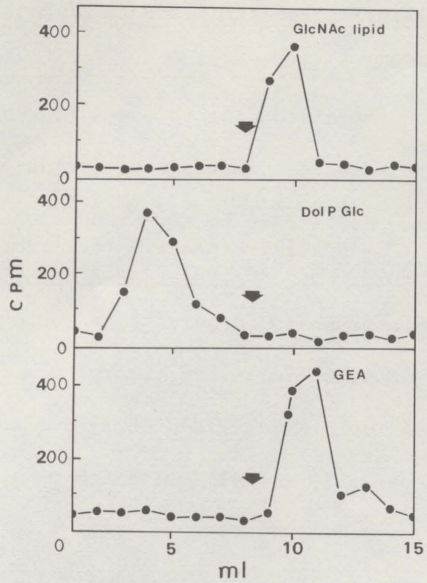


Figure 3. The lipids were poured into a DEAE-cellulose acetate column (1.5 x 0.6 cm) in Cl_3CH-CH_3OH-H_2O, 1:1:0.3 and eluted with 0.7 ml portions of the same solvent. The arrows indicate where ammonium formate (10 mM) was added.

The slow peak from a double incubation with labelled and then unlabelled UDP-N-acetylglucosamine as in Fig. 1 B, gave rise mainly to glucosaminitol showing that in this case the radioactivity appeared mainly in the reducing N-acetylglucosamine residue (Fig. 2 C).

DEAE-cellulose chromatography. It was previously reported (5,9) that some prenyl monophosphate sugars can be separated from the pyrophosphates by DEAE-cellulose chromatography. Similar experiments have been carried out now with the N-acetylglucosamine containing compounds. As shown in Fig. 3 dolichol monophosphate glucose was not retained by DEAE-cellulose under these conditions while the N-acetylglucosamine containing lipid and GEA[1] required salt for elution. In an experiment not shown it was observed that the N,N'-diacetylchitobiose containing lipid behaved like that of N-acetylglucosamine.

[1]GEA stands for the substance formed by the transfer of glucose from dolichol monophosphate glucose to an endogenous acceptor of liver microsomes. It appears to contain a 17-20 glycosyl unit oligosaccharide bound to dolichol through a pyrophosphate bridge (5).

The results of alkaline decomposition are consistent with the idea that the N-acetylglucosamine containing lipid may be pyrophosphate. Alkaline treatment (0.1 N KOH at 64° for 40 min) followed by electrophoresis in pyridine-acetate pH 6.5 showed that two substances were formed, one neutral and another negatively charged, which migrated like an hexosemonophosphate. Furthermore treatment with E. coli phosphatase of the negatively charged substance lead to the formation of a substance which migrated like N-acetylglucosamine with solvent A. This behaviour is similar to that of GEA (5) and different from dolichol monophosphate glucose which yields only 1-6 anhydro-glucosan (4).

A polyprenol pyrophosphate N-acetylglucosamine compound has been isolated by Hussey and Baddiley from Staphylococcus lactis (10).

Discussion. The incorporation of radioactivity in the N,N'-diacetylchitobiose containing lipid from UDP-$[^{14}C]$-N-acetylglucosamine may be increased either by addition of a crude lipid extract, which appears to contain the unlabelled N-acetylglucosamine lipid, or by reincubation of the labelled lipid with unlabelled UDP-N-acetylglucosamine.

That the compound formed really contains N,N'-diacetylchitobiose seems to be well proven by the chromatographic data, and by the fact that by strong acid treatment only glucosamine is liberated.

In a previous report it was assumed that the N-acetylglucosamine containing compound was a monophosphate (3). This conclusion was based on the similitude with the glucose and mannose containing lipids. However, Molnar et al. (2) using $[^{32}P]$ labelled UDP-N-acetylglucosamine and endogenous acceptor observed that one phosphate was also transferred to the lipid so that if the acceptor already contained one phosphate a pyrophosphate would be formed. On the basis of the behaviour of the product in DEAE-cellulose columns and of the pattern of alkaline decomposition, the reaction may be written as follows:

$$\text{UDP-GlcNAc} + \text{dolichol-P} \longrightarrow \text{UMP} + \text{dolichol-P-P-GlcNAc}$$

The formation of the N,N'-diacetylchitobiose derivative presumably would occur as follows:

dolichol-P-P-GlcNAc + UDP-GlcNAc ⟶ dolichol-P-P-N,N'-diacetylchitobiose + UDP

that is, the second transfer would take place directly from UDP-N-acetylglucosamine. If the donor were dolichol-P-P-GlcNAc, one would expect that the labelling of the two N-acetylglucosamine residues in N,N'-diacetylchitobiose would be equal, and this does not occur as proved by the fact that reduction with borohydride and hydrolysis of the free disaccharide showed unequal distribution of the radioactivity.

The N,N'-diacetylchitobiose containing lipid is of great interest because it may be involved in the biosynthesis of some glycoproteins. Asparagine linked N,N'-diacetylchitobiose has been found in thyroglobulin (6), ovalbumin (11), Aspergillus oryzae α-amylase (11), pineapple bromelain (12), ribonuclease B (13) and is probably present in many other glycoproteins (14-17). Furthermore GEA, which was found to be an intermediate in the biosynthesis of an unidentified glycoprotein (18), appears to contain also two hexosamine residues (19). It seems probable that GEA is built up on N,N'-diacetylchitobiose by successive addition of glycose residues.

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