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Glucose Transfer from Dolichol Monophosphate Glucose: The Product Formed with Endogenous Microsomal Acceptor

(dolichol monophosphate-*N*-acetylglucosamine/UDPG/liver microsomes/glycolipid/polyprenol)

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ABSTRACT **The product formed by incubation of dolichol monophosphate glucose with liver microsomes was studied. It is insoluble in most solvents, but is soluble in a chloroform–methanol mixture with a high content of water. Treatment with ammonia gave rise to the formation of a water soluble, negatively charged compound of molecular weight 3550. The negative charge could be removed by treatment with phosphatase.**

Acid hydrolysis of the original compound led to the liberation of an uncharged, water-soluble compound (molecular weight 3550). Acetolysis of the latter gave rise to the formation of a series of products, which appeared to be oligosaccharides when chromatographed on paper or silica plates. The original substance behaved like a polyprenol pyrophosphate when chromatographed on DEAE-cellulose.

Molecular weight measurements of the deoxycholate inclusion compound gave a value of 14,300, while dolichol monophosphate glucose under the same conditions gave 11,300.

It is tentatively suggested that the compound is dolichol joined through a phosphate or pyrophosphate bridge to an oligosaccharide containing about 20 monosaccharide residues.

Undecaprenol has been found to be involved in the biosynthesis of bacterial cell-wall compounds such a lipopolysaccharides (1), murein (2), and mannan (3). Animal tissues contain a different polyprenol that has been named dolichol. It contains about 20 isoprene residues, the one carrying the alcohol group being saturated (4).

In a previous paper (5) evidence was presented indicating that liver microsomal enzymes catalyze the transfer of glucose from UDPG to dolichol monophosphate (DMP) to form dolichol monophosphate glucose (DMPG). Similar reactions were subsequently found to occur with UDP-*N*-acetylglucosamine and GDP-mannose as donors (6, 7). On incubation of labeled DMPG with microsomal enzymes, another compound was formed (GEA: glucosylated endogenous acceptor), which was believed to be a glycoprotein because it is insoluble in lipid solvents, such as chloroform-methanol, and is precipitated by trichloroacetic acid.

Further work on the identification of the compound is now presented. The compound has not been obtained in sufficient amounts to analyze it by the usual methods, so that its properties have been studied by following the radioactivity of the compound labeled in the glucose residue. It appears to be an oligosaccharide bound to a lipid through a phosphate or pyrophosphate bridge.

Abbreviations: DMP, dolichol monophosphate; DMPG, dolichol monophosphate glucose; DMP-GlcNAc, dolichol monophosphate *N*-acetyl glucosamine; GEA, glucosylated endogenous acceptor; UDPG, uridine diphosphate glucose

METHODS

The methods used were described (5, 6). DMPG was prepared by incubation of UDPG, crude DMP extracted from liver, Mg^{++}, and detergent with liver microsomes (5). GEA was prepared by incubation of 0.5 mg of liver microsomes with DMPG (4000 cpm, 207 Ci/mol), 0.1 M Tris-maleate (pH 7.8), 0.3% deoxycholate, 40 mM 2-mercaptoethanol, and 10 mM Na-EDTA (total volume, 50 μl), for 20 min at room temperature. The reaction was stopped by the successive addition of 0.4 ml of methanol, 0.15 ml of 4 mM $MgCl_2$, and 0.6 ml of chloroform. The residual protein precipitate was washed again with the same solvents, but with 0.2 ml of 4 mM $MgCl_2$, and dried. Finally, GEA was extracted from the precipitate with 1 ml of chloroform–methanol–water 1:1:0.3. The usual yield of GEA was about 60-80% of the added DMPG. The same proportions of reagents and solvents were used for larger scale preparations.

Lipid monophosphate galactose, lipid pyrophosphate glucose, and lipid pyrophosphate trisaccharide (from *Acetobacter xylinum*) labeled in the carbohydrate residues were generously provided by M. Dankert. It is believed that these compounds are polyprenol derivatives. [1-^{14}C]Stearic acid from the Radiochemical Centre (Amersham) was a gift from Dr. R. Brenner. *Escherichia coli* alkaline phosphatase, type III was purchased from Sigma.

RESULTS

The solubility of GEA

It was reported that when reaction mixtures in which labeled DMPG was the glucosyl donor were analyzed after addition of chloroform–methanol–water 3:2:1, glucose appeared in the water phase, DMPG in the chloroform–methanol phase, and the rest of the radioactivity was at the interphase together with denatured protein.

The radioactive compound present in the interphase fraction could be extracted with dimethylsulfoxide, 6 M pyridine acetate (pH 4.4) or, preferably, with chloroform–methanol–water 1:1:0.3. As shown in Table 1, the solubility of GEA is higher in the mixture containing more water. The compound is insoluble in water, but may be solubilized with detergents such as 0.2% Na dodecyl sulfate or 0.2% Triton X-100. Several other solvents were tested by chromatography on

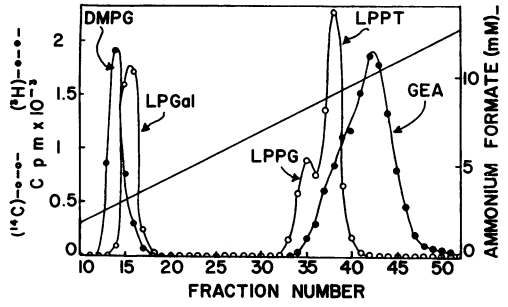

FIG. 1. Chromatography on DEAE-cellulose. A mixture containing DMPG and GEA (both labeled with tritium) and lipid monophosphate-galactose (LPGal), lipid-pyrophosphate-glucose (LPPG), and lipid-pyrophosphate trisaccharide (LPPT) from *Acetobacter xylinum* labeled with ^{14}C, was passed through a 27 × 1.1 cm column of DEAE-cellulose (acetate form) in chloroform–methanol–water 1:1:0.3, and eluted with a linear gradient of 0–13.5 mM ammonium formate in the same solvent. DMPG and GEA were identified by previous chromatography, in which they were run separately, to determine their positions of elution. Fractions of 5.4 ml were collected. The cpm values for the LPGal and GEA peaks were multiplied by 0.33 and 3.3, respectively.

small paper strips. The substance remained at the origin when run with various mixtures of chloroform–methanol without water, 1-butanol–pyridine–water 6:4:3, and aqueous ethanol with or without ammonium acetate. It moved slightly with butanol–water–acetic acid 4:5:1, (upper phase), and appeared near the solvent front with chloroform–methanol–water 1:1:0.3.

Anion-exchange chromatography

GEA could be purified by chromatography on DEAE-cellulose with chloroform–methanol–water 1:1:0.3 as solvent (Fig. 1). With a linear gradient of ammonium formate, DMPG emerged at 3 mM and GEA at 10.4 mM salt concentration. Thus, the two substances could be neatly separated.

For comparison, samples of lipid monophosphate galactose, lipid pyrophosphate glucose, and lipid pyrophosphate trisaccharide from *A. xylinum* were chromatographed under the same conditions. These compounds emerged at 3.5, 8.6, and 9.2 mM ammonium formate, respectively. Therefore, GEA behaved more like the pyrophosphates.

A similar separation of DMPG and GEA was also obtained by silica-gel thin layer chromatography (TLC) with 1-propanol–water 7:3. The R_f values for GEA and DMPG were 0.15 and 0.6, respectively. When GEA was acetylated, its R_f increased considerably.

The effect of alkali

GEA was not affected by an alkaline treatment somewhat stronger than the one usually used for saponification of glycerides (8). After 2 hr at 37°C in 0.1 N NaOH in methanol or in chloroform–methanol–water 1:1:0.3, all the radioactivity remained in the interphase after neutralization, followed by the addition of chloroform–methanol–water 3:2:1 and microsomal protein as a carrier.

However, at a higher temperature (0.1 N alkali in 1-propanol at 64°C) GEA was decomposed at about the same rate as DMPG and DMP-GlcNAc (Table 2). The water-soluble products were not studied in detail. In the case of DMPG, it was shown previously that the product is 1,6-anhydroglucosan.

After GEA was heated for 3 hr at 100°C in 10% aqueous ammonia, about 60% of the radioactivity appeared in the upper phase after the sample was dried and chloroform–methanol–water 3:2:1 was added. This decomposition product was negatively charged, as judged by paper electrophoresis in pyridine acetate (pH 6.5) at 1000 V for 3 hr. A similar alkaline treatment of DMPG and DMP-GlcNAc produced uncharged, water-soluble substances. In order to find out if the substance obtained by heating GEA with ammonia contains phosphate, it was incubated in 0.1 M Tris·HCl (pH 8.1) with purified *E. coli* alkaline phosphatase (0.01 mg of protein) for 30 min at 37°C. Electrophoresis of the reaction product obtained showed that it had no net charge.

Acid hydrolysis

Treatment of radioactive GEA with acid led to the formation of a water-soluble labeled compound. For instance, treatment with 0.1 N acid in methanol gave the results shown in Fig. 2. The half-life was about 8 min. The water-soluble product did not migrate on paper chromatography with the following solvents: ethanol–ammonium acetate (pH 7.4 or 3.8); 1-butanol–pyridine–water 6:4:3, or on paper electrophoresis in pyridine acetate (pH 6.5).

In preliminary experiments, it was found that DMPG, DMP-GlcNAc, and GEA decomposed faster in water-free solvents. Table 2 shows the half-lives of GEA, DMPG, and DMP-GlcNAc under different conditions. It may be observed that in order to obtain comparable values with 0.8 and 12% water, the temperature had to be increased from 18 to 30°C, and the acid concentration from 0.1 to 0.5 N. Under both sets of conditions, the half-life of GEA is several times higher than that of DMPG. In the water-rich solvent, the $t_{1/2}$ of GEA is about 10 times that of DMPG. In the nearly water-free solvent, GEA is not soluble, so that the significance of the values is doubtful.

Table 2 also shows the half-lives for DMP-GlcNAc. These are 2–3 times higher than those of DMPG. The difference in

TABLE 1. *Extraction of GEA from the protein precipitate by different solvent mixtures*

Chloroform	Methanol	Water	Percent extracted
2	1	—	1.5
1	2	—	14
1	1	—	10
1	1	0.085	26
2	1	0.17	25
1	2	0.17	30
1	1	0.17	57
1	1	0.25	87
1	1	0.33	97
1	1	0.415	85*
3	2	1	2*

Denatured protein precipitate containing GEA was prepared as described in Methods up to the second chloroform–methanol–4 mM $MgCl_2$ 3:2:1 wash. The pellet was dried and extracted once with 1 ml of the indicated solvent mixture and the radioactivity in the supernatant fluid was measured. The radioactivity in the unextracted protein precipitate was taken as 100%.

* Two phases formed, they were pooled and counted.

$t_{1/2}$ is smaller than was previously reported, according to which the GlcNAc compound was about eight times more stable (6). The erroneous results were probably due to the fact that the water content of the sample was not controlled.

Molecular weights of the water-soluble products

The molecular weight of the labeled product obtained by acid methanolysis (0.1 N HCl in methanol at 30°C for 60 min) was measured by gel filtration in a 93 × 1.1 cm column of Sephadex G-50 (medium) equilibrated with 0.1 M sodium phosphate (pH 6.2). Adrenocorticotropic hormone and bacitracin were used as internal standards. A value of 3550 was obtained. The methanolysis product was compared with that obtained by treatment with ammonia by running both compounds jointly under the above mentioned conditions. One of them was labeled with ^3H and the other with ^{14}C. The two substances emerged together from the column. It is likely that one substance is the methyl derivative and the other a phosphorlyated one, but the difference in molecular size is too small to be detected by this procedure.

Acetolysis

The labeled product of methanolysis was subjected to acetolysis. The conditions used were those described by Ballou (9), with minor changes (1.7 N H_2SO_4 in acetic acid–acetic anhydride 1:1 for 23 hr at 55°C). The acetylated products gave four peaks on silica-gel TLC with benzene–methanol 95:5. The deacetylated products were chromatographed on paper with unlabeled malto-oligosaccharides as internal standards. The solvent used was 1-butanol–pyridine–water 9:5:7. Glucose and eight other radioactive peaks were observed.

The $R_{glucose}$ values were as follows:

Radioactive peaks: 0.83, 0.74, 0.55, 0.45, 0.295, 0.18, 0.115, and 0.075

TABLE 2. *Acid and alkaline hydrolysis*

	Experiment		
	1	2	3
Temperature, °C	64	30	18
Acid or base	0.1 N NaOH	0.5 N HCl	0.1 N HCl
Water content, %	0.5	12	0.8
Solvent	1-propanol	chloroform–methanol–water 1:1:0.3	chloroform–methanol 2:1
Substance	Half-life (min)		
DMPG	8	6	5–6
DMP-GlcNAc	12	18	10–12
GEA	11–15*	70	140*

GEA was estimated after neutralization of the acid or alkali with a slight excess of triethanolamine or acetic acid, respectively. An aliquot was then spotted on a Whatman No. 1 paper strip of 6 × 2.5 cm and developed with water. The origin was cut out and counted in a scintillator. For DMPG and DMP-GlcNAc, the same method or separation by partition in chloroform–methanol–water 3:2:1 was used.

* The reaction mixture was dried after neutralization, dissolved in chloroform–methanol–water 1:1:0.3, and spotted on the paper.

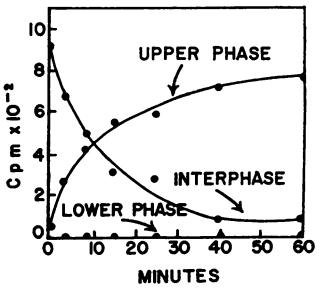

FIG. 2. Acid methanolysis. GEA was treated with 0.1 N HCl in methanol at 30°C for the indicated times, after which chloroform, water, and concentrated microsomal protein were added. The final proportion was chloroform–methanol–water 3:2:1.25. The lower and upper phases were counted in a flow counter. The interphases were dried, suspended in Bray's solution, and counted in a liquid scintillation counter.

Malto-oligosaccharides: 0.79, 0.59, 0.43, 0.29, 0.21, and 0.156

These results indicate that the water-soluble product of methanolysis is a large oligosaccharide which, according to its molecular weight, would have about 20 monosaccharide units.

Other treatments of GEA

The product of methanolysis was treated for various periods of time with α- or α-plus β-amylase. No degradation was detected as judged by paper chromatography in 1-butanol–pyridine–water 6:4:3:.

When GEA was heated with 52% aqueous phenol at 65°C for 6 min, followed by an extraction of the phenol with ether and partition in chloroform–water 3:2:1, no radioactivity appeared in the aqueous phase.

Molecular weight of GEA

Indirect evidence on the nature of the lipophilic moiety of GEA was obtained by measurements of its molecular weight in deoxycholate. It is known that this bile salt forms inclusion compounds (choleic acids) with lipids. The compounds are stoichiometric and the number of deoxycholate molecules combined depends on the chain length of the fatty acids (10).

Measurements of the molecular weight of DMPG by gel filtration in 0.5% deoxycholate gave a value of 11,300 (Fig. 3*A*). Since the theoretical molecular weight of DMPG is about 1,500, it can be concluded that 24 molecules of deoxycholate associate with each molecule of DMPG. The molecular weight obtained for stearic acid under the same conditions was 3800 (Fig. 3*B*), that is, 8.4 molecules were fixed per molecule of fatty acid. This value agrees with data obtained by other methods (10).

Measurements of the molecular weight of GEA gave a value of 14,300. The difference from DMPG is 3000, which is nearly the same as the difference in molecular weight of their hydrophilic moieties.

DISCUSSION

The insolubility of GEA in most solvents seems to be the result of its having large lipophilic and hydrophilic residues. The compound is insoluble in water and in water-free mixtures of chloroform–methanol, but is soluble in chloroform–methanol mixtures with a high content of water.

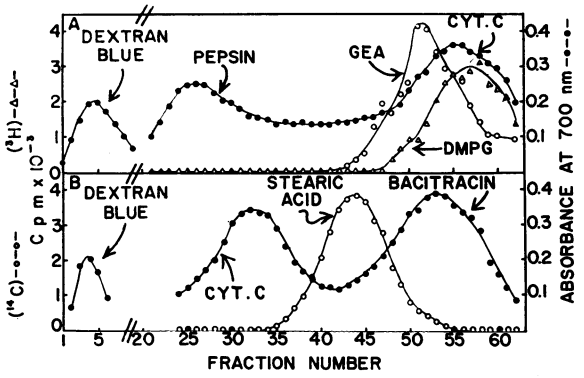

FIG. 3. The molecular weight of DMPG, GEA, and stearic acid in 0.5% deoxycholate. (A) DMPG and GEA labeled with tritium and ^{14}C, respectively, were passed through a 105 × 1.7 cm column of Sephadex G-150 equilibrated with 0.05 M sodium phosphate (pH 7.2) containing 0.5% deoxycholate. Fractions of 1.45 ml were collected. Pepsin and cytochrome-*c* used were detected by protein estimation. In order to avoid gel formation by the deoxycholate solution, the column was warmed to 37°C with a jacket. (B) [^{14}C]Stearic acid was passed through a 72 × 1.7 column of Sephadex G-100. Cytochrome *c* and bacitracin were used as standards. Other conditions were those of *A*.

The hydrophilic residue of GEA is liberated by mild-acid treatment. The rate of hydrolysis is about ten times slower than that of DMPG in a solvent in which both compounds are soluble (0.5 NHCl in chloroform–methanol–water). The substance liberated has the properties of a large oligosaccharide. It is water soluble and has no charge, as judged by paper electrophoresis. Its molecular weight measured in Sephadex columns is about 3550. After acetolysis, deacetylation, and paper chromatography, various peaks were observable as is the case when an oligosaccharide is degraded. Alkaline hydrolysis of GEA led to the formation of a charged compound that became neutral after treatment with purified phosphatase. It seems, therefore, that the hydrophilic portion of GEA is a saccharide of about 20 monosaccharide residues joined through a phosphate group to a lipophilic part.

Measurements of the molecular weight of GEA with Sephadex requires the addition of detergents in order to solubilize the substance. Deoxycholate is known to form inclusion compounds with fatty acids that are often crystalline. The number of deoxycholate molecules bound depends on the chain length of the fatty acids. For instance, the number of deoxycholate molecules bound to C_4–C_8 fatty acids is 4. This number increases to 6 and 8 for the C_9–C_{14} and C_{15}–C_{20} acids, respectively.

The difference found between the molecular weights of GEA and DMPG in 0.5% deoxycholate is in good agreement with the difference in the molecular weights of their hydrophilic moieties, which is about 3350. Therefore, the lipid residues of GEA and DMPG have the same molecular weights when combined to deoxycholate. Thus, the lipid residue in GEA might also be dolichol.

Experiments designed to decide whether the compound is a phosphate or a pyrophosphate have not yielded conclusive evidence. The fact that alkaline treatment yields a charged hydrophilic derivative, while 1,6-anhydroglucosan is formed from DMPG, may be interpreted in favor of a pyrophosphate. The elution pattern of GEA on DEAE-cellulose chromatography also points in the same direction. Treatment with hot phenol, which decomposes undecaprenyl pyrophosphates (11), did not affect GEA.

The evidence gathered from the behaviour of the radioactive compound is indirect, but agrees with a structure in which a saccharide of molecular weight 3550 is joined through a phosphate or pyrophosphate bridge to dolichol. This structure would be in accord with what has been found to occur in bacteria (12), where polysaccharides seem to be built up while joined to polyprenol pyrophosphates in preparation for transfer to the final acceptor.

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