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FURTHER STUDIES ON A GLYCOLIPID FORMED FROM DOLICHYL-D-GLUCOSYL MONOPHOSPHATE*

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ABSTRACT

Incubation of liver microsomes with dolichyl-D-glucosyl-^{14}C monophosphate led to the labelling of an endogenous acceptor. This compound seems to be also a dolichol derivative. It contains a high-molecular weight oligosaccharide bound to dolichol through a phosphate or pyrophosphate bond. Various treatments of the labelled oligosaccharide afforded further information on its structure: Reduction with sodium borohydride, followed by acid hydrolysis gave only radioactive D-glucose indicating that the labelled D-glucose is not incorporated at the reducing end of the oligosaccharide. The percentage of radioactivity, liberated as formic acid after periodate oxidation, indicates that more than one molecule of D-glucose is incorporated and that at least one of them is inside the oligosaccharide chain. Alkaline treatment of the otherwise neutral oligosaccharide gave two positively charged derivatives which could be neutralized by *N*-acetylation, indicating the presence of two hexosamine residues. The oligosaccharides isolated from different tissues by the same method as that used for rat liver, were similar as judged by their migration in paper chromatography and by the pattern of products liberated by acetolysis.

INTRODUCTION

Polyprenol phosphates have been found to be important intermediates in the biosynthesis of bacterial polysaccharides[1]. Work carried out in this laboratory has dealt with the role of similar compounds in animal tissues. In previous papers[2–4] it was reported that incubation of radioactive UDPG** (**1**) with rat liver microsomes and dolichyl phosphate leads to a series of transformations which are shown in Fig. 1.

*Dedicated to Professor V. Deulofeu, in honor of his 70th birthday. Several generations of Argentine chemists, including most of the authors of this paper, are indebted to Professor V. Deulofeu for his teachings.

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**The following abbreviations are used: UDP, uridine 5′-pyrophosphate; UDPG, uridine 5-(α-D-glucopyranosyl pyrophosphate); DolPG, dolichyl-D-glucosyl monophosphate; GEA, D-glucosylated endogenous acceptor.

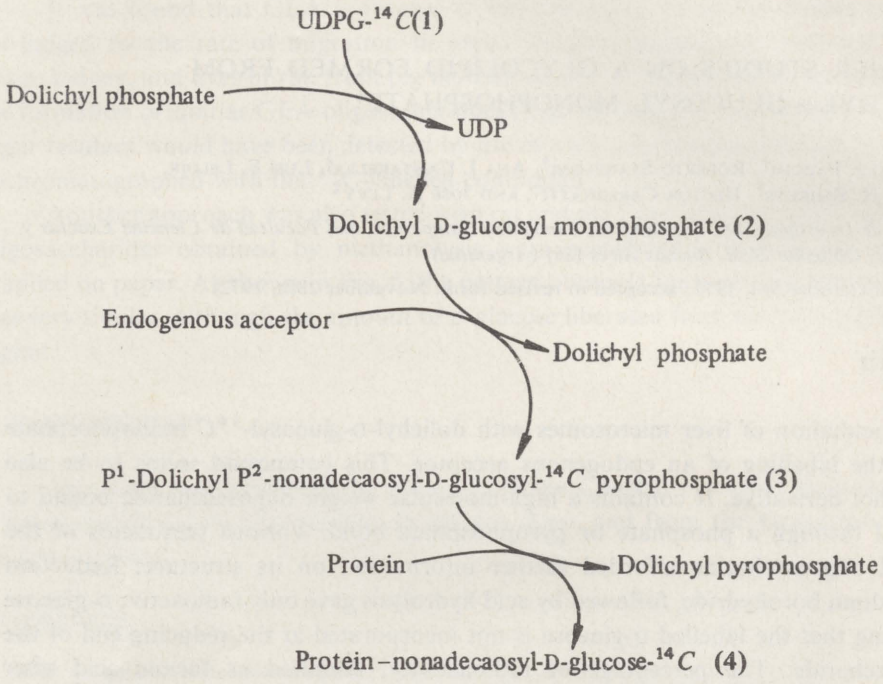

Fig. 1. Tentative scheme of the series of reactions catalyzed by liver microsomes.

The evidence available makes it fairly certain that compound **2** is in fact a derivative of dolichol, a long chain polyprenol which has been found in animal tissues[5]. Compounds similar to DolPG* (**2**) but containing, instead of D-glucose, D-mannose[6] or 2-acetamido-2-deoxy-D-glucose[7] have also been detected. The compound represented as **3** is presumably formed by transfer of D-glucose from DolPG (**2**) to an endogenous acceptor and is therefore referred[3] to as glucosylated endogenous acceptor (GEA)*. It has not yet been obtained in sufficient amount to be analyzed by the usual methods, but some evidence on its structure was obtained by studying the properties of the D-glucose-labelled compounds. The substance is insoluble in 2:1 chloroform–methanol but soluble in a more polar mixture (10:10:3 chloroform–methanol–water). On treatment with 0.1M hydrogen chloride in methanol it decomposes with a half-life of 8 min to yield a water-soluble product which has the properties of an oligosaccharide composed of about 20 glycose units. Thus, the molecular weight measured by filtration on gel columns[3] is 3,500 and, on paper chromatography, the mobility is equal to that of a maltooligosaccharide of 17 units[8]. As shown in Fig. 1, liver microsomes also catalyze the transfer of the oligosaccharide of GEA to a protein acceptor[4]. The work reported in this paper was undertaken in order to obtain further information on the structure and distribution of GEA.

*See note** page 1.

EXPERIMENTAL

Materials and methods. — Radioactive GEA labelled in the D-glucose residue was obtained by incubation of UDPG with liver microsomes in the absence of a detergent[9] as follows: Dolichyl phosphate containing about 65 nmoles of organic phosphate was obtained from pig liver and purified as far as the DEAE-cellulose absorption step[2] and then dried under vacuum in the presence of 5 μmoles of magnesium ethylene diaminetetraacetate and 5 μmoles of magnesium chloride. The dried material was suspended in a reaction mixture containing 0.1M 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) maleate (pH 7.7), 0.04M 2-hydroxy-1-ethanethiol, 5×10^5 counts/min UDPG-^{14}C (290 Ci/mole), and 15 to 18 mg of microsomal enzyme[4] in a final volume of 0.05 ml. In some experiments tritium-labelled UDPG was used. The reaction mixture was incubated for 20 min at 30°. Methanol (0.8 ml) and chloroform (1.2 ml) were then added, and the isolation of GEA was carried out as described previously[3]. The usual yield of GEA corresponded to about 20 percent of the added UDPG.

Concanavalin A was purchased from Calbiochem. Methyl α-D-glucopyranoside-^{14}C was prepared as described by Bollenback[10] and purified by paper chromatography in 6:4:3 butyl alcohol–pyridine–water[11]. The labelled oligosaccharide was prepared from GEA by methanolysis[3] or by hydrolysis[12] at pH 2 for 5 min at 100°.

The labelled glycopeptide was prepared by incubating GEA with microsomes as described previously[4], and the glycoprotein obtained was digested with papain (Sigma) for 20 h under the conditions described by Spiro[13]. The reaction mixture was precipitated with 10% trichloroacetic acid. After centrifugation, the supernatant fluid was freed from trichloroacetic acid by extraction with ether. The glycopeptide solution was desalted by passage through a column of P-2 resin (50–100 mesh, Bio-Rad) and further purified by paper electrophoresis in 5% formic acid. The only radioactive spot was eluted from the paper and used for binding with concanavalin A.

Rat and pig liver microsomes were prepared as described previously[4], and those of other tissues were obtained by the method of Moulé *et al.*[14], slightly modified by preparing a 20% instead of a 10% initial homogenate in 0.88M sucrose.

Chromatography. — Paper chromatography was performed in 4:3:4 butyl alcohol–pyridine–water in a well-saturated chamber. This solvent gave a fairly good separation of oligosaccharides[8]. Paper electrophoresis was performed in 2% sodium molybdate[15] at pH 5 or in 5% formic acid.

Sodium borohydride reduction. — The labelled oligosaccharide obtained by mild acid hydrolysis of GEA was treated with 0.1M sodium borohydride for 60 min at 30°. After the excess of sodium borohydride had been destroyed with acetic acid, the reduced product was hydrolyzed for 2 h at 100° with M hydrochloric acid. Boric acid was removed by addition of methanol and repeated evaporations.

Periodate oxidation. — The labelled oligosaccharide was treated with sodium periodate according to Fales[16] and the formic acid produced was extracted with ether as described by Hers[17].

Alkaline treatment. — The oligosaccharide was treated with 2M potassium hydroxide at 100°. The solution was neutralized with perchloric acid and the supernatant fluid was spotted on paper. *N*-Acetylation was carried out according to Roseman and Ludowieg[18].

Acetolysis. — Acetolysis of oligosaccharides was performed as described by Ballou[19] with the modification mentioned in a previous paper[3].

RESULTS AND DISCUSSION

Treatment with sodium borohydride. — In the formation of GEA from DolPG and endogenous acceptor, the mechanism of the reaction can be visualized in two ways. In one, the D-glucose residue of DolPG-^{14}C is added to the oligosaccharide moiety of the endogenous acceptor, so that the phosphate-bound D-glycosyl residue would remain unlabelled. The other possibility is that the oligosaccharide of the endogenous acceptor is added to DolGP-^{14}C so that the labelled D-glucose residue would remain bound to dolichyl phosphate and would carry the reducing group after mild acid hydrolysis. This second alternative did not seem probable because it would lead to the formation of a dolichyl oligosaccharide monophosphate and not to a pyrophosphate[3,12]. However, since the presence of the latter is not certain, an experiment was designed in order to test this possibility.

The labelled oligosaccharide was obtained by mild acid hydrolysis and treated with sodium borohydride. The reduced sample was hydrolyzed and submitted to paper electrophoresis in sodium molybdate for 75 min at 20 V/cm. A sample of labelled D-glucose was treated similarly at the same time. The scanning of the paper showed that the reduced D-glucose standard migrated a distance of 10.3 cm while the labelled sugar obtained from the oligosaccharide was found at 0.7 cm together with a D-glucose standard. This means then that the labelled D-glucose residue is not located at the reducing end of the oligosaccharide.

Periodate oxidation. — The oligosaccharide obtained by methanolysis of GEA was subjected to periodate oxidation and the formic acid liberated was measured in order to find out how much labelled D-glucose was present in the form of nonreducing end-groups.

TABLE I

FORMIC ACID PRODUCTION BY PERIODATE OXIDATION OF ^{14}C-LABELLED GEA AND METHYL α-D-GLUCOPYRANOSIDE[a]

Compound	*Formic acid*[b] (% *c.p.m.*)	*End groups* (%)
Methyl α-D-glucopyranoside	16.7 (17.0)	101
GEA oligosaccharide[c]	8.0 (8.2)	49
GEA oligosaccharide[c,d]	6.7 (6.9)	41

[a]The conditions of oxidation are described in Experimental. [b]Values in parenthesis represent duplicate measurements. [c]The oligosaccharides were obtained from GEA by methanolysis. [d]In the preparation of this sample, three times the normal proportion of UDPG was used.

The results presented in Table 1 show that the oligosaccharide is composed of two different ^{14}C-labelled D-glucose residues, about one half having free hydroxyl groups at C-2, C-3, and C-4, which presumably corresponds to nonreducing end groups, and another half having one of these groups blocked. These results indicate the possible addition of two D-glucose residues in succession or a mechanism similar to the formation of bacterial polysaccharides[1] where several glycosyl residues are added to polyprenol intermediates; these, in turn, form di- or oligosaccharides which are then transferred as repeating units to the growing polysaccharide chains. Such a mechanism, however, would require the intermediate formation of labelled polyprenol-bound oligosaccharides of less than 20 monosaccharide units. Since such units have not been detected, it may be concluded that the D-glucose residues are contiguous. The possibility that more than two D-glucose residues can be added is indicated by the experiment where the proportion of UDPG was trebled and this increase resulted in a decrease of the relative content of nonreducing end groups (Table 1).

Binding by concanavalin A. — Concanavalin A is known to combine with various polysaccharides having terminal α-D-glucosyl or α-D-mannosyl residues[20] and to a receptor site of mammalian cells[21]. As GEA is also formed by plasma membranes[22], it might contribute to concanavalin A binding in intact cells. Since no precipitate was formed by mixing the labelled oligosaccharide with concanavalin A, a method of separation of the free oligosaccharide from concanavalin A-bound

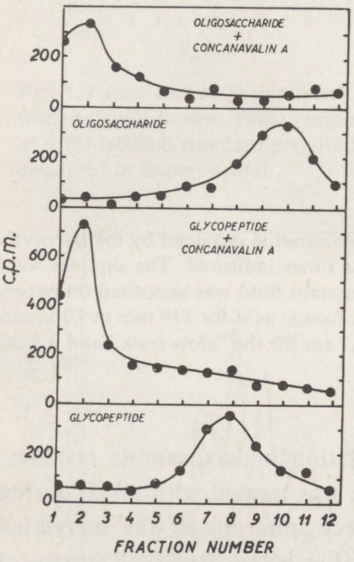

Fig. 2. Gel filtration of GEA oligosaccharide and glycopeptide in the presence or absence of concanavalin A. The samples with or without concanavalin A (0.5 mg) in 0.02M imidazol buffer (0.2 ml, pH 6.2) containing 3M sodium chloride and 2mM magnesium chloride were placed on a column (16 × 0.8 cm) of Bio-Gel P 60 (BioRad). The same buffer was used for development. Fractions (0.5 ml) were collected starting from the peak of Blue Dextran and counted with Bray's[24] solution in a liquid scintillator.

oligosaccharide by gel filtration was developed. A polyacrylamide resin was used since Sephadex contains D-glucosyl residues which may bind concanavalin A. As shown in Fig. 2, the free oligosaccharide of GEA is retained by the column, whereas, in the presence of concanavalin, it emerges with the void volume.

In a similar experiment, the glycopeptide formed by enzyme-catalyzed transfer from GEA to protein was treated with Pronase (Fig. 2). The results indicate that the terminal D-glucosyl or, eventually, D-mannosyl residues are bound by α-D-linkages to the rest of the oligosaccharide. Similar tests with a phytohemagglutinin from *Phaseolus vulgaris*, which combines with 2-acetamido-2-deoxy-D-galactose residues, gave negative results.

Alkaline treatment. — The oligosaccharide from GEA is neutral, as judged by paper electrophoresis, under both alkaline and acid conditions[4]. However, when the oligosaccharide obtained by methanolysis of GEA was treated with 2M potassium hydroxide at 100° and then subjected to electrophoresis, two positively charged peaks appeared. As shown in Fig. 3, a slow peak appeared first and then disappeared gradually as the fast peak increased.

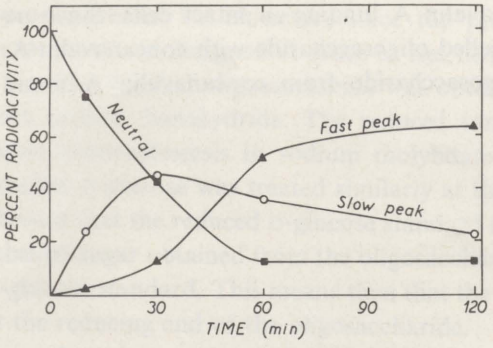

Fig. 3. Alkali treatment of the GEA oligosaccharide. The oligosaccharide obtained by methanolysis of GEA was heated at 100° in 2M potassium hydroxide for the times indicated. The samples were neutralyzed with perchloric acid and centrifuged, and the supernatant fluid was deposited on paper. The compounds were separated by paper electrophoresis in 5% formic acid for 210 min at 20 V/cm. Under these conditions, the migration toward the cathode was 6.7 cm for the "slow peak" and 9.5 cm for the "fast peak", in respect to the neutral compounds.

These results were attributed to a de-*N*-acetylation of hexosamine residues; in order to prove this hypothesis, the oligosaccharide was treated with alkali during 60 min and, after desalting, was *N*-acetylated[18]. Electrophoresis of the acetylated products gave only an uncharged peak. In order to verify whether the alkali treatment produced a degradation of the oligosaccharide, the alkali-treated product was chromatographed on paper, before or after *N*-acetylation. The migration of both products was the same as that of the original substance. Therefore, it seems that alkali treatment deacetylates first one, and then a second 2-acetamido-2-deoxyhexose, and leaves the rest of the molecule intact, as judged by its charge and size.

Occurrence of GEA in various tissues. — Oligosaccharides or polysaccharides have been implicated in many phenomena of cell and tissue specificity[23]. To find out whether GEA formation is limited to rat liver or whether it also occurs in other tissues and animals, microsomes from different sources were incubated with UDPG-^{14}C and GEA was isolated. The differences in GEA formation could result either from changes in the enzymes or in the endogenous acceptor.

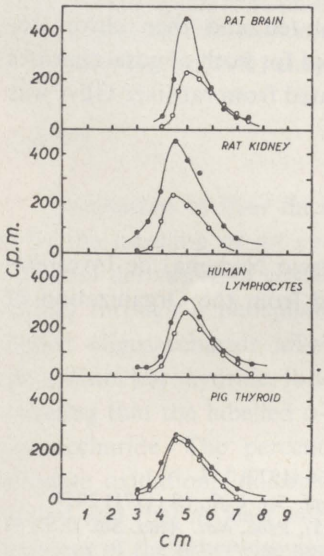

Fig. 4. Paper chromatography of the oligosaccharides of GEA prepared with enzymes obtained from different tissues. These samples were labelled with ^{14}C (●—●) and were examined in presence of a ^{3}H-labelled standard prepared with rat liver enzyme (○—○). The experimental conditions are described in Experimental.

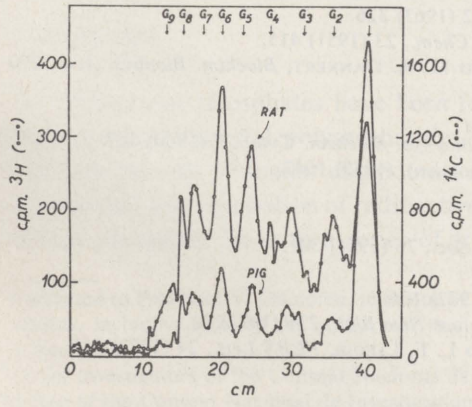

Fig. 5. Paper chromatography of the acetolyzate of the ^{3}H- and ^{14}C-labelled oligosaccharides of GEA prepared with rat liver and pig liver enzymes, respectively. The conditions of acetolysis are described in Experimental. The position on the paper of the standard maltooligosaccharides is represented by G, G_2, G_3, etc.

It was found that GEA is formed in variable yields in all the tissues tested. As judged by the rate of migration in paper chromatography, the enzymes from brain, kidney, and liver of rat, from pig thyroid, and from human lymphocytes led to the formation of similar GEA oligosaccharides (Fig. 4). Differences of more than two sugar residues would have been detected by use of a tritium-labelled internal standard cochromatographed with the ^{14}C-labelled compound.

Another approach was also tested with rat and pig liver. The differently labelled oligosaccharides obtained by methanolysis were acetolyzed and then chromatographed on paper. As shown in Fig. 5, the pattern obtained for both oligosaccharides was very similar, although the amount of D-glucose liberated from rat-liver GEA was higher.

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