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The Role of Polyprenol-Bound Saccharides as Intermediates in Glycoprotein Synthesis in Liver

(dolichol monophosphate glucose/lipid intermediates/liver microsomes)

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ABSTRACT **It has been reported that liver microsomes catalyze the transfer of glucose from uridine diphosphate glucose to dolichol monophosphate so as to produce dolichol monophosphate glucose. Dolichol is a polyprenol containing about 20 isoprene units. The glucosyl residue of dolichol monophosphate glucose is transferred to an endogenous acceptor on further incubation with liver microsomes. The glucosylated endogenous acceptor appears to be an oligosaccharide of about 20 monosaccharide units bound to dolichol through a phosphate or pyrophosphate bridge. In this paper it is reported that liver microsomes catalyze the transfer of the oligosaccharide from the glucosylated endogenous acceptor to an endogenous protein. This transfer reaction requires the presence of bivalent cations, manganese being more effective than magnesium. The presence of deoxycholate is also required. Besides the glycoprotein, several water-soluble products are also formed. Preliminary evidence indicates that they are glucose, oligosaccharides of different size, and possibly oligosaccharides bound to amino acids.**

In the first paper on the formation of dolichol monophosphate glucose (dolichol-P-Glc) it was reported that the glucosyl residue of this substance was transferred to an endogenous acceptor on further incubation with liver microsomes (1). The product thus formed was believed to be a glycoprotein because of its insolubility in certain organic solvents and in trichloroacetic acid. However, further work (2, 3) showed that most of the radioactivity transferred from glucose-labeled dolichol-P-Glc under the conditions described was recovered in an amphipatic substance, the hydrophilic residue of which appears to be an oligosaccharide of about 20 monosaccharide units. The lipophilic residue had some properties in common with dolichol-P, and evidence was obtained indicating that the bridge between the lipid moiety and the oligosaccharide is a pyrophosphate. The substance was referred to as glucosylated endogenous acceptor (Glc-acceptor). On the basis of present evidence its structure can be written as dolichol-P-P-glycose$_{19}$-Glc. Dolichol (4) is a polyprenol containing about 20 isoprene units and glycose$_{19}$ stands for an oligosaccharide of about 19 unidentified monosaccharide residues. It should be emphasized that this is a tentative formula for the Glc-acceptor. Several aspects of its structure have not been fully clarified, especially the number and identity of the monosaccharide residues and the number of phosphates in the bond between the lipophilic and hydrophilic moieties.

Abbreviations: dolichol-P-Glc, dolichol monophosphate glucose; Glc-acceptor, glucosylated endogenous acceptor.

In this paper we report that under appropriate conditions, liver microsomes catalyze the transfer of the hydrophilic moiety of the Glc-acceptor to an endogenous protein. The reactions of glucose transfer under study can be schematically written as follows:

UDP-Glc → dolichol-P-Glc → dolichol-P-P-glycose$_{19}$-Glc →
protein-glycose$_{19}$-Glc

MATERIALS AND METHODS

Radioactive Glc-acceptor labeled in the glucose residue was obtained directly from UDP-Glc as described (5).

Enzymes. Papain 2 × crystallized and α-chymotrypsin 3 × crystallized were purchased from Sigma. Pronase grade B was from Calbiochem.

Liver microsomes were prepared as follows: the 96,000 × *g* precipitate discarded in preparation of liver glycogen synthetase (6) was homogenized in two volumes of 880 mM sucrose, 5 mM EDTA (pH 7.0). The supernatant obtained after centrifugation at 16,500 × *g* for 15 min was further centrifuged at 150,000 × *g* for 180 min. The precipitate was suspended in water containing dilute Tris-maleate buffer (pH 7.7). The usual protein concentration was 60–100 mg/ml.

Chick-embryo liver microsomes were prepared as follows: about 50 livers from 11-day-old chick embryos were homogenized in 250 mM sucrose, 10 mM 2-mercaptoethanol, 5 mM EDTA (pH 7.0). The homogenate was centrifuged at 25,000 × *g* for 15 min. The precipitate was suspended in water at a protein concentration of 75 mg/ml.

Incubation Mixture. No transfer activity was obtained in preliminary experiments with rat-liver microsomes. We thought that this might be due to the absence of an appropriate acceptor for the oligosaccharide. Thus, some experiments were done with a mixture of rat and chick-embryo liver microsomes with the idea that the latter tissue might contain the appropriate acceptor. However, further experiments showed that under the conditions described below, rat-liver microsomes alone are fully active.

Radioactive Glc-acceptor (2000–4000 cpm) was dried in a test tube, and the following components were added: 80 mM Tris-maleate (pH 7.7), 0.38% (9 mM) sodium deoxycholate, 8 mM $MnCl_2$, and 2 mg of microsomal protein in a total volume of 65 μl. Equal amounts of protein of rat and chick-embryo liver microsomes were used where stated. Otherwise, the enzyme was the rat-liver preparation. The reaction was

stopped after 5 min at room temperature by successive addition of 0.4 ml of methanol, 0.16 ml of water, and 0.6 ml of chloroform. After the preparation was mixed and centrifuged, the upper phase was removed, and the interphase and lower phase were washed with 0.5 ml of chloroform–methanol–water 1:16:16. The upper phases were pooled. The lower phase was removed, and the interphase was extracted three times with 0.5 ml of chloroform–methanol–water 1:1:0.3. The extracts were pooled. The remaining denatured protein precipitate containing the insoluble product was washed with 1 ml of methanol, suspended in Bray's solution (7), and counted. The scintillation vial had to be stirred immediately before counting in order to obtain reproducible results. The other fractions were measured in a flow counter.

Proteolytic Digestions. The insoluble product was digested with Pronase and papain as described by Spiro (8) for 72 hr. The material was digested with α-chymotrypsin for 48 hr at 37° in 50 mM Tris-maleate (pH 7.7)–5 mM $CaCl_2$, with an α-chymotrypsin to total protein ratio of 0.05.

After the incubations, microsomal protein was added as carrier, and 10% trichloroacetic acid was added. The supernatant obtained after centrifugation at 16500 × *g* for 10 min was freed from trichloroacetic acid by several extractions with ether. The precipitate was washed once with 1 ml of methanol and once with 1 ml of ether.

Alkaline Degradation. The insoluble product was heated for 15 min at 100° in 2 N KOH–0.2 N $NaBH_4$. Trichloroacetic acid was then added to a 10% free acid concentration (experiments of Table 2 and Fig. 2). The fractions were centrifuged and washed as described above for the proteolytic digestions. When the products of the alkaline treatment were to be subjected to paper chromatography or electrophoresis, the alkaline solution was neutralized with perchloric acid and the precipitate was centrifuged off. Trichloroacetic acid was added to the supernatant to a 10% concentration, and the precipitate was removed. The supernatant was then freed from trichloroacetic acid with ether and dried. Boric acid was removed by several evaporations in acidified methanol. The radioactive products were then dissolved in water and passed through a 0.5 × 5-cm column of MB-3 acetate.

TABLE 1. *Requirements for formation of water-soluble and insoluble products from the Glc-acceptor*

Additions	cpm in insoluble product	cpm in water-soluble products
Deoxycholate (%)		
0.38, nonincubated	39	143
0.00	108	142
0.08	130	235
0.16	185	283
0.38	760	705
0.76	509	633
1.52	235	423
Metals		
8 mM $MnCl_2$, nonincubated	82	107
8 mM $MnCl_2$	476	333
− $MnCl_2$ + 8 mM $MgCl_2$	272	230
− $MnCl_2$ + 8 mM EDTA	93	178

The incubation mixture was as described in *Methods*, with the modifications indicated. A mixture of rat and chick-embryo liver microsomes was used in the second series.

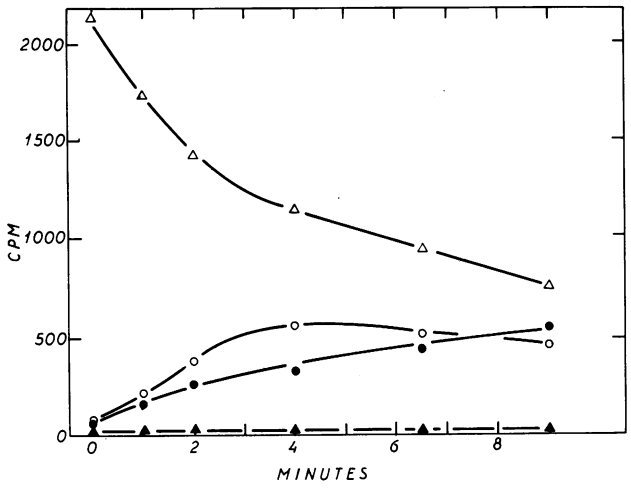

FIG. 1. Time-course of the reaction. A mixture of rat and chick-embryo liver microsomes was used here. △——△, Glc-acceptor; ○—○, insoluble product; ●——●, water-soluble products; ▲——▲, lower phase.

RESULTS

Time-course of the reaction

Incubation of liver microsomes with radioactive Glc-acceptor, deoxycholate, and manganese ions led to the gradual disappearance of Glc-acceptor and to the appearance of radioactivity in the insoluble fraction, which contains denatured protein, and in the fraction containing water-soluble substances (Fig. 1). The different substances were separated by first adding chloroform–methanol–water 3:2:1 to the reaction mixture. This gives an upper phase containing water-soluble substances, a lower phase in which dolichol-P-Glc and most lipids are soluble, and an interphase containing denatured protein and the Glc-acceptor. The latter substance can be extracted with chloroform–methanol–water 1:1:0.3, which dissolves the Glc-acceptor leaving the radioactive insoluble product together with the denatured protein precipitate (2, 3). The radioactive substance found in the precipitate will be referred to as insoluble product.

The radioactivity in the insoluble product reached a maximum and decreased thereafter (Fig. 1). Formation of the insoluble product required a bivalent cation, manganese being more effective than magnesium (Table 1). The reaction also required deoxycholate. Its optimal concentration was 0.38% under the conditions used in Table 1. Deoxycholate cannot be replaced by Triton X-100. Conditions that led to a most effective formation of the insoluble product also produced higher amounts of the water-soluble products. No incorporation into the denatured protein precipitate was found if the radioactive oligosaccharide obtained by acid methanolysis of the Glc-acceptor (2) was used as substrate. This experiment was performed in order to check the possibility that the transfer of radioactivity observed could be due to a glucosyl exchange reaction between the hydrophilic moiety of the Glc-acceptor and a preformed glycoprotein.

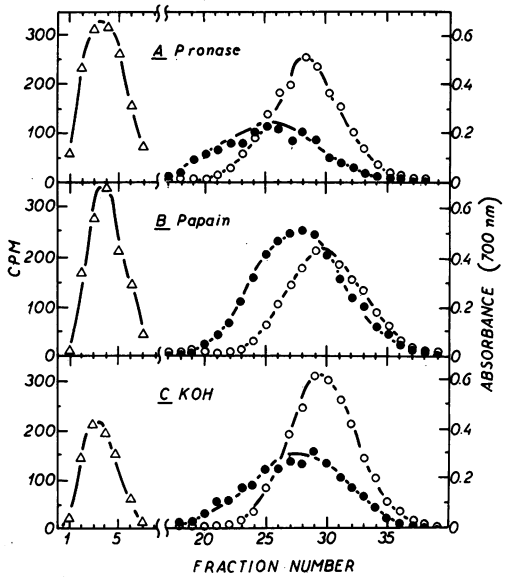

FIG. 2. Gel filtration of the proteolytic and alkaline digestion products. The digestion products labeled with ^{14}C (●——●) were prepared as described in *Methods*. They were passed through a 94 × 1.2-cm column of Sephadex G-50 medium equilibrated with 20 mM LiCl. The tritiated oligosaccharide obtained by acid methanolysis of the Glc-acceptor (2) was added as internal standard (○——○). The volume of the fractions was 1.45 ml. △——△, dextran blue.

Properties of the insoluble product

Proteolytic Digestions. The insoluble product that remained together with the denatured protein precipitate after the extraction with chloroform–methanol–water 1:1:0.3 could be dissolved in water containing sodium dodecyl sulfate and urea. The product thus dissolved was retained by DEAE-cellulose and could be eluted with a salt-sodium dodecyl sulfate–urea mixture. It emerged together with most of the protein. When the dissolved product was run in Bio-Gel P-150 columns equilibrated with a sodium dodecyl sulfate–urea solution, it emerged with the void volume. The dissolved product was included in Bio-Gel P-300 but gave a very broad peak.

After proteolysis of the insoluble product that remained together with the denatured protein precipitate, most of the radioactivity was soluble in trichloroacetic acid (Table 2). The soluble products obtained after Pronase and papain digestions were run in Sephadex G-50 with the hydrophilic moiety of the Glc-acceptor as internal standard (Fig. 2*A* and *B*). It was previously reported that the latter substance has a molecular weight of 3600 (2). The molecular weights obtained for the Pronase and papain digestion products were 4000 and 3950, respectively. The trichloroacetic acid-soluble product obtained by treatment with papain migrated to the cathode in paper electrophoresis in 5% formic acid and to the anode in 0.1 M glycine–NaOH buffer (pH 8.7). It behaved, therefore, as an amphoteric substance. The oligosaccharide of the Glc-acceptor was neutral under both conditions.

Alkaline Degradation of the Insoluble Product. The radioactivity in the denatured protein precipitate could also be solubilized by alkaline treatment (2 N KOH–0.2 N $NaBH_4$ for 15 min at 100°) (Table 2). The trichloroacetic acid-soluble product thus obtained had a molecular weight of 3800 (Fig. 2*C*).

When this trichloroacetic acid-soluble substance was chromatographed on paper with butanol–pyridine–water 4:3:4, two approximately equal peaks appeared with R_{Glc} values of 0.08 and 0.028. The first has the same mobility as the oligosaccharide liberated from the Glc-acceptor by mild acid hydrolysis (3, 9). Charged substances are retarded in this solvent. In accordance with this, negatively or positively charged peaks, besides a neutral one, appeared when the product obtained by alkaline treatment was subjected to paper electrophoresis in alkaline or acid buffers, respectively. As mentioned above, the oligosaccharide of the Glc-acceptor was neutral under both conditions. It seems, therefore, that alkaline treatment of the insoluble product gives rise to the formation of a substance indistinguishable from the oligosaccharide moiety of the Glc-acceptor and to another one that has, in adddition, charged groups. These presumably correspond to amino acids, since the papain digestion product had similar amphoteric properties and migrated in paper chromatography in butanol–pyridine–water 4:3:4 with a $R_{Glc} = 0.032$.

The oligosaccharide liberated from the Glc-acceptor by mild acid hydrolysis, treated for 15 min at 100° with 2 N KOH–0.2 N $NaBH_4$, also produced a pattern of two peaks on paper chromatography in butanol–pyridine–water 4:3:4. It behaved therefore in this solvent as the substance obtained by alkaline treatment of the insoluble product. However, the alkaline treatment of the oligosaccharide from the Glc-acceptor produces only positively charged and uncharged substances, as detected by paper electrophoresis in both acid and alkaline buffers. The positive charge might have arisen from a deacetylation of *N*-acetyl glucosamine residues present in the oligosaccharide.

Other Properties of the Insoluble Product. In order to ascertain some properties of the insoluble product not denatured by chloroform and methanol, we developed several other procedures for its isolation from the incubation mixture. These procedures and that described in *Methods* gave similar amounts of radioactivity in the insoluble product.

In the first procedure, up to a 10% concentration of trichloroacetic acid was added to the incubation mixture. The precipitate contained the insoluble product and the Glc-acceptor. The latter substance was removed with chloroform–methanol–water 1:1:0.3. The precipitate was then washed with methanol and counted.

TABLE 2. *Digestion of the insoluble product by proteolytic and alkaline treatments*

Treatment	cpm	
	Trichloroacetic acid-insoluble	Trichloroacetic acid-soluble
—	1903	252
Pronase	458	2150
Papain	400	2228
—	3013	53
α-chymotrypsin	299	2125
—	1323	100
2 N KOH– 0.2 N $NaBH_4$	120	1283

The second procedure was like the preceding one, except that the reaction mixture was heated in trichloroacetic acid for 5 min at 100°. The precipitate was not extracted with chloroform–methanol–water 1:1:0.3 because the Glc-acceptor is completely decomposed by the hot acid treatment, producing soluble radioactive products. The insoluble product appears not to be acid-labile and remains with the denatured protein precipitate.

In the third procedure, water-saturated phenol was added to the reaction mixture. The phenolic phase was separated. It contained the Glc-acceptor and the insoluble product. The phenol was removed with ether. A denatured protein interphase thus appeared. It was extracted with chloroform–methanol–water 1:1:0.3, washed with methanol, and counted.

These experiments showed, therefore, that the undenatured insoluble product is insoluble in trichloroacetic acid, resistant to mild acid treatment, and soluble in water-saturated phenol.

The water-soluble products

As shown in Fig. 1, incubation of the Glc-acceptor with liver microsomes gives rise to the formation of water-soluble products besides the insoluble product. Paper chromatography in butanol–pyridine–water 6:4:3 (10) of the soluble products showed two peaks of radioactivity, one of which had the mobility of glucose while the other remained at the origin. The proportion of the two peaks was as follows:

Min of incub:	% cpm: Origin	Glucose
5	48	52
40	27	73

that is, the proportion of glucose increased with time.

Four peaks appeared when the radioactivity remaining at the origin was subjected to further paper chromatography in butanol–pyridine–water 4:3:4 (Fig. 3) (9). The second peak beginning from the origin migrated like the oligosaccharide liberated from the Glc-acceptor by mild acid hydrolysis (R_{Glc} = 0.08). The other faster-running peaks migrated as malt-oligosaccharides of 13 and 15 glucose units.

No clear information on the nature of the slower-migrating peak was obtained. As mentioned above, charged substances are retarded in this solvent. At least two negatively charged peaks, besides a neutral one, appeared when the substance that remains at the origin in butanol–pyridine–water 6:4:3 was subjected to paper electrophoresis in an alkaline buffer [0.1 M glycine–NaOH buffer (pH 8.7), 180 min at 20 V/cm]. A similar pattern, but of neutral and positively charged peaks, was obtained in an acid medium (5% formic acid, 210 min at 20 V/cm). Therefore, it seems likely that the slowest migrating peak of Fig. 3 is an oligosaccharide bound to different numbers of amino acids.

DISCUSSION

The results obtained show that the Glc-acceptor is an intermediate in the synthesis of a glycoprotein in liver. Thus, when the Glc-acceptor labeled in the glucose residue was incubated as described, the label was transferred to a reaction product insoluble in trichloroacetic acid and in several organic solvent mixtures. However, the insoluble product was soluble in water-saturated phenol and in water containing sodium dodecyl sulfate and urea.

The insoluble product was degraded by treatment with proteases or alkali. The fact that the molecular weights of the proteolytic and alkaline digestion products were only slightly higher than the molecular weight of the oligosaccharide of the

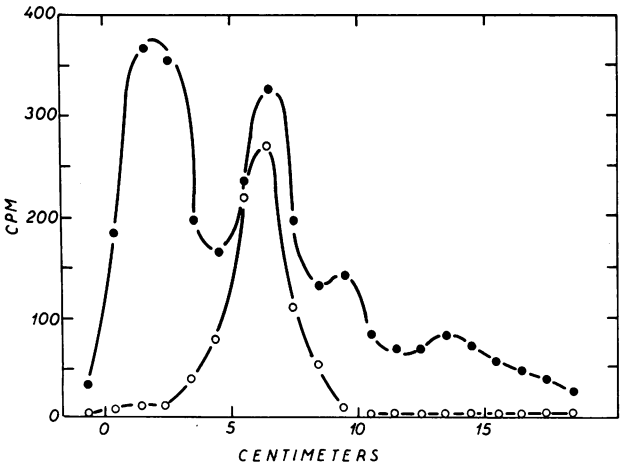

FIG. 3. Paper chromatography of the water-soluble products. The upper phase from the chloroform–methanol–water 3:2:1 extraction of the [^{14}C]Glc-acceptor to protein transference reaction mixture was analyzed. After drying, it was desalted with a Sephadex G-50 column, spotted on a Whatmann 1 paper, and developed for 24 hr in butanol–pyridine–water 6:4:3 (10). The paper was then dried and further developed with butanol–pyridine–water 4:3:4 (9). ●——●, water-soluble products. The tritiated oligosaccharide obtained by acid hydrolysis of the Glc-acceptor (3) was used as internal standard (O——O). The paper was then cut in strips and counted in a toluene scintillation mixture.

Glc-acceptor suggests that the whole oligosaccharide is transferred.

The conditions that enhanced transfer of the oligosaccharide to the protein also led to an increased formation of the water-soluble products. This result might indicate that the latter products could be produced at least in part by a degradation of the glycoprotein. The decrease in radioactive glycoprotein with time showed in Fig. 1 points in the same direction.

The nature of the carbohydrate–protein linkage of the glycoprotein has not been elucidated. An amidase that breaks the bond between asparagine and *N*-acetyl glucosamine in glycoproteins has been described in liver (11). The production of a substance with the same mobility in butanol–pyridine–water 4:3:4 as that of the oligosaccharide of the Glc-acceptor could be explained by the action of an enzyme with similar properties. The action of endo or exo glycohydrolases may be the cause of the production of glucose and of several oligosaccharides shorter than that of the Glc-acceptor. As for the slowest migrating peak of Fig. 3, preliminary evidence suggests that it could be produced by proteolysis of the glycoprotein.

Polyprenol-bound sugars are involved in the biosynthesis of several bacterial cell-wall polysaccharides (12, 13). The role of similar compounds as intermediates in glycoprotein synthesis in mammalian cells has also been suggested. Thus, Caccam *et al.* (14) found that upon incubation of liver, oviduct, or myeloma tumor microsomes with guanosine diphosphate mannose, a mannosyl lipid and a mannosyl protein were formed, although no clear relation between the two products was reported. Tetas *et al.* (15) have communicated that incubation of liver microsomes with uridine diphosphate *N*-acetyl glucosamine led to the transfer of the sugar residue to a lipid and to a protein. From "pulse-chase" experiments they concluded that the lipid derivative could be a precursor of the

glycoprotein. More recently, Helting and Peterson (16) described the transfer of galactose from galactosyl retinol to a fraction insoluble in chloroform–methanol catalyzed by microsomes from mouse mastocytomes, and De Luca *et al.* (17) have reported that glucosyl retinol is an intermediate in the glucosylation of collagen catalyzed by human platelet membranes.

The oligosaccharide moieties of glycoproteins could be synthesized in a stepwise manner by the successive transfer of monosaccharide units from sugar nucleotides or from lipid-bound monosaccharides to the protein acceptor. Alternatively, the oligosaccharide moiety could be built up in an intermediate compound, as seems to be the case reported here. The latter mechanism does not seem to be general, since there is solid evidence that in certain cases there is a sequential addition of monosaccharide units to proteins. For instance, the incorporation of xylose, two galactosyl residues, and a uronic acid residue to the protein of chondroitin sulfate catalyzed by embryonic chick cartilage enzymes, occurs by transfer of one monosaccharide at a time (18–20). A similar mechanism has been described for incorporation of the galactosyl and glucosyl residues to collagen (21) and for incorporation of sialic acid, galactosyl, and *N*-acetyl glucosaminyl residues to orosomucoid (22–24).

The nature of the glycoprotein synthesized by the liver microsomal system described here has not been elucidated.

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