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Dolichyl sulphate and *H*-phosphonate: Enzymatic reactions with activated sugars*

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Two phosphate-modified analogues of dolichyl phosphate were evaluated as substrates or inhibitors of the reactions catalyzed by mammalian microsomal enzymes. Dolichyl *H*-phosphonate could serve as an efficient acceptor for mannosyl and glucosyl transfer. The reaction products were chromatographically different from those formed from dolichyl phosphate. Lower activity of the *H*-phosphonate was observed for the reaction of *N*-acetylglucosaminyl phosphate transfer from UDP-GlcNAc. Dolichyl sulphate was shown not to serve as a substrate for the transfer of mannosyl (from GDP-Man), glucosyl (from UDP-Glc) or *N*-acetylglucosaminyl phosphate (from UDP-GlcNAc) residues in the presence of rat liver microsomes. Weak inhibitory properties of this analogue were demonstrated.

The crucial role of dolichyl phosphate in formation of the oligosaccharide portion of glycoproteins and glycosyl phospho-inositol anchors is well established for eukaryotic systems, and search for antimetabolites of dolichyl phosphate seems very interesting.

Since the pioneer studies of Mankowski *et al.* [1, 2], specificity of enzymes participating in formation of lipid-linked sugars towards the structure of polyprenyl residue was thoroughly investigated (for reviews see [3, 4]). Particularly, for dolichyl phosphate (Dol-P)

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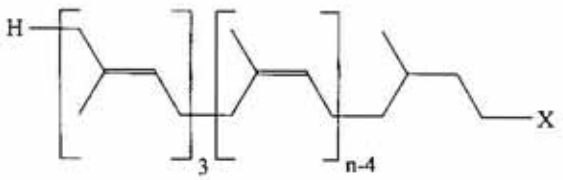
Abbreviations: GDP-Man, guanosine diphosphate mannose; UDP-Glc, uridine diphosphate glucose; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; Dol-P, dolichyl phosphate.



1 X = $OPO_3H_2 \cdot 2HNH_3$, n = 18–21

2a X = $O-\overset{\overset{O}{\|}}{\underset{\underset{H}{|}}{P}}-OH \cdot NH_3$, n = 15–18

2b X = $O-\overset{\overset{O}{\|}}{\underset{\underset{H}{|}}{P}}-ONa$, n = 18–21

3 X = OSO_2ONa, n = 15–18

Scheme 1

(see Scheme 1) the following essential structural features required for efficient interaction with eukaryotic glycosyltransferases were established: (a) presence of dihydroisoprene unit at the α-end of the chain [1, 2, 5–7]; (b) (*S*)-configuration of the dihydroisoprene unit [8–10]; (c) presence of at least 5–6 isoprene units in the chain – the reaction velocity gradually decreasing with chain-length below n = 11 [11–14]. For the synthesis of Dol-P-Man, no essential difference was noted with a change of n from 11 to 32 [6] and the reaction with some low-molecular phosphates was demonstrated [15]. The prokaryotic enzymes differ from eukaryotic enzymes in their requirements towards polyprenyl structure [2, 16, 17].

At the same time the effect of phosphate group modification in dolichol on enzymatic reaction with activated sugar remains unexplored. In this paper, we report biological properties of two phosphate-modified analogues of Dol-P, recently prepared by chemical synthesis [18, 19], namely, dolichyl *H*-phosphonate (Scheme 1; **2a**, and **2b**, **a** and **b** refer to different chain length of dolichyl residue) and dolichyl sulphate (**3**).

Both substances were tested as sugar acceptors for the transfer of mannosyl (from UDP-Man), glucosyl (from UDP-Glc) or *N*-acetylglucosaminyl phosphate (from UDP-GlcNAc) residues in the system of rat liver microsomes, the results being compared with those obtained with natural dolichyl phosphates.

MATERIALS AND METHODS

Enzyme preparation. The microsomal fractions from rat liver or brain were prepared from 3 months old rats of Wistar strain as described by Dallner [20]. The suspension of microsomes in 0.25 M sucrose/1 mM EDTA (37.2 mg/ml protein from the liver and 7.3 mg/ml protein from the brain microsomes) was stored at –80°C until used in enzymatic reactions. Protein was estimated by the Lowry method.

Enzyme assays. *Mannosyltransferase* (EC 2.4.1.83). The solution of dolichyl derivatives (as indicated in particular experiments) in chloroform/methanol (2:1, v/v) was added first to the tube. After evaporation of the organic solvents under a stream of nitrogen, the residue was dissolved in 0.5% Triton X-100 (10 μl). The suspension of microsomes (20 μl) was added, and the mixture was vortexed and preincubated for 15 min at 0°C. The buffer (70 μl) was then added; it contained Tris/HCl, pH 7.5 (30 mM), $MgCl_2$ (10 mM), 2-mercaptoethanol (5 mM) and EDTA (5 mM). The reaction was started by addition of GDP-[^{14}C]Man (0.4 nmole, 200000 c.p.m. in the case of liver and 0.2 nmole, 100000 c.p.m. in the case of brain) and incubation was continued for 3 min at 30°C. The reaction was stopped by addition of 2 ml of chloroform/methanol (2:1, v/v) and 0.1 ml of water. The mixture was shaken at 40°C for 60 min. The protein precipitate was separated by centrifugation and the chloroform/methanol extract was washed three times with 0.02 M KCl in the mixture of chloroform/methanol/water (3:48:47, by vol.). The lower, chloroform phase which con-

tained lipids was evaporated to dryness in scintillation vials and radioactivity was counted in LKB 1209 liquid scintillation counter.

Glucosyltransferase (EC 2.4.1.117). The assay was performed as above but pH of the buffer was 6.8 (optimal value, as found in preliminary experiments), the reaction was started by addition of UDP-[^{14}C]Glc (0.35–0.7 nmole, 200000–400000 c.p.m.) and the mixture was incubated for 20 min at 37°C.

N-*Acetylglucosaminylphosphotransferase* (EC 2.7.8.17). The assay was performed as above with the following differences: (a) All mixtures contained, in addition to specified dolichyl derivatives, dolichyl phosphate mannose (150 pmoles), prepared by enzymatic reaction as described above; (b) pH of the buffer was 7.0 (optimal value for this reaction); (c) the reaction was started by addition of UDP-[^{14}C]GlcNAc (0.38 nmoles, 100000 c.p.m.) and the mixture was incubated for 20 min at 37°C.

Thin-layer chromatography was carried out on Kieselgel G plates for Nano-DC (Merck). The developing solvent used was chloroform/methanol/water (60:25:4, by vol.) (A). Thin-layer reversed phase chromatography was performed on RP-18 HPTLC plates (Merck, 0.25 mm thick) using 50 mM H_3PO_4 in acetone as a solvent (B). Lipids were located on thin-layer chromatograms with iodine vapour; the radioactive spots were detected using X-ray films.

Radioactive nucleoside diphosphate sugars. GDP-[^{14}C]mannose (sp. act. 251 mCi/mmol), UDP-[^{14}C]glucose (sp. act. 287.4 mCi/mmol) and UDP-*N*-acetyl-[^{14}C]glucosamine (sp. act. 265 mCi/mmol) were from the Radiochemical Centre, Amersham, England.

Polyisoprenoids and their derivatives. The natural mixture of mammalian liver dolichyl phosphates obtained by the method of Danilov & Chojnacki [21] was from the "Collection of Polyprenols" at the Institute of Biochemistry and Biophysics (Warsaw, Poland). The proportion of individual dolichols, D18, D19, D20, D21 in the mixture (dolichol **A**) was 1:4:3:1; the mixture contained also smaller amounts of longer and shorter chain prenologues. The mixture of dolichols of chain length around 16 isoprene units (D15, D16, D17, D18 in the approximate ratio 1:4:3:2, dolichol **B**) was prepared chemically (Veselovsky V.V. *et al.*, unpublished) from the mixture of fully unsaturated polyprenols isolated from spruce [22]. The synthesis of **2a** and **2b** was performed as described [18] with dolichols **A** or **B**. Sulphate **3** was synthesized in the reaction of dolichol **B** with pyridine·SO_3 in dimethylformamide [19]. The products were characterized by ^{1}H NMR.

All other chemicals were purchased from commercial sources.

RESULTS AND DISCUSSION

Interaction of dolichyl phosphate and its analogues with GDP-Man in the presence of microsomes from rat liver or brain

Dolichyl phosphate analogues (**2a**, **2b**, and **3**, Scheme 1) were studied as substrates for glycosyltransferases from rat liver or brain.

In accordance with literature data [23], we observed that rat liver microsomes incubated with natural dolichyl phosphate (**1**) and GDP-[^{14}C]Man brought about a rapid incorporation of [^{14}C]mannose into the lower lipid phase (see Fig. 1; curve **1**), up to 37% of the label could be found in the form of lipid. When the incubation mixture contained analogues **2a** or **2b** instead of Dol-P, incorporation of radioactivity into the lipid phase was also observed, though its maximum was about 2.5 times lower than that observed with natural dolichyl phosphate (Fig.1, curves **2a, 2b**).

When dolichyl phosphate (**1**) and GDP-[^{14}C]Man were incubated with rat brain microsomes, we observed that up to 78% of the label could be found in the form of lipid.

As one can see the mannosyltransferase from brain microsomes (Fig. 2) is more active

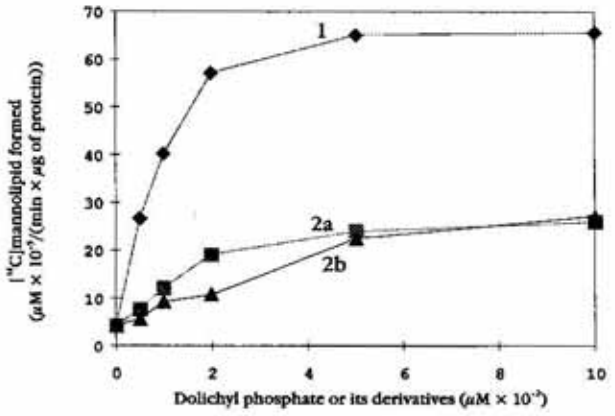


Figure 1. Formation of lipid-linked sugars from GDP-[^{14}C]Man by rat liver microsomes in the presence of varied concentration of dolichyl phosphate (1) and *H*-phosphonates (2a) or (2b).

◆, Dol-P; ■, 2a; ▲, 2b.

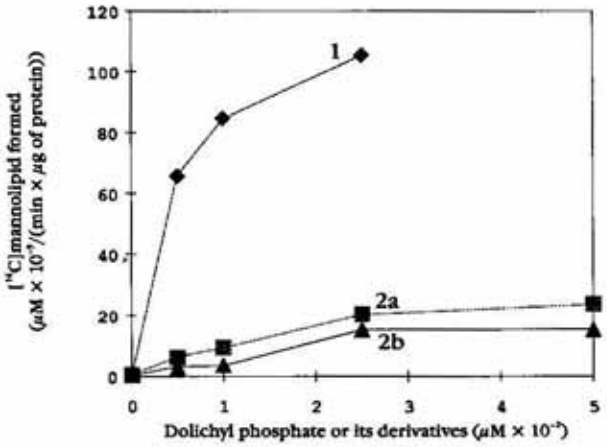


Figure 2. Formation of lipid-linked sugars from GDP-[^{14}C]Man by rat brain microsomes in the presence of varied concentrations of dolichyl phosphate (1) and *H*-phosphonates (2a) or (2b).

◆, Dol-P; ■, 2a; ▲, 2b.

than the enzyme from rat liver (Fig. 1). The transfer of [^{14}C]mannose from GDP-[^{14}C]Man upon adding exogenous dolichyl phosphate (1) is 100–200 times higher (Fig. 2, curve **1**) than the transfer to endogenous acceptors in rat brain microsomes. The transfer rate is significantly lower when *H*-phosphonates (**2a**, **2b**) are added instead of 1 (Fig. 2, curves **2a**, **2b**). The data obtained allow to conclude that mannosyltransferase from brain is significantly more sensitive to changes in substrate structure than the similar enzyme from rat liver.

The formation of [^{14}C]mannolipids from dolichyl phosphate and *H*-phosphonates under conditions of enzymic assay was confirmed by thin-layer chromatography with autoradiographic detection (see Fig. 3, lanes 1–6). The mannolipids formed from the *H*-phosphonates (**2a**, **2b**) were found to migrate slightly behined the dolichyl phosphate mannose formed in the reaction with dolichyl phos-

Table 1. The effect of dolichyl sulphate concentration on the formation of lipid-linked sugars by rat liver microsomal enzymes

Concentration of 3	Radioactivity of lipid-linked sugars after incubation, c.p.m.				
(μM)	GDP-[^{14}C]Man			UDP-[^{14}C]Glc	UDP-[^{14}C]GlcNAc
	Dolichyl phosphate added, concentration (μM)				
	0	100	250	0	0
	1	2	3	4	5
0	5547	54213	67317	2960	1264
50	–	46457	69870	3203	940
100	3848	52723	66725	2602	1189
200	3124	42784	62900	2246	–
500	3046	43893	54700	1722	1082
1000	–	30473	42117	–	–

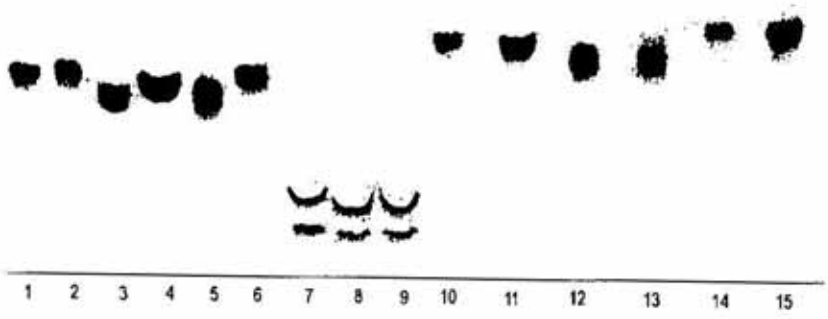


Figure 3. Autoradiography of thin-layer chromatograms of glycolipids formed from exogenous dolichyl phosphate (1) (1, 2, 7, 10, 11) and *H*-phosphonates (2a) (3, 5, 8, 12, 13) and (2b) (4, 6, 9, 14, 15) by rat liver (2, 5–15) or brain (1, 3, 4) microsomes in the presence of GDP-[^{14}C]Man (1–6), UDP-[^{14}C]GlcNAc (7–9) and UDP-[^{14}C]Glc (10–15).

Thin-layer chromatography was performed on Kiesel gel G plates as described in Materials and Methods.

phate (1). Thus, mobility of dolichyl phosphate mannose relative to dolichyl phosphate (R_{DP}) was 0.91 (cf. 0.95 in Ref. [24]), whereas R_{DP} were 0.81 for the product from **2a** and 0.88 for the product from **2b**; thus, the mobility seems to depend on the chain length of dolichyl radical.

On the contrary, in the case of dolichyl sulphate (3) no evidence was obtained for the formation of labeled lipid-bound sugars from GDP-[^{14}C]Man by rat liver microsomes. When 3 was incubated at increased concentrations with microsomes and GDP-[^{14}C]Man, slight inhibition of transfer of [^{14}C]Man onto endogenous dolichyl phosphate was observed (see Table 1, column 1). The inhibition of dolichyl phosphate mannose formation from added dolichyl phosphate was also rather weak (Table 1, columns 2 and 3) and became significant only at 1 mM concentration of the sulphate 3. Only dolichyl phosphate [^{14}C]mannose was observed on thin-layer chromatograms.

Interaction of dolichyl phosphate and its analogues with UDP-Glc in the presence of rat liver microsomes

The stimulation of lipid-linked glucose formation by dolichyl phosphate in the presence

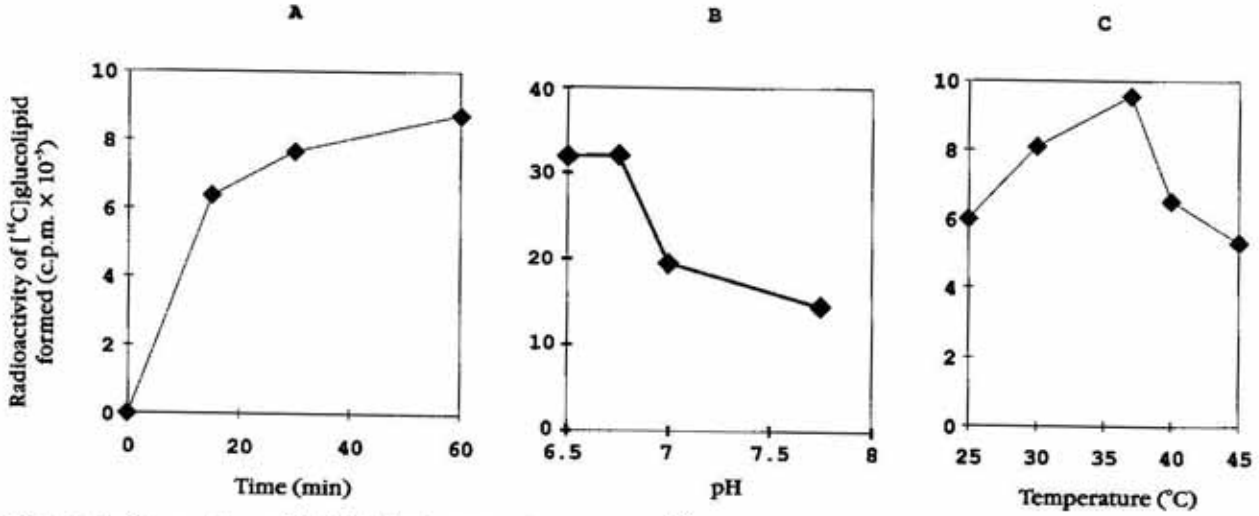


Figure 4. Formation of lipid-linked sugar from UDP-[^{14}C]Glc and dolichyl phosphate (100 μM) in the presence of rat liver microsomes (372 μg of protein) under various experimental conditions.

The incubations were performed as described under "Materials and Methods", with variation of incubation time (A), pH (B), and temperature (C). In the case of (A) and (C) pH was 7.7.

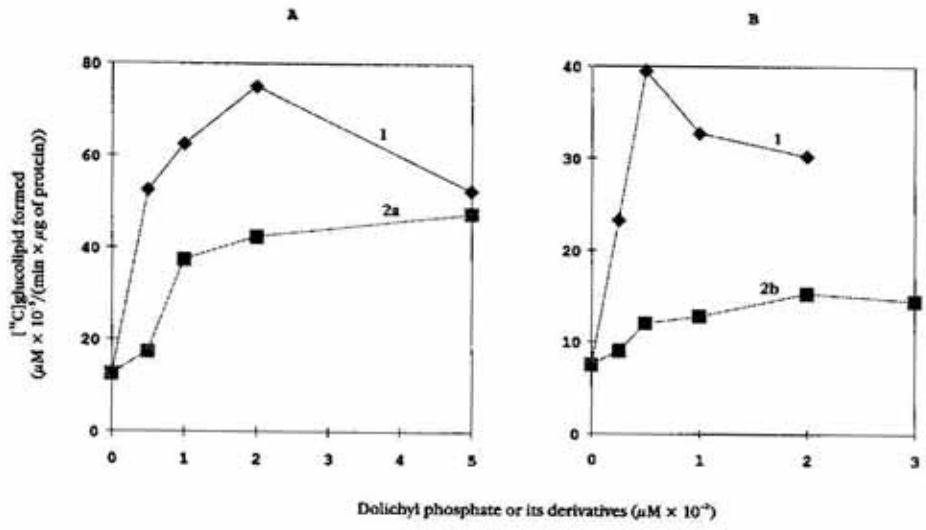


Figure 5. Formation of lipid-linked sugars from UDP-[^{14}C]Glc by rat liver microsomes (372 μg of protein) in the presence of dolichyl phosphate (1) and *H*-phosphonates (2a) and (2b).

A, Reaction with 2a, UDP-[^{14}C]Glc concentration 7 μM. B, Reaction with 2b, UDP-[^{14}C]Glc concentration 3.5 μM.

of labeled UDP-glucose and rat liver microsomes under standard conditions [23] was found to be rather low as compared with synthesis of glycolipids from GDP-mannose. Therefore we have studied the influence of reaction conditions on formation of lipid-linked sugars from UDP-glucose (Fig. 4).

According to the obtained results, further experiments with glucosyltransferase were performed in Tris/HCl buffer (pH 6.8) for 20 min at 37°C.

The dependence of the reaction rate on the formation of lipid-linked sugars from UDP-[^{14}C]Glc in the presence of rat liver microsomes, on dolichyl phosphate or dolichyl *H*-phosphonate concentration is presented in Fig. 5.

Both molecular species of the *H*-phosphonate, **2a** and **2b**, show considerable acceptor activity, although the rate of the reaction is lower than that with the natural dolichyl phosphate. Under the conditions used, inhibition of dolichyl phosphate glucosylation by an excess of substrate is evident, a phenomenon not observed in the case of the *H*-phosphonates **2a** and **2b**.

Glucosylation products of **1**, **2a** and **2b** were separated by thin-layer chromatography and detected by autoradiography as described previously for the mannosyl derivatives. Like in that case, the products formed from the *H*-phosphonates **2a** (R_{DP} 0.83, lanes 12 and 13 in Fig. 3) and **2b** (R_{DP} 0.93, lanes 14 and 15 in Fig. 3) could be distinguished from dolichyl phosphate glucose (R_{DP} 0.89, lanes 10 and 11 in Fig 3). It is interesting to note that for the dolichyl phosphate glucose derivatives the influence of chain length of the dolichyl residue

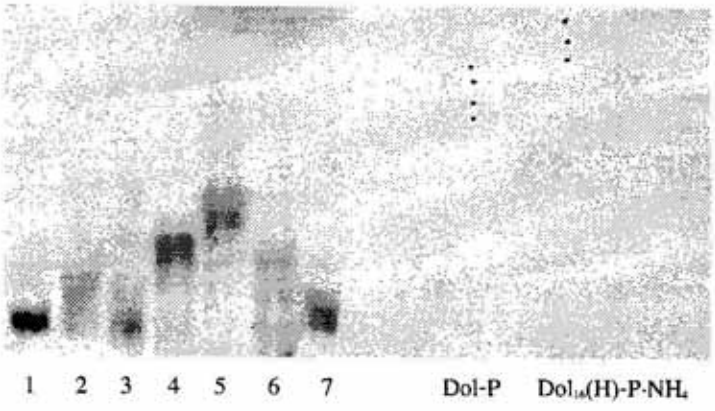


Figure 6. Autoradiography of thin-layer reversed phase chromatogram of glycosylation products of exogenous dolichyl phosphate (1) (1, 4) and *H*-phosphonates (2a) (6) and (2b) (2, 3, 5) by rat liver microsomes in the presence of GDP-[^{14}C]Man (1–3) and UDP-[^{14}C]Glc (4–6).

Thin-layer reversed phase chromatography was performed on RP-18 plates as described in Materials and Methods.

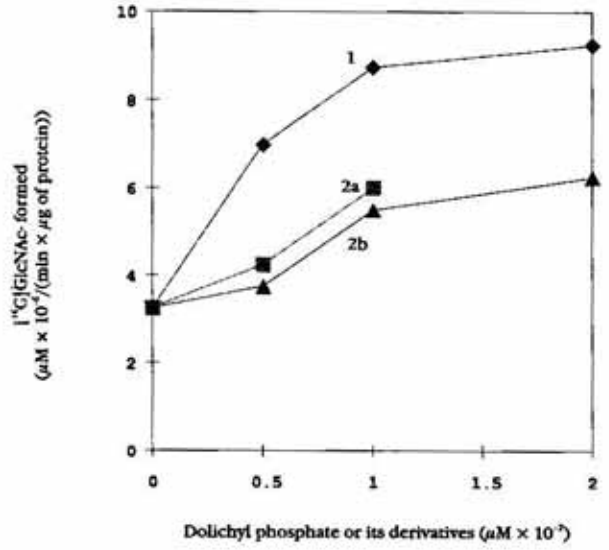


Figure 7. Formation of lipid-linked sugars from UDP-[^{14}C]GlcNAc by rat liver microsomes (372 μg of protein) in the presence of dolichyl phosphate (1) and *H*-phosphonates (2a) and (2b).

on chromatographic mobility is not the same as in the case of dolichyl phosphate mannose derivatives. A similar effect was observed on reversed phase thin-layer chromatography (Fig. 6) where the products obtained from UDP-[^{14}C]Glc and **1**, **2a** or **2b** (lanes 4, 5 and 6, respectively) are clearly different, whereas similar products formed from GDP-[^{14}C]Man (lanes 1, 2 and 3) are visibly rather closer to each other.

Glucosyl transfer from UDP-[^{14}C]Glc to endogenous dolichyl phosphate was slightly inhibited by addition of dolichyl sulphate (**3**) (Table 1, column 4). This inhibition was less significant than in the case of an analogous reaction with GDP-[^{14}C]Man.

Interaction of dolichyl phosphate and its analogues with UDP-GlcNAc in the presence of rat liver microsomes

It has been shown previously that addition of dolichyl phosphate mannose stimulates the biosynthesis of dolichyl pyrophosphate-*N*-acetylglucosamine [25]. To achieve a more efficient formation of GlcNAc-containing glycolipids we included dolichyl phosphate mannose into the incubation mixtures for the assay of *N*-acetylglucosaminylphosphotransferase activity. Preliminary experiments showed that optimal pH for this reaction was 7.05, and incubation time 20 min at 37°C.

Under these conditions, significant incorporation of radioactivity from UDP-[^{14}C]GlcNAc into glycolipids was observed in the presence of rat liver microsomes and dolichyl phosphate (Fig. 7). Participation of the *H*-phosphonates (**2a**, **2b**) in the reaction was less efficient.

TLC analysis followed by autoradiography detected two products formed, with a mobility similar to that of pyrophosphates, presumably dolichyl-PP-GlcNAc and dolichyl-PP-diacetylchitobiose (Fig. 3, lane 7). The monosaccharide derivative is the predominating product and initially only small amounts of dolichyl-PP disaccharide may be seen. In this case, the chromatographic mobility of dolichyl pyrophosphate derivatives and the products formed from **2a** or **2b** in the incubation mixture were quite close (Fig. 3, lanes 8, 9).

As in the case of mannose and glucose transfer reactions dolichyl sulphate (**3**) has no substrate properties in the reaction of *N*-acetylglycosaminyl phosphate transfer and behaves as a very weak inhibitor (see Table 1, column 5).

CONCLUSIONS

The postulated mechanism of conversion of dolichyl phosphate into dolichyl glycosyl phosphates includes nucleophilic attack of the phosphate anion on the C-1 hexosyl center of the nucleoside diphosphate sugar. A similar attack on the P_α-atom of the sugar nucleotide may be the key step in formation of dolichyl diphosphate sugars [4].

The compounds with *H*-phosphonate group instead of phosphate are often used as enzymes inhibitors of important biosynthetic pathways (e.g. as potent pesticides) [26]. In spite of the significant difference of glycosyltransferase systems investigated, dolichyl *H*-phosphonate analogues were found to react

rather efficiently with all three nucleoside diphosphate sugar donors in the presence of rat liver microsomes. The formation of labeled lipid-linked sugars from *H*-phosphonate analogues **2a** and **2b** was confirmed by radiochromatography. It may be suggested that the products formed from **2a** or **2b** and nucleoside diphosphosugars (GDP-Man, UDP-Glc and UDP-GlcNAc) are diesters, in which glycosyl moieties (mannosyl-, glucosyl- or *N*-acetylglucosaminyl-phosphate residues) are connected with dolichyl *H*-phosphonate residue instead of the residue of dolichyl phosphate.

The products formed from **2a** and **2b** in the case of the reaction with GDP-Man and UDP-Glc were shown to be chromatographically distinguishable from the corresponding dolichyl phosphate sugars. The different chain length of dolichyl residue in the products prepared from **2a** (15–18 residues) and **2b** (18–21 residues) is evident, thus excluding the possibility of their formation from endogenous dolichyl phosphate. Still, direct confirmation of their structure is necessary. It may be based on data of ^{31}P-NMR spectroscopy.

The same is true for the products formed from UDP-GlcNAc; in this case there is no chromatographic evidence for non-identity of these products and dolichyl pyrophosphate *N*-acetylglucosamine.

Under conditions of the enzymatic reactions studied, dolichyl phosphate should exist predominantly as dianion. The ability of monoanionic dolichyl *H*-phosphonates to participate in the reactions allows to suggest that in all cases the presence of double negative charge in the dolichyl derivative is not necessary for formation of enzyme-substrate complexes and proceeding of the enzymatic reactions.

Dolichyl sulphate is not a substrate for enzymatic reactions probably due to its low nucleophilicity – charge is distributed between three oxygen atoms. In the phosphate or *H*-phosphonate monoanions charge is distributed between two oxygen atoms. Rather weak inhibitory properties of dolichyl sulphate would imply a strict requirement for the negative charge for the binding of the anionic group of dolichyl phosphate to the enzymes. The change of phosphate for sulphate excludes interactions of this dolichyl anionic derivative with the enzymes.

Studies on the enzymes of dolichol dependent protein glycosylation with other phosphate-modified analogues of dolichyl phosphate might help in understanding the mechanism of enzymic reactions and regulation in this important process.

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