BBA 56069

GLUCOSE TRANSFER FROM DOLICHOL MONOPHOSPHATE GLUCOSE TO THE LIPID MOIETY OF THE ENDOGENOUS MICROSOMAL ACCEPTOR

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(Received February 15th, 1972)

SUMMARY

Further studies on the structure of the microsomal endogenous substance which accepts labeled glucose from dolichol monophosphate glucose are reported. In order to obtain sufficient amounts of substance for measuring bound dolichol monophosphate, a concentrate was prepared by treating liver with mixtures of chloroform–methanol–water which lead to purification of the radioactive compound.

Estimation of dolichol monophosphate, by its accelerating action on dolichol monophosphate glucose formation, showed that it is liberated by mild acid treatment of the concentrate. The dolichol monophosphate-yielding substance behaved like the labeled glucosylated acceptor when tested by its solubility properties in some solvent mixtures, thin-layer and DEAE-cellulose chromatographies and acid or alkaline treatments. Therefore the results provide further evidence that the lipid moiety of the endogenous acceptor of microsomes is dolichol phosphate.

INTRODUCTION

Dolichol, a polyprenol found in animal tissues, has been shown to be involved in the transfer of sugar residues[1]. Thus liver microsomes catalize the following reactions:

$$\text{UDP-Glc} + \text{dolichol-}P \rightarrow \text{dolichol-}P\text{-Glc} + \text{UDP} \quad (1)$$

$$\text{Dolichol-}P\text{-Glc} + \text{acceptor} \rightarrow \text{Glc-acceptor} + \text{dolichol-}P \quad (2)$$

where dolichol-*P*, dolichol-*P*-Glc and Glc-acceptor represent dolichol monophosphate, dolichol monophosphate glucose and glucosylated endogenous acceptor respectively.

The two reactions can be studied separately by using either labeled UDP-Glc or dolichol-*P*-Glc. Information on the structure of the Glc-acceptor was deduced from the properties of the radioactive compound prepared from dolichol-*P*-Glc[2]. It

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

was found that mild acid treatment leads to the liberation of what appears to be an oligosaccharide containing about 20 monosaccharide residues. The identity of the lipid moiety of the Glc-acceptor was not definitely established but studies of the deoxycholate inclusion compounds showed that it could be dolichol[2]. Deoxycholate is known to form inclusion compounds with lipids. For instance, it combines with fatty acids and the ratio of deoxycholate to lipid is related to the lenght of the parafin chain[3]. The molecular weights of the Glc-acceptor and dolichol-*P*-Glc measured in Sephadex columns equilibrated with deoxycholate were found to be 14000 and 11000, respectively. The difference (3000) is nearly the same as the difference in the molecular weights of the hydrophylic moieties of the Glc-acceptor and dolichol-*P*-Glc which is 3400. From this result it was deduced that the lipid part of the Glc-acceptor could be dolichol. Evidence was presented indicating that the oligosaccharide and lipid moieties are joined by a phosphate or pyrophosphate bridge.

Since dolichol-*P* can be estimated by its accelerating action on the rate of Reaction 1 the method has been used to detect its presence in the Glc-acceptor[1]. It has in fact been found that the breakdown of the Glc-acceptor leads to the liberation of substance which reacts like dolichol-*P*.

MATERIALS AND METHODS

Liver microsomes, dolichol-*P* and labeled dolichol-*P*-Glc and the Glc-acceptor, were prepared as previously described[1,2].

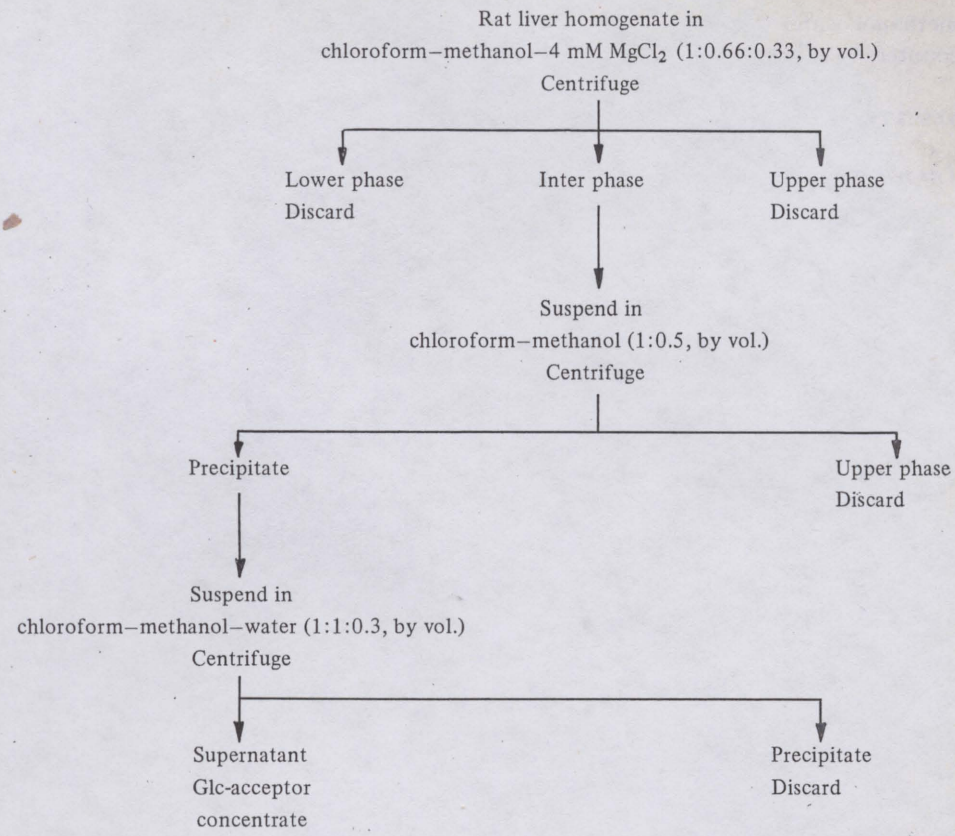
Glc-acceptor concentrate

A concentrate containing unlabeled Glc-acceptor and substances of similar solubility was prepared as indicated in the simplified diagram shown in Scheme 1. About 100 g of rat liver were suspended in 100 ml of 4 mM $MgCl_2$ and homogenized. Methanol (400 ml) and chloroform (600 ml) were successively added. The mixture was centrifuged and the protein interphase was removed and extracted twice with 1200 ml of chloroform–methanol–4 mM $MgCl_2$ (3:2:1, by vol.) and twice with the same mixture but with water instead of $MgCl_2$. The denatured protein was then dried under reduced pressure, extracted once with 300 ml of chloroform–methanol (2:1, by vol.) dried again and extracted 3 times with 350 ml of chloroform–methanol–water (1:1:0.3, by vol.). The extract was dried and dissolved in 350 ml of chloroform–methanol–water (1:1:0.3, by vol.).

Aliquots from this crude extract were further purified as follows. They were dried, a small amount of the denatured protein from the previous step was added and the whole was extracted 4 times with 6 ml of chloroform–methanol (2:1, by vol.). The residue was dried and extracted 3 times with 2 ml of chloroform–methanol–water (1:1:0.3, by vol.). The extract obtained is referred to as the Glc-acceptor concentrate. A parallel preparation performed with radioactive Glc-acceptor and dolichol-*P*-Glc added at the first step showed that the concentrate contained about 25% of the added Glc-acceptor and none of the dolichol-*P*-Glc.

Chloroform–methanol–water (3:2:1, by vol.) partition

The partition was performed by the successive addition of 0.4 ml of methanol, 0.2 ml of water, about 20 mg of denatured protein saved from the last step of the prep-



Scheme 1. Simplified diagram of the procedure for the preparation of Glc-acceptor concentrate.

aration of the Glc-acceptor concentrate and 0.6 ml of chloroform. The upper, inter and lower phases were processed as described in text.

The amounts but not the proportions of the components were sometimes modified.

Treatment with 0.15 M acid

The sample was heated for 5 min at 100 °C in 0.4 ml of 0.15 M HCl in 1-propanol after which 0.3 ml of water and 0.6 ml of chloroform were added. The lower phase was then washed three times with 0.5 ml of chloroform–methanol–water (1:16:16, by vol.). The blanks were prepared by adding the HCl after the chloroform.

Dolichol-P estimation

10 μl of 0.1 M EDTA (magnesium salt), containing 0.1 M $MgCl_2$ were added to the samples. They were dried under vacuum and the following incubation mixture was added: 0.15 M Tris–maleate (pH 7.7), 0.7% Triton X-100, 0.1 M 2-mercaptoethanol, 102000 cpm of [^{14}C]UDP-Glc (228 Ci/mol) and 1.5 mg of rat liver microsomal protein in a total volume of 0.175 μl. The samples were incubated 15 min at 37 °C. The reaction was stopped by the successive addition of 0.4 ml of methanol, 0.1 ml of water and 0.6 ml of chloroform. The lower phase was twice washed with chloroform–

methanol–water (1:16:16, by vol.) and the radioactivity was measured with a flow counter.

RESULTS

The release of dolichol-P from the Glc-acceptor concentrates by acid treatment

It was reported previously that the hydrophylic moiety of the Glc-acceptor is liberated by mild acid[2]. If dolichol-*P* were the lipid moiety of the Glc-acceptor it would be expected to be liberated simultaneously under similar conditions. Since the method of dolichol-*P* estimation is not sensitive enough to detect the small amounts present in radioactive Glc-acceptor the experiments had to be carried out on the Glc-acceptor concentrates. These were prepared as described in Methods, making use of the solubility of the Glc-acceptor known from studies with the radioactive compound[2]. The method was based on the following properties: (a) [^{14}C]Glc-acceptor remains in the interphase together with denatured protein in chloroform–methanol–water (3:2:1, by vol.), while dolichol-*P* and dolichol-*P*-Glc appear in the lower phase; (b) [^{14}C]Glc-acceptor is insoluble in chloroform–methanol (2:1, v/v) while the other two substances are soluble, (c) [^{14}C]Glc-acceptor is soluble in chloroform–methanol–water (1:1:0.3, by vol.).

The experiment presented in Table I shows that the Glc-acceptor concentrate treated with acid yields a substance which behaves like dolichol-*P* in that it stimulates Reaction 1. The experiment also shows that the solubility of the dolichol-*P* yielding substance is the same as that of [^{14}C]Glc-acceptor.

It was checked that the radioactive substance formed in the dolichol-*P* test was in fact dolichol-*P*-Glc by the following criteria: (a) it migrated like dolichol-*P*-Glc in thin-layer chromatography with chloroform–methanol–water (65:25:4, by vol.) (R_F = 0.2) and with 1-propanol–water (7:3, by vol.) (R_F = 0.63)[2], (b) it had the same half life in 0.1 M HCl in chloroform–methanol (2:1, by vol.) at room temperature[2].

Thin-layer chromatography

Radioactive Glc-acceptor and dolichol-*P*-Glc can be separated in thin-layer chromatography in silica gel plates with 1-propanol–water (7:3, by vol.) as solvent[2]. The Glc-acceptor concentrate was run in this solvent with dolichol-*P*-Glc and the

TABLE I

DOLICHOL-*P* PRODUCTION BY ACID TREATMENT OF THE Glc-ACCEPTOR CONCENTRATE

The Glc-acceptor concentrate from 8 ml of crude extract was dried and subjected to the chloroform–methanol–water (3:2:1, by vol.) partition (see Methods). The upper and lower phases were removed and the protein interphase was dried and extracted twice with 0.7 ml of chloroform–methanol–water (1:1:0.3 by vol.). The three fractions (upper, lower and interphase extract) were divided in halves and one set was treated with 0.15 M acid as described in Methods. Dolichol-*P* was then estimated. The numbers shown in this table correspond to the cpm of dolichol-*P*-Glc synthesized in the presence of the samples *minus* those of dolichol-*P*-Glc synthesized by the enzyme alone (297 cpm).

	Dolichol-P (cpm in dolichol-P-Glc)	
	Direct estimation	*After 0.15 M acid treatment*
Upper phase	0	0
Lower phase	0	211
Interphase extract	0	2478
Standard dolichol-*P*	1500	—

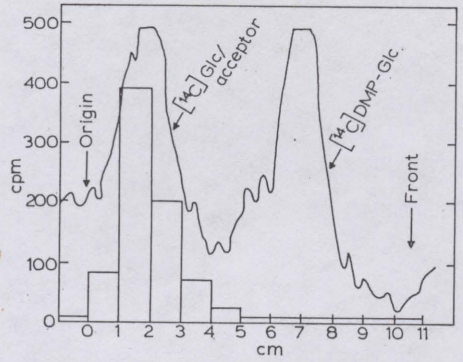


Fig. 1. Thin-layer chromatography. The Glc-acceptor concentrate from 10 ml of crude extract was spotted on a 5 cm × 20 cm silica gel G plate and developed with 1-propanol–water (7:3, by vol.) as solvent. [^{14}C]Glc-acceptor and [^{14}C]dolichol-*P*-Glc were added as internal standards. The plate was scanned (continuous line) and then twelve fractions of 1 cm width were scraped off. Each was heated for 10 min at 100 °C in 1 ml of 0.25 M HCl in 1-propanol, centrifuged and decanted. The silica gel was extracted 3 times more with 0.5 ml of 0.12 M HCl in chloroform–methanol (2:1, by vol.) at room temperature. The supernatants were pooled and chloroform and water were added so as to produce a chloroform–alcohol–water (3:2:1, by vol.) mixture. The lower phases were then washed 3 times with 1 ml of chloroform–methanol–water (1:16:16, by vol.) and dolichol-*P* was assayed. The bars represent the cpm of dolichol-*P*-Glc obtained in each fraction *minus* those of dolichol-*P*-Glc synthesized by the enzyme alone (220 cpm). DMP for all figures represents dolichol-*P*.

Glc-acceptor (both ^{14}C-labeled) as internal standards. The plate was scanned and twelve fractions of 1 cm width were scraped from it.

Since no mild method for eluting the Glc-acceptor from the silica was found, a treatment with 0.25 M acid was performed directly on the fractions. Dolichol-*P* was then extracted and estimated. The radioactivity of the internal standards did not interfere because it remained in the aqueous washings. The results are shown in Fig. 1. It can be seen that the substance that produced dolichol-*P* has the same R_F as [^{14}C]-Glc-acceptor.

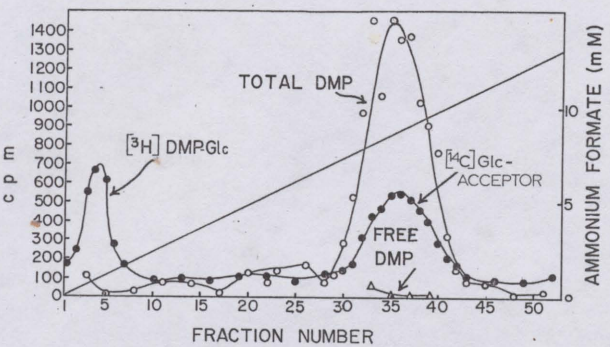


Fig. 2. DEAE-cellulose chromatography. The Glc-acceptor concentrate from 110 ml of crude extract, together with [^{14}C]Glc-acceptor and [^{3}H]dolichol-*P*-Glc was chromatographed on DEAE-cellulose as previously described[2]. 1 ml of the fractions was used for the radioactivity measurements. The remaining part of the fractions was divided in halves. All were dried and one set was subjected to the 0.15 M acid treatment. Dolichol-*P* was then assayed. ●—●, [^{3}H]dolichol-*P*-Glc and [^{14}C]Glc-acceptor; ○—○, [^{14}C]dolichol-*P*-Glc synthesized in the aliquots subjected to the acid treatment *minus* that synthesized by the enzyme alone (150 cpm); △—△, the same but without acid treatment.

DEAE-cellulose chromatography

Radioactive Glc-acceptor and dolichol-*P*-Glc can also be separated by DEAE-cellulose chromatography using chloroform–methanol–water (1:1:0.3, by vol.) as solvent and a linear ammonium formate gradient[2]. Fig. 2 shows an experiment performed with the Glc-acceptor concentrate using [^{14}C]Glc-acceptor and [^{3}H]dolichol-*P*-Glc as internal standards. Dolichol-*P* was found after the 0.15 M acid treatment only in the fractions which contained [^{14}C]Glc-acceptor.

Alkaline treatment

It was previously reported that the hydrophylic moiety of [^{14}C]Glc-acceptor can be liberated by alkaline treatment[2]. The experiment described in Fig. 3 shows that under such conditions, the rate of liberation of radioactive oligosaccharide from [^{14}C]-Glc-acceptor is the same as that of dolichol-*P* from the Glc-acceptor concentrate.

Mild acid treatments in methanolic and aqueous media

[^{14}C]Glc-acceptor is decomposed by 0.1 M HCl in methanol at 30 °C with a half

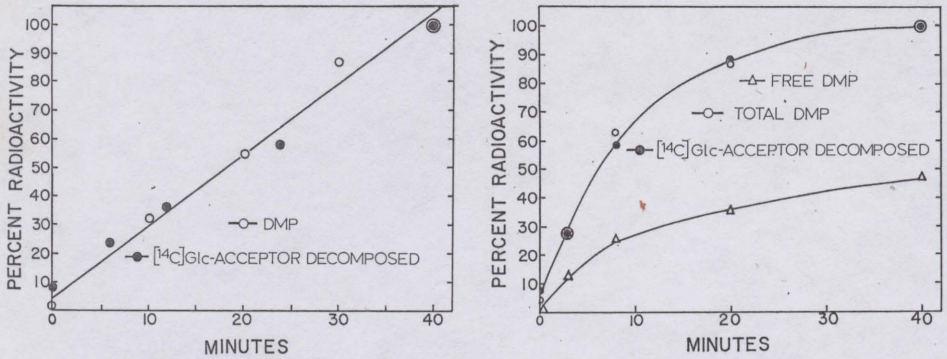


Fig. 3. Alkaline treatment. The Glc-acceptor concentrate from 75 ml of crude extract was divided in five aliquots and dried. After addition of 0.4 ml of 1-propanol and 4 μl of 10 M KOH, each aliquot was heated for different times at 64 °C after which 0.3 ml of water, 0.6 ml of chloroform and 4 μl of 11.7 M HCl were successively added. The lower phases were washed 3 times with 0.5 ml of chloroform–methanol–water (1:16:16, by vol.), dried, and subjected to the chloroform–methanol–water (3:2:1, by vol.), partition. The lower phases were removed and dolichol-*P* was estimated in them. The cpm of dolichol-*P*-Glc synthesized by the aliquot treated for 40 min with alkali *minus* the dolichol-*P*-Glc synthesized by the enzyme alone (3778–229 = 3549 cpm) were taken as 100% for this curve (○—○). A parallel experiment was performed with [^{14}C]Glc-acceptor under the same conditions with the exception that after the alkaline treatment the samples were diluted with 0.8 ml of water, neutralized with acetic acid, dried and subjected to the chloroform–methanol–water (3:2:1, by vol.) partition. The upper phases were removed and counted. The cpm found after 40 min of alkaline treatment (905 cpm) were taken as 100% for this curve (●—●).

Fig. 4. Acid methanolysis. The Glc-acceptor concentrate from 20 ml of crude extract was divided in five aliquots, mixed with [^{14}C]Glc-acceptor and dried. After adding 0.6 ml of methanol and 5 μl of 11.7 M HCl, the tubes were incubated at 30 °C for different times. The reaction was stopped by the chloroform–methanol–water (3:2:1, by vol.) partition. The upper phases were removed, neutralized with LiOH and counted. The cpm found in the 40-min aliquot (1934 cpm) were taken as 100% for this curve (●—●). The remaining inter and lower phases were washed twice with 0.5 ml of chloroform–methanol–water (1:16:16, by vol.) The lower phases were removed, divided in halves and one set subjected to the 0.15 M acid treatment. Dolichol-*P* was then estimated. ○—○, Dolichol-*P*-Glc formed in the aliquots subjected to the 0.15 M acid treatment; △—△, the same but in samples not subjected to that treatment. The cpm of dolichol-*P*-Glc synthesized by the 40-min aliquot subjected to the 0.15 M acid treatment *minus* the dolichol-*P*-Glc synthesized by the enzyme alone (1633 − 276 = 1357 cpm) were taken as 100% for these two curves.

TABLE II

ACID HYDROLYSIS OF GLC-ACCEPTOR CONCENTRATE

The Glc-acceptor concentrate from 15 ml of crude extract was divided in 3 aliquots and dried. Water (0.45 ml) and dilute HCl (to pH 2) were added to Tubes A and B. These were heated for 5 min at 100 °C after which they were subjected to the chloroform–methanol–water (3:2:1, by vol.) partition. Tube C is an unheated blank. The inter and lower phases were washed twice with 0.5 ml of chloroform–methanol–water (1:16:16, by vol.), the lower phases were removed and subjected to the 0.15 M acid treatment where indicated. Dolichol-*P* was then estimated. The cpm of dolichol-*P*-Glc synthesized by the enzyme alone (550 cpm) have been subtracted from the numbers given in this table.

Tube	*Hydrolysis at pH 2, 5 min, 100 °C*	*0.15 M acid treatment of lower phase of previous*	*Dolichol-P (cpm in Dolichol-P-Glc)*
A	+	−	909
B	+	+	2182
C	−	+	3

life of 7–8 min. A radioactive, uncharged, water soluble substance is produced which appears to be a large oligosaccharide[2].

The experiment depicted in Fig. 4 showed that under such conditions two different lower-phase soluble products were liberated from the Glc-acceptor concentrate. One of them could be estimated directly as dolichol-*P* and the other could generate dolichol-*P* after a stronger acid treatment. It can also be seen that the rate of liberation of these two substances was the same as that of radioactive oligosaccharide from [^{14}C]Glc-acceptor.

These two lower phase soluble substances were also produced by a mild acid treatment of the Glc-acceptor concentrate in a completely aqueous medium (Table II). It was found that [^{14}C]Glc-acceptor was almost completely hydrolized under these conditions and that a radioactive water soluble, uncharged molecule was produced.

DISCUSSION

The results reported in this paper show that a substance extracted from rat liver (Glc-acceptor concentrate), having the same solubility properties as [^{14}C]Glc-acceptor in chloroform–methanol–water (3:2:1 and 1:1:0.3, by vol.) mixtures, liberated dolichol-*P* by acid treatment. Moreover, this dolichol-*P* producing substance migrated in thin-layer chromatography like [^{14}C]Glc-acceptor and was eluted together with it from DEAE-cellulose. The rate by which the Glc-acceptor concentrate liberated dolichol-*P* in 0.1 M KOH in 1-propanol was the same as that of production of radioactive oligosaccharide by [^{14}C]Glc-acceptor. The Glc-acceptor concentrate liberated two lower phase soluble substances by acid methanolysis or hydrolysis. One was dolichol-*P* and the other could generate dolichol-*P* by a stronger acid treatment. The rate by which both substances were produced by acid methanolysis was the same as that of radioactive oligosaccharide liberation by [^{14}C]Glc-acceptor.

The structure previously suggested for the Glc-acceptor was that of an oligosaccharide joined to dolichol through a phosphate or pyrophosphate bridge[2]. If the latter possibility were true, dolichol pyrophosphate would be the expected lipid moiety produced by acid methanolysis or mild acid hydrolysis, since an uncharged oligosaccharide is liberated by such treatments. It is possible that under those conditions

dolichol pyrophosphate is partly degraded to dolichol-*P*. As dolichol pyrophosphate is presumably not a substrate for dolichol-*P*-Glc synthesis, the increase in dolichol-*P* that appeared after the stronger acid treatment is probably due to the conversion of the pyrophosphate to the monophosphate derivative.

The results presented in this paper provide further evidence indicating that dolichol is the lipid moiety of the Glc-acceptor. The fact that mild acid treatment leads to the formation of a substance which has the solubility of dolichol-*P* and which yields dolichol-*P* on further acid treatment, indicates the presence of a pyrophosphate bridge joining the hydrophylic and lipophylic moieties. This strengthens the previous evidence based on the elution pattern of the Glc-acceptor in DEAE-cellulose columns[2].

ACKNOWLEDGEMENTS

This investigation was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina). A.J.P. and N.H.B. are Career Investigators of this Institution.

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