Proceedings of the National Academy of Sciences
Vol. 66, No. 1, pp. 153–159, May 1970

Dolichol Monophosphate Glucose: An Intermediate in Glucose Transfer in Liver*

Nicolás H. Behrens† and Luis F. Leloir‡

INSTITUTO DE INVESTIGACIONES BIOQUÍMICAS "FUNDACIÓN CAMPOMAR" AND FACULTAD DE CIENCIAS EXACTAS Y NATURALES, BUENOS AIRES, ARGENTINA

Communicated February 9, 1970

Abstract. The microsomal fraction of liver has been found to catalyze glucose transfer from UDPG to a lipid acceptor which appears to be identical to the compound obtained by chemical phosphorylation of dolichol. The substance formed (dolichol monophosphate glucose) is acid labile and yields 1,6-anhydroglucosan by alkaline treatment. It can be used as substrate by the enzyme system yielding a glucoprotein which is subsequently hydrolyzed to glucose.

One of the most important developments in the field of saccharide biosynthesis has been the discovery of lipid intermediates in sugar transfer reactions. The studies of Wright *et al.*[1] on O-antigen and of Higashi *et al.*[2] on peptidoglucan synthesis in bacteria showed that polyprenol pyrophosphate sugars are formed by transfer from nucleotide sugars and subsequently act as donors for polysaccharide formation. As shown by Scher *et al.*,[3] similar events occur in *M. lysodeikticus* where mannose is first transferred from GDP-mannose to undecaprenol monophosphate and then to mannan. In animal tissues an enzyme has been described which catalyzes mannose transfer from GDP-mannose to a lipid.[4]

In the course of work with UDPG it has now been found that liver contains enzymes which catalyze the following reactions:

$$\text{UDPG} + \text{acceptor lipid} \rightarrow \text{G-acceptor lipid} + \text{UDP} \quad (1)$$

$$\text{G-acceptor lipid} + \text{protein} \rightarrow \text{acceptor lipid} + \text{G-protein} \quad (2)$$

$$\text{G-protein} \rightarrow \text{G} + \text{protein} \quad (3)$$

Since the rate of formation of glucosylated acceptor lipid by reaction (1) is proportional to the acceptor lipid added, the latter could be estimated and purified. The preparations obtained could be used as substrates for reaction (2). This direct transfer from the lipid intermediate has only been detected in few cases. Evidence is presented indicating that the acceptor lipid is dolichol monophosphate. Dolichol[5] is a polyprenol present in animal tissues which has 16 to 21 isoprene units, the first being saturated.

Materials and Methods. Reagents: UDPG labeled in the glucose with ^{14}C was prepared according to the method of Wright and Robbins[6] with slight modifications. Dolichol was prepared from pig liver. The extraction and alumina chromatography was performed as described by Burgos *et al.*[5] The fractions were analyzed by thin-layer

chromatography with chloroform as solvent. Those which contained a substance having an R_f of about 0.45 and giving a green color with the anisaldehyde reagent were pooled and purification was performed by preparative thin-layer chromatography, first with chloroform and then with petroleum ether (30°–65°)-isopropyl ether, 80:20 (ref. 7). After each run the dolichol was extracted from the silica gel with ethyl ether. The purity of the sample was checked by thin-layer chromatography and its identity by its infrared spectrum. Phosphorylation of dolichol was performed using trichloroacetonitrile as condensing agent.[8, 9] After the reaction the mixture was dried *in vacuo*, extracted with chloroform-methanol and washed.

1,6 Anhydroglucosan was prepared by heating salicin in 1 *N* NaOH for 10 hr at 100° and neutralizing with Dowex 50 H^+. This preparation contained salicin and 1,6-anhydroglucosan. After chromatography with solvent A (see below), the substances could be visualized with the alkaline silver reagent.[10] Both compounds react slowly. The $R_{glucose}$ of anhydroglucosan varied in different runs between 2.2 and 2.8. Salicin had a 20% higher mobility.

Chromatography: The following solvents were used for silica gel thin-layer chromatography: (*A*) chloroform-methanol-ammonia-water, 80:30:0.5:3; (*B*) chloroform-methanol-formic acid-water, 70:18.5:8:0.5 (ref. 11); and (*C*) chloroform-methanol-water, 65:25:4. The solvent for paper chromatography was (*D*) *n*-butanol-pyridine-water, 6:4:3. Polyprenols were visualized with the anisaldehyde reagent and lipids with iodine or fluorescein.[7] Localization of the acceptor lipid was performed by removing 1 cm zones from the plates followed by extraction with chloroform-methanol, 2:1, containing 0.6 *N* HCl. The samples were then washed and assayed for acceptor lipid as described below.

Preparation of the enzyme: Rough and smooth rat liver microsomes were prepared according to the method of Moulé *et al.*[12] The smooth fraction was usually more active than the rough but both were used and were very stable if kept frozen with 0.25 *M* sucrose–0.01 *M* glycylglycine. The concentration was adjusted so that 1 ml corresponded to 1 gm of fresh liver.

Assay for lipid glycosylation: A sample of acceptor lipid in chloroform-methanol was mixed with 5 μl of 0.1 *M* Mg-EDTA (prepared by neutralizing ethylenetetraacetic acid with MgO), mixed thoroughly, and dried *in vacuo*. The components of the reaction mixture were then added. The final concentration was: 0.2 *M* glycylglycine, pH 7.5, 0.1 *M* mercaptoethanol, 0.6% Triton X-100, ^{14}C-UDPG (125,000 counts/min, 205 μc/μmole) and 20 μl of enzyme in a final volume of 50 μl. Before adding the enzyme, the lipid was emulsified with a vortex mixer. After incubation at 37° for 15 min, 0.4 ml of methanol and 0.6 ml of chloroform were added. The protein precipitate was separated by centrifugation and after adding 0.2 ml of 4 mM $MgCl_2$, the chloroform phase was washed as described by Folch *et al.*,[13] dried on planchets, and counted.

Preparation of the acceptor lipid: Ground pig liver was treated with 2 vol of acetone and filtered. The dry residue was extracted with 3 vol of chloroform-methanol, 2:1, and filtered. The extract was made 0.1 *N* in NaOH, and incubated 15 min at 37°. Sufficient HCl was then added to give a concentration of 0.1 *N* in free acid. After refluxing for 15 min, the chloroform layer was washed according to the technique of Folch *et al.*[13] The extract was then poured into a DEAE-cellulose column in the acetate form.[14] The column was washed with chloroform-methanol, 2:1, and then eluted with 0.1 *M* ammonium acetate, pH 4, in chloroform-methanol, 2:1. Fractions which contained acceptor lipid activity were pooled, washed free from ammonium acetate, and concentrated. Further purification was achieved using silica gel thin-layer chromatography with solvent systems A and B in succession. Before preparing the plates, the silica gel was treated with 5% HCl in ethanol, filtered, and thoroughly washed with ethanol. Acceptor lipid was eluted from the chromatograms with 0.6 *N* HCl in chloroform-methanol, 2:1.

Results.—Glucose transfer to the acceptor lipid: Incubation of the enzyme preparation with UDPG labeled in the glucose moiety and acceptor lipid led to the appearance of radioactivity in the chloroform-soluble fraction. In order to

find out if phosphate is also transferred, a parallel incubation with β^{32}P-labeled UDPG was made. The results were as follows:

glucose transferred from ^{14}C-UDPG 3.7 $\mu\mu$mole
phosphate transferred from ^{32}P-UDPG 0.03 $\mu\mu$mole

It was concluded, therefore, that only glucose is transferred.

The conditions for glucolipid formation are shown in Table 1. The reaction is

TABLE 1. *Optimal conditions for lipid glucosylation.*

Reaction mixture	$\mu\mu$moles of glucose in chloroform
Complete	9
Minus acceptor lipid	0.75
Minus Mg^{++}	0.08
15 mM Mn^{++} instead of Mg^{++}	1
Minus deoxycholate	4
Minus mercaptoethanol	7.2
Complete plus 4 mM UDP	0.5

Reaction mixture and assay as described in *Materials and Methods* except that 8.5 mM EDTA was added as sodium salt, Mg^{++} was 15 mM, and Triton X-100 was replaced by 0.4% deoxycholate.

increased by Mg^{++} but hardly at all by Mn^{++}. The glucosylation is inhibited completely by EDTA added in excess of Mg^{++}, and by UDP. Deoxycholate or Triton X-100 usually doubled the yield of glucolipid.

The fact that addition of acceptor lipid gave more chloroform-soluble radioactivity was used for its estimation. As shown in Figure 1, glucosylation was proportional to the amount of acceptor lipid added.

The radioactivity in the lipid increased at first with the time of incubation and decreased subsequently due to its simultaneous disappearance through reaction (2). Therefore accurate measurements of the rate of the glucosylation reaction could not be obtained. Experiments in which the different fractions of liver homogenates were measured showed that the activity was distributed in all the particulate fractions, but the microsomes were always more active. Estimations

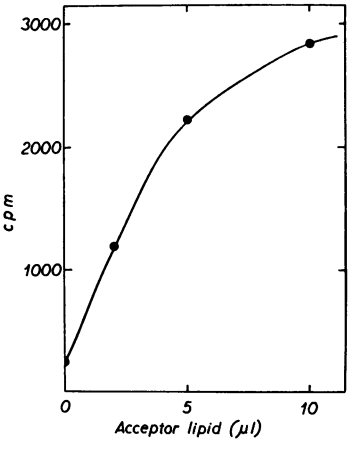

FIG. 1.—Glycosylation as a function of acceptor lipid concentration. Increasing amounts of purified acceptor lipid were added to the reaction mixture and glucosylation was assayed as described in *Materials and Methods*. A purified sample of acceptor lipid (0.53 μmole of phosphate/ml) was used.

in the smooth and rough microsome fractions showed no great differences in activity but usually more in the former.

Properties and purification of the acceptor lipid: The acceptor lipid was found to be soluble in chloroform methanol and in butanol but sparingly soluble in ethyl ether or acetone. The solubility characteristics were those of a phospholipid. Treatment with 1 *N* acid at 100° for six minutes, or 0.05 *N* alkali at 100° for five minutes did not affect acceptor activity. Since it was retained by DEAE-cellulose it was concluded that the acceptor lipid is an acid. Its behavior on thin-layer chromatography agrees with this conclusion. It remained at the origin in alkaline but moved in acid solvents. In solvents A and B the R_f was 0 and 0.75, respectively. Acetylation had no effect but methylation with diazo methane destroyed the acceptor lipid activity. Treatment with Br_2 or Fe^{+++} inactivated the acceptor lipid completely.

Purification of the acceptor lipid could be achieved by decomposition of other lipids with alkali and acid, followed by DEAE-cellulose and thin-layer chromatography. However, considerable losses were often experienced, particularly during thin-layer chromatography presumably due to the presence of Fe^{+++} in the silica gel. Most of the Fe^{+++} could be removed by first washing with ethanol-HCl. In this manner considerable purification was obtained (Table 2).

TABLE 2. *Purification of acceptor lipid.*

	mmoles of organic phosphate	mμmoles of glucose incorporated per μmole of phosphate	Recovery (%)
Crude extract	44.8	0.008	100
After alkaline and acid treatment	4.2	0.06	70
DEAE-cellulose	0.12	1.9	69
Thin-layer chromatography	0.03	6.8	55

The product of the last purification step gave only one spot after thin-layer chromatography with solvents A and B. The purified preparations contained organic phosphate in a form which was only released under drastic conditions.

Properties of the glucosylated acceptor lipid: The glucosylated acceptor lipid is acid labile. In *n*-propanol at 100° with 5 per cent trichloroacetic acid or in 0.01 *N* HCl, it is completely decomposed in five minutes. Comparable treatment but at pH 6 had no effect. The course of the breakdown at 18° in chloroform methanol containing 0.1 *N* HCl is shown in Figure 2. Under these conditions methyl glucoside is formed whereas in more aqueous solvents glucose is formed as judged by paper chromatography with solvent D. In no case was any galactose detected.

Alkaline treatment (45 min at 100° with 1 *N* NaOH, in aqueous *n*-propanol) was found to decompose the radioactive glycosylated acceptor lipid so that the radioactivity became water soluble. The compound formed did not migrate in electrophoresis and moved faster than glucose during paper chromatography with solvent D. After extracting it from the paper and heating at 100° in 0.5 *N* acid for two hours, it was transformed into a substance which had the mobility of glucose.

Since alkaline degradation in water gave the same compound as in *n*-propanol

it could be excluded that the product formed was a glucoside. Its migration during paper chromatography with solvent D was compared with that of 1,6-anhydroglucosan and it was found that both compounds behaved in the same manner. The rate of formation of 1,6-anhydroglycosan under milder conditions (0.1 *N* NaOH at 64°) is shown in Figure 2.

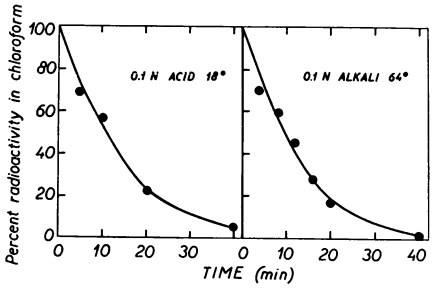

FIG. 2.

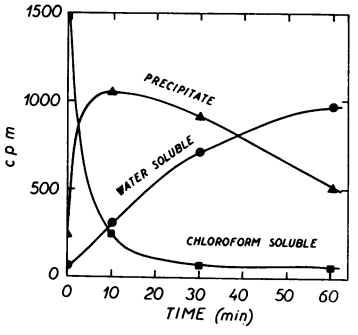

FIG. 3.

FIG. 2.—Acid and alkaline degradation of the glucosylated acceptor lipid. Acid treatment of ^{14}C-glucosyl acceptor lipid: 0.1 *M* HCl in chloroform methanol, 2:1, at 18°. At the indicated times, samples of the reaction mixture were withdrawn, washed free from water soluble products as described under *Materials and Methods*, evaporated in a scintillation vial, and the radioactivity of the remaining glucosyl acceptor lipid measured after dissolving in Bray's solution.[15] Alkaline treatment: 0.1 *M* NaOH in *n*-propanol at 64°. Samples were washed after addition of sufficient chloroform to give a twophase system and HCl to make the solution slightly acid.

FIG. 3.—Transfer from the glucosylated acceptor lipid. Glucosylated acceptor lipid was incubated at 30° with 50 μl of microsomal enzyme and the following reaction mixture: 7 mM Na-EDTA, 0.14 *M* glycylglycine, pH 7.5, 0.07 *M* mercaptoethanol, and 1.28% Triton X-100. The reaction was stopped by addition of 1 ml of chloroform methanol, 2:1, and 0.25 ml of 4 mM $MgCl_2$. After centrifugation the precipitate appeared at the chloroform-water interphase. The water was sucked off, the chloroform phase decanted, and the remaining precipitate washed with butanol, transferred to a scintillation vial, and counted with Bray's solution. Both the chloroform and the aqueous phases were counted in a gas flow counter. Counts measured in the scintillator were corrected to the flow-counter efficiency multiplying by 0.75.

Transfer from the glucosylated acceptor lipid: Incubation of the glucosylated acceptor lipid with the enzyme preparation at 30° in the presence of 1.3 per cent Triton X-100, leads to the changes depicted in Figure 3. It is apparent that the radioactivity which at the start is soluble in chloroform methanol, becomes insoluble, and then gradually becomes water soluble. These reactions do not require Mg^{++}.

The nature of the compound formed first has not been settled definitely but it appears to be a protein. Treatment with trichloroacetic acid did not solubilize the radioactive compound. Phenol at 65° extracted most of the radioactivity. Acid hydrolysis (1 *N* SO_4H_2 for 30 min at 100°) led to the liberation of glucose from the compound. Treatment for three hours with 3 *M* KOH at 100° leads to the formation of a substance which seemed to be different from the known amino acid-sugar compounds. This point will be studied further.

The reaction in which the radioactivity of the insoluble fraction becomes water

soluble (reaction 3) is presumably catalyzed by a glucosidase because the reaction product is mainly glucose.

Synthetic dolichol phosphate: The infrared spectra of purified preparations of acceptor lipid had some similarities with that of dolichol.[5] However, the preparation of pure specimens of acceptor lipid was not easy, so that a short cut was attempted. The unsaponifiable fraction of liver was phosphorylated as described in *Materials and Methods*. The preparation obtained was active in the assay for lipid glucosylation. Phosphorylation of a pure sample of dolichol also gave a product which reacted as acceptor in our test. The compound obtained by the chemical phosphorylation of dolichol is the same as the acceptor isolated from liver as judged by the following criteria: (*a*) They both acted as glucose acceptors from UDPG when incubated with the liver enzyme; (*b*) Both resisted acid treatment (5 min in 1 *N* acid at 100°); (*c*) They gave the same R_f on thin-layer chromatography with solvent B (R_f = 0.81). Comparison of the two compounds after enzymatic glucosylation showed that (*d*) both released their glucose after five minutes at 100° in 0.01 *N* HCl in 50 per cent *n*-propanol; (*e*) both were decomposed with alkali (1 *N* NaOH in 85 per cent *n*-propanol, 10 min at 100°) and gave compounds which migrated like 1,6-anhydroglucosan during paper chromatography with solvent D; (*f*) They had the same R_f on thin-layer chromatography with Solvent C (0.20); (*g*) When incubated with the enzyme as described in Figure 3 the synthetic compound behaved the same as the natural compound. That is, the radioactivity was transferred to the insoluble material and appeared afterwards in the aqueous phase.

Discussion. Incubation of the microsomal fraction of liver with UDPG leads to a series of glucose transfer reactions. The first acceptor of glucose is a lipid which has the same properties as the compound obtained by phosphorylation of dolichol. The enzyme that catalyzes this transfer can use both the natural acceptor lipid and synthetic dolichol phosphate. The glucosylated compound formed from both acceptors appears to be the same as judged by the lability to acid and alkali and by its behavior during thin-layer chromatography. Furthermore, both the natural and synthetic acceptors resist acid treatment under conditions where a pyrophosphate group would be hydrolyzed.

The compounds isolated from bacteria behave in a different manner towards acids. Thus undecaprenol phosphate which is involved in mannan synthesis loses 80 per cent of its phosphate by heating at 100° at pH 2 for 20 minutes.[16] This difference is probably due to the fact that phosphate in undecaprenol phosphate is labilized by the allyl structure to which it is bound, whereas in dolichol the first isoprene unit is saturated.

The evidence indicates therefore that the natural acceptor lipid is dolichol monophosphate and following the current nomenclature for nucleotide sugars, the glucosylated compound can be called dolichol monophosphate glucose (DMPG). Many free polyprenols differing in chain length, degree of saturation, and in the number of *cis* and *trans* groups have been isolated from different organisms[17] so that it seems likely that, as in the case of sugar nucleotides, a large family of polyprenol phosphosugars containing various sugar and polyprenol moieties will be found. Furthermore, compounds with either one or two phosphates have already been found, so that the variety of possible intermediates is even larger.

Information on the type of linkage of the glucose in the glucosylated lipid can be deduced from the alkaline degradation. It is known that aryl glycosides yield 1,6-anhydro sugars by alkaline treatment and that enhanced reactivity is found when the OH group at C_2 of the sugar is *trans* to the aglycon.[18] Since the glucosylated acceptor lipid yields 1,6-anhydroglucosan even faster than salicin (β-glucosyl-O-hydroxybenzyl alcohol) it seems likely that it also has a β-linkage.

Data on the nature of the products formed by transfer from the glucosylated acceptor lipid (reactions (2) and (3)) are preliminary. One of the few proteins which contain glucose is collagen and transfer from UDPG has been studied.[19, 20] However, in our case, no glucosyl-galactosyl-lysine could be detected as alkaline degradation product of G protein as would be expected if the product of reaction (2) were collagen.

Studies on the nature of the products formed from dolichol monophosphate glucose and similar transfer reactions with other sugars are in progress.

We wish to thank Dr. M. Dankert for having suggested the initial experiments and for his constant advice.

* Supported in part by grants from the U.S. Public Health Service (GM 03442) and the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

† Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

‡ Reprints may be addressed to Dr. Leloir, Instituto de Investigaciones Bioquímicas "Fundación Campomar," 2490 Obligado, Buenos Aires (28), Argentina.

[1] Wright, A., M. Dankert, P. Fennesey, and P. W. Robbins, these PROCEEDINGS, **57**, 1798 (1967).

[2] Higashi, Y., J. L. Strominger, and C. C. Sweeley, these PROCEEDINGS, **57**, 1878 (1967).

[3] Scher, M., W. J. Lennartz, and C. C. Sweeley, these PROCEEDINGS, **59**, 1313 (1968).

[4] Caccam, J. F., J. J. Jackson, and E. H. Eylar, *Biochem. Biophys. Res. Commun.*, **35**, 505 (1969).

[5] Burgos, J., F. W. Hemming, J. F. Pennock, and R. A. Morton, *Biochem. J.*, **88**, 470 (1963).

[6] Wright, A., and P. W. Robbins, *Biochim. Biophys. Acta*, **104**, 594 (1965).

[7] Dumphy, P. J., J. D. Kern, J. F. Pennock, K. J. Whittle, and J. Feeney, *Biochim. Biophys. Acta*, **136**, 136 (1967).

[8] Cramer, F., and H.-J. Böhm, *Angew. Chem.*, **71**, 775 (1959).

[9] Popják, G., J. W. Cornforth, R. H. Cornforth, R. Ryhage, and D. S. Goodman, *J. Biol. Chem.*, **237**, 56 (1962).

[10] Trevelyan, W. E., D. P. Procter, and J. S. Harrison, *Nature*, **166**, 444 (1950).

[11] Hooghwinkel, G. J. M., P. Borri, and J. C. Riemersma, *Rec. Trav. Chim.*, **83**, 576 (1964).

[12] Moulé, Y., J. Chauveau, and C. Rouiller, *J. Biophys. Biochem. Cytol.*, **7**, 547 (1960).

[13] Folch, J., M. Lees, and G. H. Sloane Stanley, *J. Biol. Chem.*, **226**, 497 (1957).

[14] Rouser, G., G. Kritchevsky, and A. Yamamoto, in *Lipid Chromatographic Analysis*, ed. G. V. Marinetti (New York: M. Dekker, Inc., 1967), vol. 1, p. 99.

[15] Bray, G., *Anal. Biochem.*, **1**, 279 (1960).

[16] Lahav, M., T. H. Chin, and W. J. Lennartz, *J. Biol. Chem.*, **244**, 5890 (1969).

[17] Hemming, F. W., *Biochem. J.*, **113**, 23P (1969).

[18] Capon, B., *Chem. Rev.*, **69**, 407 (1969).

[19] Bosmann, H. B., and E. H. Eylar, *Biochem. Biophys. Res. Commun.*, **30**, 89 (1968).

[20] Spiro, R. G., *J. Biol. Chem.*, **242**, 4813 (1967).