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THE SYNTHESIS OF P^1-2-ACETAMIDO-4-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-DEOXY-α-D-GLUCOPYRANOSYL P^2-DOLICHYL PYROPHOSPHATE, (P^1-DI-*N*-ACETYL-α-CHITOBIOSYL P^2-DOLICHYL PYROPHOSPHATE)*†

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ABSTRACT

2-Acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl phosphate, pure according to thin-layer and gas–liquid chromatography, optical rotation, and treatment with alkaline phosphatase and 2-acetamido-2-deoxy-β-D-glucosidase, was prepared by treatment of 2-methyl-[4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline with dibenzyl phosphate, followed by the removal of the benzyl groups by catalytic hydrogenolysis, and *O*-deacetylation. In contrast, a sample prepared by the phosphoric acid procedure was shown to consist mainly of the β anomer. 2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate was treated with P^1-diphenyl P^2-dolichyl pyrophosphate to give a fully acetylated pyrophosphoric diester, which was *O*-deacetylated to give P^1-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate. This compound could be separated from the β anomer by t.l.c., and its behavior under dilute acid and alkaline conditions was investigated.

INTRODUCTION

As a result of investigations in several laboratories into the role of "lipid intermediates" in glycoprotein biosynthesis, compounds have been isolated that are lipid-bound derivatives of 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucose (di-*N*-acetylchitobiose)[2–6], and lipid-bound derivatives of

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oligosaccharides having a 2-acetamido-2-deoxy-D-glucose residue at the "reducing" end[4,7,8]. In one case, proof of the presence of a di-*N*-acetylchitobiose residue at the "reducing" end of the oligosaccharide has been obtained[3,11], whereas in other systems formation of lipid-bound oligosaccharides from the di-*N*-acetylchitobiosyl lipid has been shown[5,12,13]. It is known that many glycoproteins contain a di-*N*-acetylchitobiose residue linked to an asparagine residue[14], and recent studies indicate that the biosynthesis of at least a part of the oligosaccharide moiety of such glycoproteins occurs via a lipid intermediate, with subsequent transfer of the oligosaccharide from the lipid to the protein moiety[15].

The identity of the lipid component of these compounds has not been conclusively established, although it has often been assumed to be dolichol because the formation of the di-*N*-acetylchitobiosyl lipid is stimulated by dolichyl phosphate[2] and that of the oligosaccharide lipid by P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate[13]. The identification of the lipid-bound di-*N*-acetylchitobiose compound as a pyrophosphate diester is also tentative, being based on the low mobility in thin-layer chromatography, adsorption to DEAE-cellulose and elution by high concentrations of salt, lability to mild-acid hydrolysis, and hydrolysis by mild alkali to give a product having the properties of a sugar phosphate. During studies in this laboratory on the biosynthesis of glycoproteins by calf pancreas, a labeled, lipid-bound derivative of di-*N*-acetylchitobiose was formed from UDP-2-acetamido-2-deoxy-D-[^{14}C]-glucose[16]. This biosynthetic product was compared by thin-layer chromatography with the chemically synthesized P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate previously described in a preliminary communication[13]. The two compounds were expected to cochromatograph, and indeed, this was found to be the case when the solvent system was chloroform–methanol–water. However, when the chromatographic system was chloroform–methanol–aqueous ammonium hydroxide, a small difference in migration rate was consistently observed, indicating the presence of an unexpected structural difference between the chemically synthesized[13] and biosynthetic[16] compounds.

The first synthesis of P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate[13] was not considered completely satisfactory, because the product contained some of the corresponding monosaccharide derivative[17], and because the procedure employed, fusion of a peracetyl sugar with phosphoric acid[18] usually gives a mixture of anomeric phosphates when applied to hexosamines. As shown in this paper, the previously synthesized compound[13] unexpectedly consists mainly of the β anomer, rather than a $\sim$1:1 mixture, as was reported earlier, so another method of synthesis was devised.

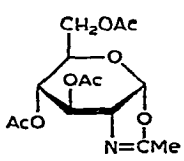
RESULTS AND DISCUSSION

The previous chemical syntheses of polyisoprenyl pyrophosphate diesters derived from 2-acetamido-2-deoxy-D-glucose[17,19] and di-*N*-acetylchitobiose[13], were based on the phosphoric acid fusion-method[18] for the preparation of the glycosyl

phosphates. This procedure might be expected to cause either hydrolysis or isomerization of the glycosidic linkage of a disaccharide, as well as possible *O*- or *N*-deacetylation. A further complication with 2-acetamido-2-deoxy sugars is the formation of an anomeric mixture of phosphates[19,20]. For these reasons, and the observation that the synthetic P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate prepared earlier[13] did not cochromatograph in one solvent system with the ^{14}C-labeled compound biosynthesized by calf pancreas[16], an alternative, milder method of phosphorylation, (the "oxazoline procedure"), was developed in the present study. In a preliminary communication, Khorlin *et al.*[21] showed that protected hexosamine phosphates could be obtained by treatment of an oxazoline with dibenzyl or diphenyl phosphate. The unprotected 2-acetamido-2-deoxy-D-glucose phosphate ester was subsequently obtained by catalytic hydrogenolysis of the aromatic groups, but this step was not performed for di-*N*-acetylchitobiose. Surprisingly, this method[21] gave the α anomer, whereas nucleophilic attack on a 1,2-oxazoline is expected to yield a product having the phosphate group in the *trans* position relative to the acetamido group[22], namely, the β anomer for derivatives of 2-acetamido-2-deoxy-D-glucose. The aim of the present study was to confirm the formation of the α-D-linked glycosyl phosphate by use of the readily available oxazoline **1** derived from 2-acetamido-2-deoxy-D-glucose, to modify the method in order to obtain 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (**8**), and finally to employ **8** for the synthesis of the dolichyl pyrophosphate diester **12**.

Treatment of 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline[23] (**1**) with dibenzyl phosphate in toluene[21] gave a single phosphate derivative, together with many unphosphorylated by-products derived from **1**, which could be removed by chromatography. Hydrogenation of the crude product gave a mixture containing a single, major glycosyl phosphate, corresponding on thin-layer chromatography (t.l.c.) to authentic 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate[19] (**4**).

As 2-methyl-[4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline (**6**) is only slightly soluble in toluene, Khorlin *et al.*[21] performed the phosphorolysis in toluene–nitromethane. In our hands, however, nitromethane was found to be an inhibitor of the phosphorolysis. Reaction of the oxazoline **1** with dibenzyl phosphate or diphenyl phosphate in 1,2-dichloroethane (or dichloromethane) was as efficient as



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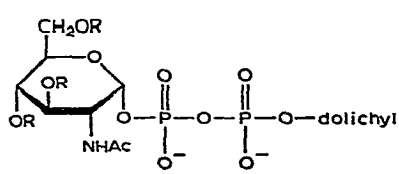

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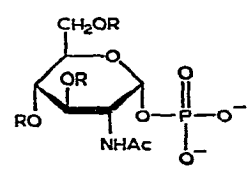
3 R = H



4 R = Ac
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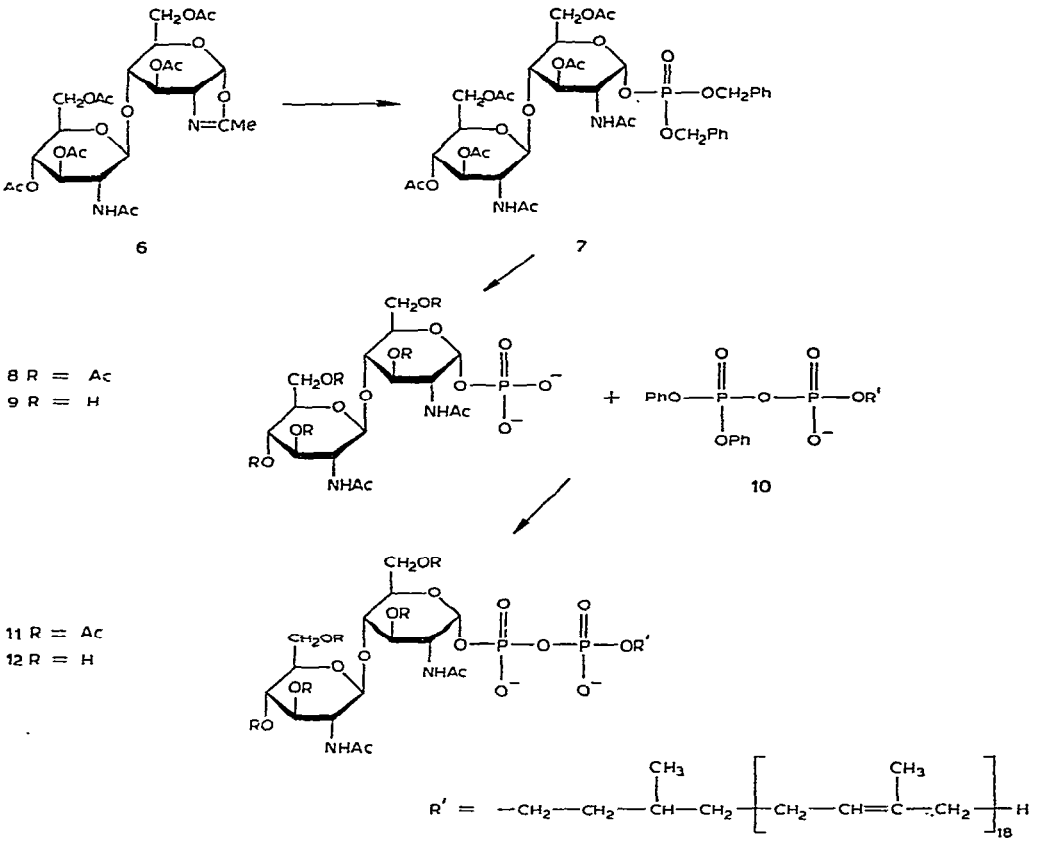

reaction in toluene, but other solvents gave very poor yields of the required compound. Although Khorlin *et al.*[21] treated **1** with dibenzyl phosphate, the disaccharide oxazoline **6** was treated with diphenyl phosphate. In our hands, however, dibenzyl phosphate proved to be a more effective reagent in both cases. When **6**, prepared as previously described[24], was treated with a small excess of dibenzyl phosphate, under the conditions developed in this study for the treatment of **1**, a major, phosphate-containing product (**7**) was obtained, together with a small proportion of the monosaccharide phosphate[21], which was readily removed by preparative-layer chromatography (p.l.c.). Catalytic hydrogenation of the dibenzyl phosphate **7** gave the peracetylglycosyl phosphate **8**, which was purified by p.l.c. The α anomeric configuration was attributed to **8** by analogy with the results obtained with the oxazoline **1**, and this was confirmed by treatment of the *O*-deacetylated compound **9** with 2-amino-2-2-deoxy-β-D-glucosidase (see later). Treatment of **8** with sodium methoxide in methanol under the conditions previously[17] used to *O*-deacetylate glycosyl phosphates, gave, unexpectedly, two major compounds (t.l.c.), but under more rigorous conditions, only a single major compound **9** was obtained; acetyl groups resistant to saponification have been encountered in other disaccharide derivatives of 2-acetamido-2-deoxy-D-glucose[25].

Condensation of the tributylammonium form of **8** with P^1-diphenyl P^2-dolichyl pyrophosphate[17] (**10**) gave the peracetyl pyrophosphate diester **11**, which was isolated by p.l.c. It did not exhibit the unusual instability of the previously synthesized compound[13] and could be handled in a way similar to that of the 2-acetamido-2-deoxy-D-glucose derivative[17] **2**. Compound **11** could be separated from **2** by t.l.c. and had the same mobility as the previously prepared chitobiose compound **13**. *O*-Deacetylation of **11** was performed as for peracetylchitobiosyl phosphate (**8**) (see preceding paragraph), and gave a single major compound (t.l.c.) together with traces of other compounds (including dolichyl phosphate) that could be readily removed by p.l.c. to yield pure P^1-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (**12**). In order to determine whether the di-*N*-acetylchitobiosyl phosphate prepared earlier[13] by the phosphoric acid procedure[18] has a chemical structure different from that of that prepared by the "oxazoline procedure", the compounds obtained by both methods were compared. The peracetyl phosphate derivatives produced by the two methods were found not to cochromatograph on t.l.c. In the earlier study[13], the peracetylchitobiosyl phosphate gave, on treatment with a dilute solution of sodium methoxide in methanol, two spots on t.l.c. of approximately equal intensity and closely similar R_F values (see B, Fig. 1). It was concluded that these spots corresponded to α and β anomers, and that the anomers of the peracetyl compound were not resolved by t.l.c. These conclusions were consistent with the method of formation, low optical rotation, i.r. spectra, and elementary analysis.

In order to explain the discrepancies between the chromatographic properties of the compounds obtained by the two methods, and in view of the surprisingly severe conditions required for the *O*-deacetylation of **8** and other 2-acetamido-2-deoxy-D-glucose-containing disaccharide derivatives[25], a sample of peracetylchitobiosyl phosphate prepared by the phosphoric acid procedure[18] was *O*-deacetylated under rigorous conditions. T.l.c. now showed that most of the compound originally considered[13] to be the α anomer was converted into the compound having a lower mobility, considered[13] to be the β-D anomer (see A, Fig. 1). A small residual spot remained unaffected, and corresponded to **9**, obtained from **6** (see C, Fig. 1). The product also contained some 2-acetamido-2-deoxy-D-glucopyranosyl phosphate (see D, Fig. 1). These observations suggest that the phosphoric acid procedure had unexpectedly given mainly the β-D anomer, and not a 1:1 mixture of anomers as had been originally concluded[13]. In our experience[19], the phosphoric acid procedure[18], when employed for the preparation of 2-acetamido-2-deoxy sugar phosphates, has given a mixture of anomers with the α-D anomer preponderant. Although other workers[20] obtained mixtures containing slightly more of the β-D than of the α-D anomer, in no case reported until now has the β-D anomer been the only major product. These conclusions were supported by comparison of optical rotations and i.r. spectra, and t.l.c., and by the results of alkaline phosphatase treatment; the compounds obtained by *O*-deacetylation under harsh conditions gave only di-*N*-acetylchitobiose, whereas the original product obtained by the phosphoric acid

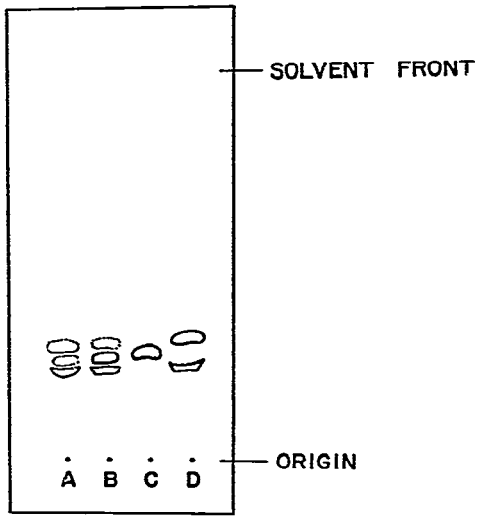

Fig. 1. T.l.c. on silica gel plates (F 254, Merck) of 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α,β-D-glucopyranosyl phosphate and of **9** (lanes A–C), and of 2-acetamido-2-deoxy-α,β-D-glucopyranosyl phosphate (lane D). Sample A was prepared by the phosphoric acid procedure and subjected to severe *O*-deacetylation conditions, whereas sample B was prepared in the same way and subjected to mild *O*-deacetylation conditions. Sample C (**9**) was prepared by the oxazoline procedure. The solvent system was 1:1 PrOH–15M NH_4OH, and compounds were detected with the anisaldehyde spray. The positions of the spots were ascertained by tracing their outline on the back (glass side) of the chromatograph plate, and the average R_F values were 0.27 (**9**), 0.25 (di-*N*-acetyl-β-chitobiosyl phosphate), 0.42 (**5**), and 0.26 (2-acetamido-2-deoxy-β-D-glucopyranosyl phosphate).

method[13] gave, after *O*-deacetylation under mild conditions, a second compound, presumably containing a resistant *O*-acetyl group and moving faster on chromatograms than di-*N*-acetylchitobiose. These studies also confirmed that compound **9** was free of monosaccharide **5**, whereas the product made earlier[13] contained 2-acetamido-2-deoxy-D-glucopyranosyl phosphate.

Additional proof of the anomeric configuration of the di-*N*-acetylchitobiosyl phosphates made by the two methods was provided by treatment with 2-acetamido-2-deoxy-β-D-glucosidase. On treatment with this enzyme, di-*N*-acetylchitobiose was completely hydrolyzed to 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate[19] (**5**) was resistant to the enzyme, and a ~1:1 mixture (t.l.c.) of 2-acetamido-2-deoxy-α- and β-D-glucopyranosyl phosphates[26] was only hydrolyzed to 50%. Under identical conditions, the compound prepared by the phosphoric acid procedure gave 2-acetamido-2-deoxy-D-glucose as the only major product, whereas the compound made by the oxazoline procedure gave a ~50% yield of 2-acetamido-2-deoxy-D-glucose, and a second major compound that was identified as 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate (**5**). These results therefore confirmed that the di-*N*-acetylchitobiosyl phosphate prepared by the phosphoric acid procedure was mainly the β-D anomer, whereas the compound

TABLE I

THIN-LAYER CHROMATOGRAPHY[a] ON SILICA GEL OF **3** AND **12**

Solvent systems	R_F *of compounds*	
	3	**12**
60:25:4 $CHCl_3$–MeOH–H_2O (*A*)	0.23	0.11
60:35:6 $CHCl_3$–MeOH–H_2O (*B*)	0.42	0.31
65:35:4:4 $CHCl_3$–MeOH–15M NH_4OH–H_2O (*G*)	0.27	0.23 (α); 0.20 (β)
6:3:1 Me_2CHOH–15M NH_4OH–H_2O (*F*)	0.77	0.67
30:15:4:2 $CHCl_3$–MeOH–AcOH–H_2O (*I*)	0.42	0.27
8:5:1 2-Methyl-4-heptanone–AcOH–H_2O (*E*)	0.30	0.23

[a]The R_F values (anisaldehyde spray) are the averages of those obtained in several different runs. As the values vary from one batch of plates to the next, with different lengths of plate, with the method of drying the samples, and with the presence of other compounds in the sample, it is essential for the compounds being compared to be chromatographed on the same plate; R_F values are especially variable in solvents *B* and *I*.

TABLE II

EFFECT OF DILUTE ACID ON THE α (**12**) AND β ANOMER[a] OF P^1-2-ACETAMIDO-4-*O*-(2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL)-2-DEOXY-D-GLUCOPYRANOSYL P^2-DOLICHYL PYROPHOSPHATE

Solvent and condition[b]	*Time* (*h*)	*Temp.* (°)	*Extent of reaction* (%) α	β	*Compounds identified*[c]
Cation-exchange resin[d] in 1:1 MeOH–CH_2Cl_2	24	20–23	0	>50	Dol-*PP*, Dol-*P*
10:10:3 $CHCl_3$–MeOH–0.08M HCl	0.5	20–23	0	80	Dol-*PP*, Dol-*P*
10:10:3 $CHCl_3$–MeOH–0.08M HCl	1	20–23	0	>90	Dol-*PP*,Dol-*P*, $(GlcNAc)_2$
10:10:3 $CHCl_3$–MeOH–0.08M HCl	3	20–23	0	>90	Dol-*PP*, Dol-*P*
10:10:3 $CHCl_3$–MeOH–0.08M HCl	18	20–23	10	100	Dol-*PP*, Dol-*P*
10:10:3 $CHCl_3$–MeOH–0.08M HCl	0.08	93	>90	>90	Dol-*PP*,Dol-*P*, $(GlcNAc)_2$ Me-$(GlcNAc)_2$
1:1 Me_2CHOH–0.05M HCl	0.08	93	>90	>90	Dol-*PP*,Dol-*P*, $(GlcNAc)_2$

[a]The samples of the β anomer contained a small proportion of the α anomer.
[b]The starting compound (0.1 mg) was treated with the appropriate solvent or reagent (0.1 ml) in a tube sealed with Teflon-lined screw top. At the end of the treatment, the approximate extent of reaction, and nature of products, were ascertained by t.l.c. (for details of solvent systems, see Table I). All proportions of solvents or reagents are v/v.
[c]Abbreviations: Dol-*PP*, dolichyl pyrophosphate; Dol-*P*, dolichyl phosphate; $(GlcNAc)_2$, di-*N*-acetylchitobiose; Me-$(GlcNAc)_2$, methyl glycoside of di-*N*-acetylchitobiose. The ratio of Dol-*PP* (R_F 0.18) to Dol-*P* (R_F 0.29) was estimated from t.l.c. (Solvent G) to be ~5:1 in each experiment. $(GlcNAc)_2$ (R_F 0.48) and Me-$(GlcNAc)_2$ (R_F 0.50) were identified by t.l.c. in solvent *F*.
[d]Samples of peracetyl compounds were *O*-deacetylated with methanolic sodium methoxide, and the solutions treated with an excess of cation-exchange resin (pyridinium$^+$).

TABLE III

EFFECT OF ALKALI ON **12**

Solvent and reagent[a]	*Time (h)*	*Temp. (°)*	*Extent of reaction (%)*	*Compounds identified*[b]
1:10 M NaOH–PrOH	20	20–23	10	Dol-*P*
1:10 M NaOH–PrOH	2	70	50	Dol-*P*
1:10 M NaOH–PrOH	0.25	85	80	Dol-*P*, **9**, products of *N*-deacetylation[c]
15M NH_4OH	1	100	20–25[e]	Dol-*P*, **9**[d]
1.5M NH_4OH	1	100	0	
1:1 15M NH_4OH–PrOH	1	100	0	
1:1 Et_3N–PrOH	3	100	20–40[e]	Dol-*P*, **9**[d]

[a]For experimental details, see Table II. All proportions of solvents and reagents are v/v. [b]Abbreviations: see Table II. Products were identified by t.l.c., Dol-*P* (R_P 0.29) in solvent *G*; **9** (R_P 0.25) and products of *N*-deacetylation (R_P 0.23), in solvent *F*. (For details of solvent systems, see Table I). [c]The solution was neutralized (10% acetic acid), evaporated (N_2), the residue dissolved in H_2O (50 μl) and treated with cation-exchange resin, before examination by t.l.c. with anisaldehyde and ninhydrin as spray reagents. [d]The solution was evaporated, and the residue dissolved in water (50 μl) before examination by t.l.c. [e]Variation in different experiments.

prepared by the oxazoline procedure was the α-D anomer. Gas–liquid chromatography of the per-*O*-trimethylsilyl derivatives of the α-D and β-D anomers of di-*N*-acetylchitobiosyl phosphate did not separate them, but both compounds were well separated from 2-acetamido-2-deoxy-D-glucopyranosyl phosphate, thus confirming the purity of compound **9**.

The formation of a pyrophosphate diester from **8** is not expected to change the proportion of α- and β-D anomers, so it is reasonable to conclude that **12** is the α-D anomer, whereas the compound reported earlier[13] is mainly the β-D anomer. The two anomers were not separated by t.l.c. in most solvent systems, but in chloroform–methanol–aqueous ammonium hydroxide, the α-D anomer shows a higher mobility. In this solvent system, the biosynthetic glycolipid isolated from pancreas cochromatographed[16] with the synthetic α-D anomer **12** but not with the β-D anomer[13]. T.l.c. of the two synthetic products in the same solvent system also confirmed that the previously synthesized material[13] contained only a small proportion of the α-D anomer **12**. Compound **12** was well separated from the corresponding monosaccharide derivative[17] **3**, by chromatography in a variety of solvent systems (see Table I).

Compound **12** and the corresponding β-D anomer were also readily distinguished by their different stabilities when stored in chloroform–methanol solutions, and by the stability of the α-D anomer **12** under acid conditions that converted the β-D-linked compound into a mixture of dolichyl pyrophosphate and di-*N*-acetylchitobiose (see Table II). Stronger acid-treatment was required to convert **12** into the same mixture.

Under alkaline conditions, the pyrophosphate bond of **12** was cleaved to yield a mixture of dolichyl phosphate and **9** (see Table III). Treatment with hot,

dilute alkali in 1-propanol gave the most complete hydrolysis of **12**, but the reaction was complicated by *N*-deacetylation. Compound **12** was inert to treatment with hot, dilute aqueous ammonium hydroxide, in contrast to the behavior of the lipid-bound oligosaccharide formed by the oviduct[3] that has a di-*N*-acetylchitobiosyl phosphate residue at its "reducing" terminus[11]. However, this may have been due to the greater solubility in an aqueous medium of the biosynthetic compound, which contains 7–9 residues, and to the much smaller quantities of material being treated.

It was previously reported[13] that synthetic P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate, now known to have been mainly the β-D anomer, stimulated the formation of lipid-bound oligosaccharides in lymphocytes, presumably by acting as an acceptor of D-mannose residues. It seems possible that the compound was active in this sytem because the small amounts of α-D anomer were sufficient, or it may be that the mannosyl transferases involved in the formation of the oligosaccharide lipids do not exhibit specificity for the anomeric configuration of the di-*N*-acetylchitobiosyl phosphate residue in the lipid–disaccharide.

EXPERIMENTAL

General methods. — Optical rotations were determined in 1-dm, semimicro tubes with a Perkin–Elmer polarimeter, Model 141. I.r. spectra were recorded with a Perkin–Elmer spectrophotometer, Model 237. The cation-exchange resin used was AG50W-X8 (200–400 mesh, Bio-Rad Laboratories, Richmond, CA 94804). In all cases, the amount of resin used was in at least a two-fold excess over the quantity necessary to obtain complete ion-exchange. Evaporations were conducted *in vacuo* with the bath temperature kept below 30°. Evaporations of toluene (for drying purposes) were conducted in a high vacuum obtained with an oil pump and carbon dioxide–acetone trap. All proportions of solvents are v/v. The microanalyses were performed by Dr. W. Manser, Zurich, Switzerland. Dolichyl phosphate and dolichyl pyrophosphate were prepared by the methods of Wedgwood *et al.*[27], and Warren and Jeanloz[28], respectively.

Chromatographic methods. — Thin-layer chromatography (t.l.c.) was performed on precoated plates of Silica Gel G (E. Merck A.G., Darmstadt, Germany). The plates supplied were 20 × 20 cm; they were cut to a length of 6 cm and used without pretreatment. Preparative-layer chromatography (p.l.c.) was performed on precoated PLC plates, Silica Gel 60 (Merck). The spray reagent, unless otherwise stated, was 1:1:18 anisaldehyde–sulfuric acid–ethanol[29], and the plates were heated to 125°. Unsaturated compounds were detected with a 1% solution of potassium permanganate in 2% aqueous sodium carbonate. Phosphate groups were detected with the spray reagent described by Dittmer and Lester[30]. Solvents *A*, *B*, and *C* were 60:25:4, 60:35:6, and 10:10:3 chloroform–methanol–water, respectively, solvent *D* was 20:15:2 2,6-dimethyl-4-heptanone–acetic acid–water, solvent *E*, 8:5:1 2,6-dimethyl-4-heptanone–acetic acid–water, solvent *F*, 6:3:1 2-propanol–15M ammonium hydroxide–water, solvent *G*, 65:35:4:4 chloroform–methanol–15M

ammonium hydroxide–water, solvent *H*, 1:1 1-propanol–15M ammonium hydroxide, and solvent *I*, 30:15:4:2 chloroform–methanol–acetic acid–water. The R_F values were calculated from measurement of the distance from the origin of the chromatogram to the point of maximum intensity of the spot after development. Gas–liquid chromatography was performed with a Perkin–Elmer Model 900 instrument, equipped with a flame-ionization detector.

*2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-*D*-glucopyranosyl dibenzyl phosphate* (**7**). — A mixture of 2-methyl-[4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline[24] (**6**, 55 mg) and dibenzyl phosphate (95 mg, Aldrich Chemical Co., Milwaukee, WI 53233) was dissolved in 1,2-dichloroethane (2 ml), and the solution was kept for 24 h at room temperature. Examination by t.l.c. (5:1 chloroform–methanol) showed a major phosphorylated compound (R_F 0.71), a minor phosphorylated compound having a higher R_F value, and a large number of unphosphorylated by-products arising from **1**, several of which were present in major proportions. The mixture was chromatographed on two p.l.c. plates (20 × 20 cm), in 5:1 chloroform–methanol, and the compounds were detected with the phosphate–specific spray reagent. The silica gel of the band containing **8** was extracted by stirring overnight with solvent *C*, and filtration and evaporation of the resulting solution gave a residue that was extracted with 2:1 chloroform–methanol. Filtration and evaporation gave the dibenzyl phosphate derivative **7** (30 mg), which was free of any phosphate derivative of 2-acetamido-2-deoxy-D-glucose on t.l.c., and showed a single phosphate-positive spot (R_F 0.71, 5:1 chloroform-methanol), but contained several minor contaminants as detected by anisaldehyde. For characterization purposes, a sample of this compound (30 mg) was purified by trituration with 1:1 hexane–ether to give a solid (21 mg, 25%), m.p. 132–137°, $[\alpha]_D^{20}$ +32° (*c* 0.8, methanol), $[\alpha]_D^{20}$ +34° after correction for residual water content; ν_{max}^{KBr} 3320, 2950, 1740, 1655, 1540, 1380, 1230, 1125, 1050, 950 (broad), 730, and 680 cm^{-1}.

Anal. Calc. for $C_{40}H_{51}N_2O_{19}P$: C, 53.69; H, 5.76; for $C_{40}H_{51}N_2O_{19}P \cdot 3H_2O$: C, 50.62; H, 6.07. Found: C, 50.37; H, 5.41.

*2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-*D*-glucopyranosyl phosphate* (**8**). — Compound **7** (30 mg, used without purification) was dissolved in methanol (2 ml) and hydrogenated in the presence of 10% palladium-on-charcoal (25 mg, Fluka A.G., Buchs, SG, Switzerland) for 2.5 h at 2 atm. Examination by t.l.c. showed no phosphate-positive spot in the position of the starting material (5:1 chloroform–methanol) but an intense spot at the origin. T.l.c. (solvent *B*) showed a single, major phosphate–positive compound having R_F 0.22. The solution was treated with pyridine (0.5 ml), filtered, and evaporated, after which two 2-ml portions of toluene were evaporated from the residue. The product was purified by chromatography on a p.l.c. plate (20 × 8 cm) in solvent *C* and detected with the phosphate-specific spray. Extraction from the plate was performed with solvent *C* as for the dibenzyl phosphate **7**, and after filtration and

evaporation of the resulting solution, the residue was extracted with methanol, to give **8** (13 mg, 80% based on purified **7**) as an amorphous solid, m.p. 228–229°, $[\alpha]_D^{20}$ +22° (*c* 0.65, methanol), $[\alpha]_D^{20}$ +22.5° after correction for residual water content; ν_{max}^{KBr} 3350, 2950, 1745, 1655, 1550, 1375, 1240, 1120, 1045, 950, 905, 845, and 720 cm^{-1}.

Anal. Calc. for $C_{26}H_{39}N_2O_{19}P$: C, 43.70; H, 5.51; for $C_{26}H_{39}N_2O_{19}P \cdot H_2O$: C, 42.63; H, 5.65. Found: C, 42.59; H, 5.32.

The peracetylchitobiosyl phosphate prepared by the phosphoric acid procedure[13] as the monoammonium salt showed $[\alpha]_D^{25}$ −4° (*c* 1.05, methanol), and an i.r. spectrum lacking the peaks at 950, 905, and 720 cm^{-1}.

On t.l.c. in solvents *B* and *C*, the R_F values of **8** (0.22 and 0.66, respectively) were very close to those[17] of **4** but the colors of the spots (anisaldehyde) after the plate had cooled were different, green for **8** and brown for **4**. Also, the compounds did not cochromatograph when applied to the plate together. Compound **8** did not cochromatograph with peracetylchitobiosyl phosphate prepared by the phosphoric acid procedure[13], the R_F values for the latter product being: 0.21 (*B*) and 0.63 (*C*), and the two products were also distinguished by the different colors of the spots, green for **8** and brown for the sample obtained by the earlier procedure[13].

P^1-*2-Acetamido-4*-O-(*2-acetamido-2-deoxy-β*-D-*glucopyranosyl*)-*2-deoxy-α*-D-*glucopyranosyl* P^2-*dolichyl pyrophosphate* (**12**). — Compound **8** (20 mg) was converted into the tributylammonium form, as described previously[17] for 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl phosphate (**4**). A mixture of **8** and P^1-diphenyl P^2-dolichyl pyrophophosphate (**10**, derived from 14 mg of pig-liver dolichol by the method of Warren and Jeanloz[17]) was dried by several additions and evaporations of toluene, and dissolved in 1:10 1,2-dichloroethane–pyridine (4.4 ml). After being kept for 48 h at room temperature, examination of the mixture by t.l.c. (solvent *A*, anisaldehyde and phosphate sprays) showed the presence of a major compound (R_F 0.7), a compound having the same mobility as that of **10** and diphenyl phosphate (R_F 0.8), small amounts of dolichyl phosphate (R_F 0.63), and residual **8** (near the origin). If desired, unreacted **8** could be recovered by extraction as described for the recovery of **4**, in the preparation[17] of **2**. Otherwise, the mixture was applied directly to a preparative-layer plate (8 × 20 cm), which was chromatographed with solvent *A*. The band containing the compound was located with the potassium permanganate and the phosphate-specific sprays (it was the lower of the two phosphate-positive bands), and the silica gel was extracted by stirring with solvent *C* overnight. The resulting solution was filtered through Celite and evaporated, and the residue was extracted with 2:1 chloroform–methanol to give **11** (tributylammonium form, 6 mg, 9% based on **8**, 32% based on C_{95} dolichol) as a syrup; ν_{max}^{film} 3340, 2905, 2930, 2860, 1745, 1655, 1545, 1450, 1375, 1230, 1135, and 930 cm^{-1}. Compound **11** was pure according to t.l.c. in solvents *A*, *G*, and *D*. In solvents *A* and *G*, it had the same mobility as that[17] of **2** (R_F 0.70 in both solvents), but in solvent *D* **11** had R_F 0.52 and **2** had R_F 0.60. Compound **11** (tributylammonium form) was stored at −15° in chloroform–methanol solution and was stable indefinitely (t.l.c.).

Compound **11** (1 mg) was dissolved in dichloromethane (0.25 ml) and treated with 3% sodium methoxide in methanol until an excess was present (pH paper). The reaction was followed by t.l.c. (solvent *G*), which showed formation after 1–2 h of a single, major product (R_F 0.23). Sodium ions were removed by addition of cation-exchange resin (pyridinium form), which was filtered off and washed with 2:1 chloroform–methanol. Evaporation of the combined filtrates gave **12**, which showed a single major spot on t.l.c. in solvents *A*, *B*, *E*, *F*, *G*, and *I* (for R_F values, see Table I). The product contained traces of dolichyl phosphate, which were present in **11**, but there was no sign of decomposition as a result of the resin treatment. The product did not contain any dolichyl pyrophosphate, or any of the monosaccharide[17] **3**, in contrast to the product obtained via the peracetylchitobiosyl phosphate prepared by the phosphoric acid procedure[13]. Compound **12**, without further purification, was suitable for studies of the action of acid and alkali (see Tables II and III). It could be successfully stored for one week at −15° in chloroform–methanol, after which gradual decomposition into dolichyl pyrophosphate and di-*N*-acetylchitobiose took place, the half-life being approximately 5 weeks.

To obtain a completely pure sample according to t.l.c., compound **11** was *O*-deacetylated as just described but, instead of treating with a cation-exchange resin, the alkaline solution of **12** was chromatographed on a thin-layer plate (20 × 4 cm) in solvent *B*. The band containing **12** (potassium permanganate and the phosphate-specific reagents) was extracted from the silica gel as described for **11**. The product was pure according to t.l.c. in six solvent systems (see Table I) when detected with spray-reagents specific for lipids, carbohydrates, unsaturated compounds, and phosphate esters.

It was separated by t.l.c. in solvent *G* from the major component (namely, the β-D anomer) of the corresponding compound as prepared by the alternative procedure[13], but the separation was only successful when both products had been purified by preparative t.l.c. to eliminate salts. This t.l.c. also confirmed that the latter product contained a small proportion of the α-D anomer (**12**).

The phosphate content (10-μg sample) of **12** was determined by (*a*) the method of Ames and Dubin[31], or (*b*) by the method of Chen *et al.*[32] after digestion with conc. sulfuric acid. The 2-acetamido-2-deoxy-D-glucose content (200-μg sample) was determined by hydrolysis with 4M hydrochloric acid (250 μl) for 4 h at 100°. After evaporation (nitrogen gas) and several additions and evaporations of water, the residue was treated with 1:2 acetic anhydride–pyridine (200 μl) overnight at room temperature. Water (100 μl) was added, and after evaporation, followed by three additions and subsequent evaporations of toluene, the residue was *O*-deacetylated with a saturated solution of ammonia in methanol and analyzed by the method of Reissig *et al.*[33].

Anal. Calc. ratio of P to 2-amino-2-deoxyglucose: 1:1. Found (*a*) 1:1.02; (*b*) 1:1.11.

2-Acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl phosphate (**9**). — Compound **8** (17 mg) was treated with 1% methanolic

sodium methoxide until an excess was present (pH paper). After 2 h at room temperature, examination by t.l.c. (solvent *F* or *H*) revealed two compounds having similar R_F values: 0.25, 0.23 (solvent *F*) and 0.27, 0.25 (solvent *H*). Therefore, an equal amount of 3% sodium methoxide in methanol was added, and the reaction was monitored by t.l.c. When only a single spot remained, the solution was diluted with abs. ethanol and kept overnight at −15°. The precipitate was filtered off to give the sodium salt of **9** (11 mg, 67%). This product was pure according to t.l.c. (anisaldehyde and phosphate sprays), but elementary analysis and its i.r. spectrum revealed that it contained sodium salts; $[\alpha]_D^{20}$ +32° (*c* 0.45, 1:1 methanol–water), $[\alpha]_D^{20}$ +41° after correction for salt content; ν_{max}^{KBr} 3350, 1650, 1560, 1450 (Na_2CO_3), 1120, 950, and 870 cm^{-1}.

Anal. Calc. for $C_{16}H_{28}Na_2O_{14}P$: C, 34.97; H, 5.16; for $C_{16}H_{28}N_2Na_2O_{14}P \cdot Na_2CO_3 \cdot NaOCH_3$: C, 30.50; H, 4.41. Found: C, 30.78; H, 3.93.

A sample of di-*N*-acetylchitobiosyl phosphate, as prepared by the phosphoric acid procedure[13] (that contained residual water but no salt) had $[\alpha]_D^{26}$ −2° (*c* 0.55, 1:1 methanol–water) and an i.r. spectrum that lacked peaks at 1450 and 870 cm^{-1} but, instead, had peaks at 1376, 1325, 975, and 800 cm^{-1}. There was no definite peak at 1745 cm^{-1} (*O*-acetyl group).

Compound **9** had the same R_F values in solvents *F* and *H*, namely 0.25 (*F*) and 0.27 (*H*), as the sample of di-*N*-acetylchitobiosyl phosphate prepared previously by the phosphoric acid procedure[13]. However, when a sample of the earlier product was treated with 3% sodium methoxide in methanol (1 h), t.l.c. showed that most of the compound having R_F 0.27 (solvent *H*) had been converted into the slower-migrating compound having R_F 0.25 (see A, Fig. 1). This t.l.c. also showed that the product obtained previously[13] contained some 2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate (**5**) (R_F 0.42); the β-D anomers of the di- and mono-saccharide phosphate were not separated. However, **9** was free of either anomer of the monosaccharide phosphate.

Action of alkaline phosphatase on **9** *and on di-*N*-acetylchitobiosyl phosphate prepared by the phosphoric acid procedure.* — The samples treated with the enzyme consisted of di-*N*-acetylchitobiosyl phosphate (100 μg) prepared in three different ways: (*a*) by the "oxazoline procedure" followed by *O*-deacetylation with 3% sodium methoxide in methanol (**9**), (*b*) by the phosphoric acid procedure[13] followed by *O*-deacetylation with 1% sodium methoxide in methanol, and (*c*) by the same procedure as (*b*) except that the strength of the sodium methoxide solution used for *O*-deacetylation was 3%. The samples were dissolved in water (100 μl) and the pH was adjusted to ~8 with 0.1M sodium hydroxide. The solutions were incubated for 2 h at 37° with alkaline phosphatase (0.12 unit, from *Escherichia coli*, EC 3.13.1, Sigma Chemical Co., St. Louis, MO 63118). Examination by t.l.c. (solvent *B*, 3 developments) showed that the only product from **9** was di-*N*-acetylchitobiose. Both of the other samples (*b* and *c*) gave rise to 2-acetamido-2-deoxy-D-glucose in addition to di-*N*-acetylchitobiose. The sample that had been prepared by mild deacetylation treatment (*b*) also yielded a third substance, having an R_F value slightly higher than

that of di-*N*-acetylchitobiose (solvents *F* or *H*). The solutions were diluted with methanol (5 ml), clarified by filtration, and evaporated, and the residues subjected to trimethylsilylation with Tri-Sil (Pierce Chemical Co., Rockford, IL 61105), and examination by g.l.c., performed on a 30-cm stainless-steel column packed with 0.1% OV-17 on Corning glass beads, the temperature being programmed to increase from 80 to 300° at 10°/min. 2-Acetamido-2-deoxy-D-glucose was eluted as a double peak centered at 155°, whereas di-*N*-acetylchitobiose was eluted as a single peak at 244°. This examination confirmed that the product from **9** was di-*N*-acetylchitobiose only, and showed that the approximate ratio of di-*N*-acetylchitobiose to 2-acetamido-2-deoxy-D-glucose, in the product from di-*N*-acetylchitobiosyl phosphate as prepared previously[13], was 4:1.

Action of 2-acetamido-2-deoxy-β-D-glucosidase on di-N-acetylchitobiosyl phosphate (prepared by both the phosphoric acid procedure and the oxazoline method) and on 2-acetamido-2-deoxy-D-glucopyranosyl phosphate. — Incubations of each compound (50 μg) in 100 μl of phosphate–citrate buffer pH 4.5 (made by mixing 27.25 ml 0.1M citric acid and 22.7 ml of disodium hydrogenphosphate and diluting to 100 ml with water) were performed with 25 μg of enzyme (2-acetamido-2-deoxy-β-D-glucosidase from beef kidney EC 3.2.1.30, Boehringer–Mannheim Corporation, New York, N.Y. 10017) overnight at 37°. In each case, a second sample was treated in the same way except that no enzyme was present. 2-Acetamido-2-deoxy-D-glucose was identified by t.l.c. in solvent *B* (3 developments). Glycosyl phosphates were not identifiable in this way owing to interference by salt and protein. Therefore, to determine the identity of the second product resulting from the degradation of **9**, a larger-scale incubation was performed, with 200 μg of substrate and 100 μg of enzyme, in 400 μl of buffer. After being kept overnight at 37°, the solution was evaporated to dryness and the residue treated with 1:2 acetic anhydride–pyridine (0.2 ml). The mixture was stirred overnight, treated with water (100 μl), and evaporated to dryness, to give a residue that was extracted with 5:1 chloroform–methanol. T.l.c. of the resulting solution revealed the presence of a compound corresponding to **4** having R_F 0.24 (solvent *B*), and 0.75 (solvent *C*), together with noncarbohydrate substances. Therefore, the crude product was chromatographed on a thin-layer plate (5 × 20 cm) in solvent *B*. To detect the carbohydrate-containing band, a 1-cm strip was cut from the plate and sprayed (anisaldehyde). The silica gel was extracted with solvent *C*, filtered off, and the filtrate evaporated, to give a product that was treated with 1% sodium methoxide in methanol for 30 min at room temperature to effect *O*-deacetylation. After removal of sodium ions with a cation-exchange resin, the compound present was identified as **5** by t.l.c. (solvent *F*). The β-D anomer was not present.

Gas–liquid chromatography of the trimethylsilyl ethers of α and β anomers of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate and di-N-acetylchitobiosyl phosphate. — Samples (100 μg) of the compounds were converted into trimethylsilyl ethers by treatment with a mixture of pyridine-hexamethyldisilazane–chlorotrimethylsilane (Sylon-HTP, Supelco Inc., Bellefonte, PA 16823) for 24 h at room temperature. Chromatography was performed on (*a*) a column (3 m, stainless steel)

packed with 3% OV-17 on Gas Chrom Q (80–100 mesh, Applied Science Labs., State College, PA 16801) or (*b*) a column (30 cm, stainless steel) packed with 0.1% OV-17 on Corning glass beads GLC 110 (120–140 mesh, Supelco Inc.). In method (*a*) the temperature of the column was programmed to increase from 120 to 310° at 10°/min and the trimethylsilyl ether of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate was eluted at 262° and that of di-*N*-acetylchitobiosyl phosphate after the column had been maintained for 13 min at 310°. In both cases, the compounds gave a single peak with no separation of α and β anomers. In (*b*), the column temperature was programmed to increase from 80 to 310° at 10°/min. The trimethylsilyl ether of 2-acetamido-2-deoxy-D-glucopyranosyl phosphate was eluted at 187° and that of di-*N*-acetylchitobiosyl phosphate at 255° (single peaks). For comparison, the derivative of inositol was eluted at 160° and that of sucrose at 220°.

Action of alkali on **9**. — Compound **9** (100 μg) was treated with 1:10 M sodium hydroxide–1-propanol (0.1 ml) and the mixture kept for 15 min at 85°. The alkali was neutralized by addition of a small excess of 10% aq. acetic acid, and the solvents evaporated (nitrogen). The residue was dissolved in water (50 μl), and the resulting solution treated with cation-exchange resin (pyridinium form) and then examined by t.l.c. (solvent *H*). This showed that most of **9** had not been affected, and so the experiment was repeated under stronger conditions (1:1 M sodium hydroxide–1-propanol). T.l.c. now showed that some of **9** (R_F 0.27) had been converted into a new product having the same R_F as di-*N*-acetyl-β-chitobiosyl phosphate[13] (R_F 0.25). However, the new product gave a positive reaction with ninhydrin spray, whereas neither anomer of di-*N*-acetylchitobiosyl phosphate gave a positive reaction. When even stronger conditions were employed (M sodium hydroxide alone, for 1 h), t.l.c. showed that most of **9** was converted into a substance having a lower R_F value and giving a double spot with ninhydrin. This partially *N*-deacetylated material cochromatographed with the product obtained from the alkaline treatment of **12** (Table III).

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