PHOSPHATE MIGRATION IN SOME PHOSPHATE MONOESTERS AND DIESTERS OF METHYL α-D-MANNOPYRANOSIDE

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ABSTRACT

The syntheses of methyl α-D-mannopyranoside 4- and 6-monophosphate and monobenzyl (methyl α-D-mannopyranoside 6-phosphate) are described. Condensation of tetra-*O*-acetyl α-D-mannopyranosyl phosphate with methyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside by *N,N'*-dicyclohexylcarbodiimide afforded methyl α-D-mannopyranoside 6-(α-D-mannopyranosyl phosphate) after removal of protecting groups. Methyl α-D-mannopyranoside 4-(α-D-mannopyranosyl phosphate) was prepared by a similar condensation between the fully protected mannopyranosyl phosphate and methyl 2,3,6-tri-O-benzoyl-α-D-mannopyranoside. These new sugar phosphates have been hydrolysed in acid and in alkali and the extent of phosphate migration between the O-4 and O-6 of the methyl α-D-mannopyranoside residue determined.

INTRODUCTION

The extracellular phosphomannan from the yeast *Hansenula holstii* has been reported[1] to contain phosphate diester groups that link C-1 of one D-mannose residue with C-6 of another D-mannose residue. Similar structures have also been proposed for the phosphate diester groups in the mannans released from cell walls of *Saccharomyces cerevisiae* by chemical[2] or enzymic[3,4] methods. In the case of the mannan from *Kloeckera brevis*[5], the phosphate groups were thought to be attached to O-3 or O-4 of D-mannose residues in the (1 → 6)-linked backbone. Dr. Ballou has informed us that this structure may be in error, since subsequent study in his laboratory suggests that the polysaccharide of this mannan contains α-D-mannosyl phosphate groups attached to position 6 of D-mannose residues in the side chain, a structure analogous to that reported here and elsewhere for other mannans. It is possible that the phosphate group may migrate during the course of isolation and degradation of the mannans. Such migrations are known to take place during the degradation of phospholipids[6] and nucleic acids[7] with alkali. A requirement for migrations of this type is the proximity of a hydroxyl group to allow the formation of a cyclic phosphate. In the case of yeast phosphomannans, the C-2 hydroxyl group of each D-mannopyranosyl residue is unlikely to be able to form a cyclic phosphate by nucleophilic

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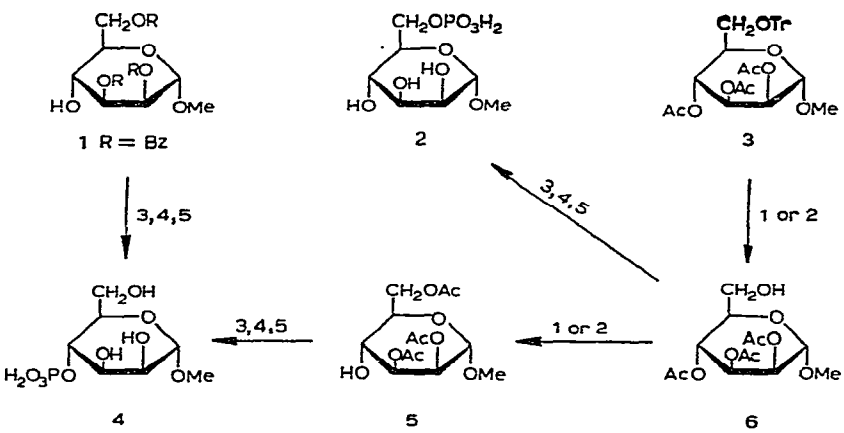
attack on the phosphorus atom attached on C-1 because in the *C1* (D) conformation as the phosphate group is antiparallel to the adjacent hydroxyl group. This situation exists in α-D-mannopyranosyl phosphate (**9b**), which does not form a cyclic phosphate on treatment with *N,N'*-dicyclohexylcarbodiimide (DCC)[8]. On the other hand, if the phosphate group is esterified to O-6 of a D-mannose residue, then cyclisation with the equatorial C-4 hydroxyl group would be feasible, as similar 4,6-cyclic phosphates have been synthesized in the D-glucopyranose[8,9] and D-galactopyranose series[10]. For our investigations on formation of cyclic phosphates and phosphate-group migration in the D-mannopyranose series, we have synthesized the following new compounds: methyl α-D-mannopyranoside 6- and 4-phosphate (**2** and **4**), monobenzyl (methyl α-D-mannopyranoside 6-phosphate) (**7**), and methyl α-D-mannopyranoside 4- and 6-(α-D-mannopyranosyl phosphate) (**10** and **11**).

RESULTS AND DISCUSSION

Synthesis of monoester phosphates and diester phosphates of methyl α-D-mannopyranoside. — The reactions used to prepare methyl α-D-mannopyranoside 6-phosphate (**2**) and the corresponding 4-phosphate (**4**) are summarised in Scheme 1.

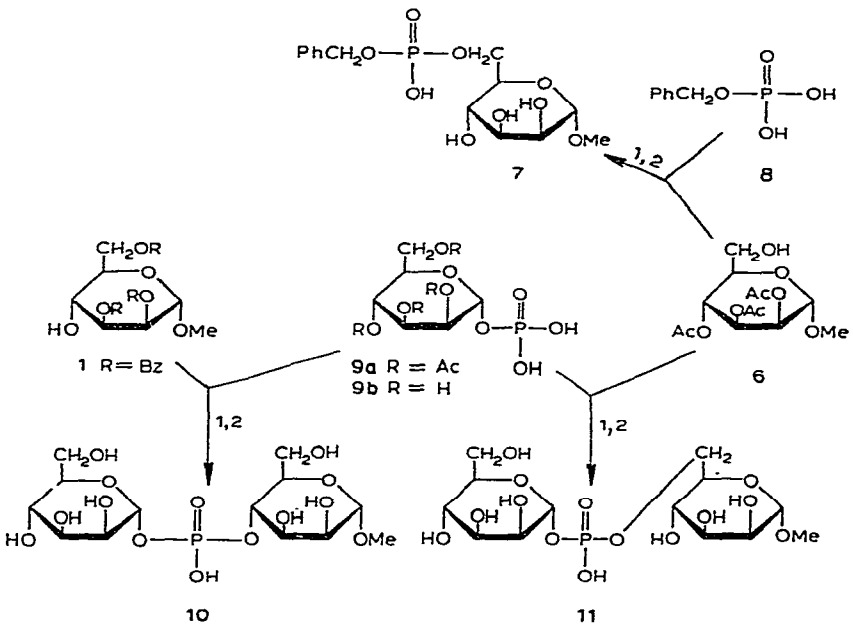


Scheme 1. Reagents: 1, HBr, HOAc; 2, silicic acid; 3, $(PhO)_2POCl$; 4, H_2, Pt; 5, OH^-.

Treatment of 2,3,6-tri-*O*-benzoyl-α-D-mannopyranoside[11] (**1**) with diphenyl phosphorochloridate, followed by removal of protecting groups, gave the 4-phosphate (**4**). Similar treatment of 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside[12] (**6**) afforded the 6-phosphate (**2**). In some preparations of **2** we found[13] substantial amounts (10–20% of total P) of the 4-phosphate (**4**). This impurity results probably from the migration of the acetyl group from the O-4 to the O-6 in **6** during the detritylation of **3** with hydrogen bromide in acetic acid. The product (**5**) from this migration would then give the impurity **4** in the subsequent reactions. Detritylation, by using silicic acid[14]

gave similar results. The impurity in preparations of **2** was eliminated when the detritylated product (**6**) was isolated after a brief exposure to silicic acid (2–3 h).

The syntheses of monobenzyl (methyl α-D-mannopyranoside 6-phosphate) (**7**), methyl α-D-mannopyranoside 4-(α-D-mannopyranosyl phosphate) (**10**), and methyl α-D-mannopyranoside 6-(α-D-mannopyranosyl phosphate) (**11**) were achieved by condensing a phosphate monoester (**9a** or **8**) with an alcohol (**1** or **6**) by reaction with DCC in anhydrous pyridine[15,16]. The reactions are outlined in Scheme 2.



Scheme 2. Reagents: 1, *N,N'*-dicyclohexylcarbodiimide; 2, OH^-.

Methyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl phosphate (**9a**) was a key compound in this work. It was prepared by the procedure of Posternak and Rosselet[17] with the exception of the hydrogenation step, in which we used Adam's catalyst for the removal of benzyl protecting groups. Compound **9a** was not isolated by Posternak and Rosselet, as it was required only as an intermediate in their synthesis of D-mannopyranosyl phosphate (**9b**). The condensations with DCC in anhydrous pyridine were quantitative when large excesses of DCC (5–10 moles to 1 mole or sugar phosphate) were used. In initial experiments, two phosphorylated products were formed when **9a** was condensed with **6** in the presence of DCC in anhydrous pyridine by using molar ratios of sugar phosphate:alcohol:DCC of 1:1:2. The major product from this reaction had the same paper-chromatographic properties as **2** and had a mannose: phosphorus ratio of 1:1. Hydrolysis of this compound at the pH of its free acid solution gave mainly inorganic phosphate. It seems probable that this product was the

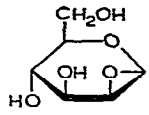
symmetrical pyrophosphate formed by condensing two moles of **9a** followed by removal of protecting groups. The minor product was the required diester **11**.

Migration of phosphate groups. — Treatment of methyl α-D-mannopyranoside 6-phosphate (**2**) and its derived phosphate diester (**11**) with mild acid gave small amounts of α-D-mannopyranose 6-phosphate (**13**). The main product from the acidic treatment of **11** was the 6-phosphate (**2**). The 4-phosphate (**4**) was not detected. Hydrolysis of **11** by 2N sodium hydroxide at 100° also gave the 6-phosphate (**2**) as the main product (85%), the other product being the 4-phosphate (15%). Thus the migration of the phosphate group from the 6- to the 4-hydroxyl groups in the D-mannopyranose series does not operate to any great extent either under acidic or under alkaline conditions. Similar treatment of the 4-phosphatemonoester (**4**) and its derived phosphate diester (**10**) did not produce any of the 6-phosphate (**2**). It seems that the phosphate group does not migrate from the O-4 to O-6 in the D-mannopyranose series under the conditions mentioned. These latter results are a little unexpected, as we have already cited evidence in this paper suggesting that, under acidic conditions, an acetyl group may migrate from the O-4 to O-6 in the D-mannopyranose series.

Mechanism of hydrolysis of phosphate diesters by alkali. — It appears likely that the alkaline hydrolysis of **11** proceeds mainly by rearside attack by the 2-hydroxyl group adjacent to the phosphate diester group, to form an epoxide (**12**) with displacement of the main product (**2**). Formation of a cyclic phosphate by nucleophilic attack of the 4-hydroxyl group on the phosphorus atom must also take place to a lesser extent, giving **15** as an intermediate product. Both of these mechanisms, involving epoxide and cyclic phosphate intermediates, have been demonstrated by Brown and Usher[18] in their studies on the alkaline hydrolysis of cyclohexyl esters of 2-hydroxylalkyl phosphates.

Some evidence that supports the epoxide mechanisms for hydrolysis of **11** by alkali comes from studies on the hydrolysis of monobenzyl (methyl α-D-mannopyranoside 6-phosphate) (**7**). This diester is very stable to treatment by 0.5M sodium hydroxide for 4 h at 100°, being converted to an extent of 10% to the 6-phosphate (**2**). No 4-phosphate (**4**) was formed. The relative stability of **7** as compared with **11** suggests that, in the latter compound, the presence of a free hydroxyl group in an axial–axial (*trans*) relationship to the phosphate diester group facilitates hydrolysis.

Two cyclic phosphates (**14** and **15**), which may be intermediates in the hydrolysis of **10** and **11** by alkali, have been prepared by the reaction of the appropriate sugar phosphate (**2** and **4**) with DCC. This reaction has been used by several workers[8,19,20] for the elucidation of the structure of sugar phosphates. The cyclic phosphate, prepared from **2**, was hydrolysed by alkali to **2** (20%) and **4** (80%). This result is analogous to those obtained by Szabo and Szabo[21] for the hydrolysis of methyl α-D-glucopyranoside 4,6-(hydrogen phosphate) and for the hydrolysis of methyl α-D-galactopyranoside 4,6-(hydrogen phosphate). Compound **15** was not hydrolysed under conditions that are known to open five-membered cyclic phosphates[22] and which do not affect a six-membered cyclic phosphate. These properties are consistent with **15** being methyl α-D-mannopyranoside 4,6-(hydrogen phosphate).



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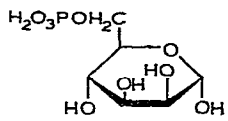

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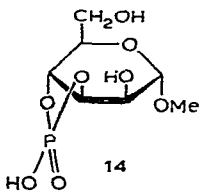

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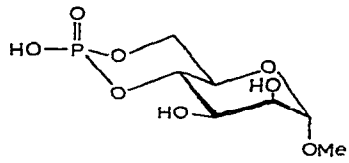

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The reaction of **4** with DCC in aqueous pyridine afforded an *N*-phosphorylurea, which is a characteristic product of the reaction between a five-membered, cyclic phosphate and DCC[8]. A cyclic phosphate [presumably methyl α-D-mannopyranoside 3,4-(hydrogen phosphate), **14**] was obtained when **4** was treated with DCC in aqueous pyridine and in the presence of triethylamine. The tertiary base prevents the formation of *N*-phosphorylureas[19,20]. This cyclic phosphate was completely hydrolysed by alkaline conditions[22] that open five-membered cyclic phosphates, and methyl α-D-mannopyranoside 6-phosphate was not a product of the hydrolysis. These results are consistent with the general observations made by other workers[8], who found that where the formation of both 5- and 6-membered cyclic phosphate rings is possible, the formation of the five-membered ring is favoured.

The evidence presented here does not support the possibility that phosphate groups migrate either from the O-4 to O-6, or to any great extent from the O-6 to O-4 of D-mannopyranose residues when phosphomannans are treated with acid or with alkali. Therefore, the identification of D-mannose 6-phosphate as a product of acidic hydrolysis of a mannan clearly indicates that the phosphate groups in the parent compound are located at O-6 of a mannopyranose residue. This evidence, taken in conjunction with results obtained previously[2,3,4,13,23], establishes that the phosphate groups in mannans from *S. cerevisiae* link C-6 of one D-mannose residue with C-1 of another D-mannose residue.

EXPERIMENTAL

General. — Solutions were concentrated under diminished pressure below 40°. Melting points are corrected. Optical rotations were measured at 20° with a Perkin–Elmer 141 Polarimeter. P.m.r. spectra were measured with a 60 MHz Perkin–Elmer R10 spectrometer at normal operating temperature, by using deuteriochloroform solutions with tetramethylsilane as internal standard. Pyridine was dried by distillation from phosphorus pentaoxide and was stored over solid potassium hydroxide. Total

phosphate, inorganic phosphate and acid-labile phosphate were determined as described by Bartlett[24]. Bio-Rad Analytical Grade cation exchange resin (AG 50W-X2:50–100 mesh) was used.

Chromatographic methods. — T.l.c. was conducted on layers of silica gel G (Merck) deposited on microscope slides; compounds were located by charring at 120° with an aqueous solution (50%) of ammonium sulphate containing sulphuric acid (10%). Phosphate esters were separated by ascending chromatography for 18 h on unwashed Whatman No. 1 paper in 60:30:10 (v/v) isopropyl alcohol–ammonia (0.88)–water R_F values are given in Table I. Sugars were detected by the silver nitrate–alkali method[25] or by the periodate–Schiff reagent[26]. Phosphates were detected with the molydate reagent[27]. The phosphorus contents of spots detected on chromatograms were determined as described previously[28].

TABLE I

PAPER CHROMATOGRAPHY[a] OF PHOSPHATE ESTERS

Compound	R_F *Values*
Pyrophosphate	0.10
Orthophosphate	0.18
α-D-Mannopyranosyl phosphate (9b)	0.24
α-D-Mannopyranose 6-phosphate (13)	0.23
α-D-Mannopyranoside 4,6-(hydrogen phosphate)	0.55
Methyl α-D-mannopyranoside 4-phosphate (4)	0.46
Methyl α-D-mannopyranoside 4-(α-D-mannopyranosyl phosphate) (10)	0.56
Methyl α-D-mannopyranoside 6-phosphate (2)	0.41
Methyl α-D-mannopyranoside 6-(α-D-mannopyranosyl phosphate) (11)	
Methyl α-D-mannopyranoside 4,6-(hydrogen phosphate) (15)	0.68
Dibenzyl phosphate	0.90
Monobenzyl phosphate (8)	0.54
Monobenzyl (methyl α-D-mannopyranoside 6-phosphate) (7)	0.87
N-Phosphorylureas	0.95–0.98

[a]Unwashed Whatman No. 1 paper. Solvent: 60:30:10 (v/v) isopropyl alcohol–ammonia (0.88)–water ascending for 18 h.

Methyl α-D-mannopyranoside 6-phosphate (2). — *Procedure A.* Diphenyl phosphorochloridate (1 g, 3.7 mmoles) was added to methyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside[12] (6; 1 g. 3.1 mmole) in anhydrous pyridine (15 ml). The mixture was kept overnight at room temperature. Water (5 ml) was added, and after 1 h the solvents were removed and the residue dissolved in chloroform. The solution was washed three times with ice-cold hydrochloric acid (1%, v/v), three times with ice-cold sodium hydrogen carbonate (2%, w/v), once with ice–water, dried over sodium sulfate, and evaporated to dryness. The residual syrup was dissolved in ethanol (30 ml) and shaken with charcoal (B. D. H. Activated Charcoal, washed to neutrality with water). The charcoal was filtered off and the filtrate was hydrogenated in the presence of Adams platinum. When the hydrogen uptake had ceased the catalyst was filtered off. Ethanol was evaporated off to give a white solid, which was dissolved in 10 ml

of 1:1 (v/v) ethanol–water and this solution was adjusted to pH 11 with 2M sodium hydroxide. After 30 min at room temperature the sample was passed through a cation-exchange column in the cyclohexylammonium form. The eluate was extracted three times with ether and evaporated to dryness. The residue was taken up in water. Addition of acetone gave 0.65 g (48%) of the dicyclohexylammonium salt of **2**, m.p. 135–142° (decomp.); $[\alpha]_D$ +64° (*c* 1.0, in water); Calc. for $C_{19}H_{41}N_2O_9P$: C, 48.3; H, 8.7; N, 5.9; P, 6.6%; Found: C, 48.1; H, 8.5; N, 6.0; P, 6.6.

Procedure B. Monobenzyl phosphate[20] (**8**; 100 mg, 0.53 mmole) was dissolved in anhydrous pyridine. Methyl 2,3,4-tri-*O*-acetyl α-D-mannopyranoside (300 mg, 1 mmole) and DCC (1 g, 5 mmole) were added and the reaction mixture was kept for 18 h at 37°. Water was added (5 ml) and the *N,N'*-dicyclohexylurea filtered off. The solution was adjusted to pH 11 with dilute sodium hydroxide and after 15 min it was passed through a cation-exchange resin (H^+ form). Paper-chromatographic analysis showed that one phosphorylated product was formed in 60% yield (based on monobenzyl phosphate). This product was isolated by preparative, paper chromatography and hydrogenated in the presence of Adams platinum to give the phosphate (**2**) isolated as its dicyclohexylammonium salt as already described (0.1 g, 33% based on monobenzyl phosphate) m.p. 136–142° (decomp.), $[\alpha]_D$ +62° (*c* 1.0, water); identical with authentic **2** by paper chromatography. The intermediate product isolated by preparative chromatography was therefore methyl α-D-mannopyranoside 6-benzyl phosphate (**7**).

Methyl α-D-mannopyranoside 6-(α-D-mannopyranosyl phosphate) (**11**). — Tetra-*O*-acetyl-α-D-mannopyranosyl chloride (2.5 g, 6.8 mmoles) was condensed with silver dibenzyl phosphate (3.5 g, 9 mmoles) in benzene under the conditions described by Posternak and Rosselet[17]. The reaction mixture was filtered through charcoal and the benzene evaporated off. The residue was dissolved in chloroform (50 ml), washed three times with cold, aqueous sodium hydrogen carbonate (5% w/v), three times with ice–water, and evaporated to dryness. The residue was dissolved in ethanol (12 ml) and the solution treated with activated charcoal. The charcoal was filtered off, the filtrate adjusted to pH 5–6 with dilute sodium hydroxide solution and hydrogenated over Adams platinum. The solution was maintained at pH 5–6 during the hydrogenation by addition of dilute sodium hydroxide solution. After 18 h the catalyst was filtered off and the filtrate (pH 6) evaporated to dryness. The residue was dissolved in warm isobutyl alcohol (15 ml) and an cooling the crude sodium salt of tetra-*O*-acetyl α-D-mannopyranosyl phosphate (**9a**) was deposited (900 mg, 28%). Attempts to purify this product were unsuccessful. However, its p.m.r. spectrum was in close agreement with that published by Onodera and Hirano[29], and on deacylation it gave α-D-mannopyranosyl phosphate (**9b**), isolated as a dilithium salt $[\alpha]_D$ +42° (*c*, 1.0, water); lit.[30] $[\alpha]_D$ +46.3°. The p.m.r. spectrum of a solution in deuterium oxide was identical with that recorded for an authentic sample[29]. The crude sodium salt (**9a**; 500 mg, 1.66 mmole) was converted into a pyridinium salt, which was dried by repeatedly evaporating pyridine from it, and it was finally dissolved in anhydrous pyridine (20 ml). Methyl 2,3,4-tri-*O*-acetyl-α-D-mannopyranoside

(**6**; 500 mg, 1.66 mmole) and DCC (3 g, 15 mmole) were added, and the reaction mixture was kept for 18 h at 37°. Water (10 ml) was added and the crystalline *N,N'*-dicyclohexylurea filtered off. The filtrate was evaporated to a volume of 5 ml and the solution adjusted to pH 11 with 2M sodium hydroxide. After 15 min the solution was passed through a cation-exchange resin (H^+ form), and the eluate was neutralised immediately with cyclohexylamine. Excess cyclohexylamine was removed by extraction (3 times) of the eluate with ether. The eluate was then evaporated to dryness and the residue dissolved in propyl alcohol. Addition of petroleum ether (b.p. 60–80°) yielded the cyclohexylammonium salt of **11** (350 mg, 62%), m.p. 205–210° (decomp.), $[\alpha]_D$ +62.5°; Calc. for $C_{19}H_{38}NO_{14}P$: C, 42.6; H, 7.2; N, 2.6; P, 5.8%; Found: C, 43.0; H, 7.3; N, 2.75; P, 5.7.

Methyl α-D-mannopyranoside 4-phosphate (**4**). — Diphenyl phosphorochloridate (1.25 g, 4.6 mmole) was added to methyl 2,3,6-tri-*O*-benzoyl-α-D-mannopyranoside[11] (**1**, 2 g, 4 mmole) and the solution was kept for 18 h at 37°. Water (5 ml) was added, and after 2 h the solvents were removed and the residue dissolved in chloroform. The solution was washed with ice-cold hydrochloric acid (1% solution), ice-cold sodium hydrogen carbonate solution (5% w/v) and ice–water, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in ethanol (30 ml) and hydrogenated over Adams platinum. When the uptake of hydrogen was complete, the catalyst was filtered off and the filtrate adjusted to pH 11–12 with 2M sodium hydroxide. After 15 min the solution was passed through a cation-exchange resin (H^+ form) and the eluate extracted five times with ether to remove benzoic acid, before neutralisation with cyclohexylamine and three further extractions with ether. The dicyclohexylammonium salt of **4** (700 mg, 38%) was precipitated from a concentrated aqueous solution on addition of acetone, m.p. 136–143° (decomp.), $[\alpha]_D$ +75.5°; Calc. for $C_{19}H_4O_9PN_2$: C, 48.1; H, 8.7; N, 5.9; P, 8.3%; Found: C, 48.5; H, 8.8; N, 6.0; P, 8.4.

Methyl α-D-mannopyranoside 4-(α-D-mannopyranosyl phosphate) (**10**). — The pyridinium salt (500 mg, 1.66 mmole) of **9a** was condensed with methyl 2,3,6-tri-*O*-benzoyl-α-D-mannopyranoside (**1**; 680 mg, 1.7 mmole) under the reaction conditions described for compound **11**. The reaction mixture was processed as for compound **11** except that the acidified solution of the debenzoylated product was extracted with ether to remove benzoic acid as described for compound **4**. The cyclohexylammonium salt of **10** (300 mg, 49%) was homogeneous on paper chromatography m.p. 135–142° (decomp.); $[\alpha]_D$ +75.5°; Calc. for $C_{19}H_{38}NO_{14}P$: C, 42.6; H, 7.2; N, 2.6; P, 5.7%; Found: C, 42.3; H, 6.9; N, 2.4; P, 5.7.

Hydrolysis of phosphate esters with acid. — *A.* Samples (5 mg) of phosphate esters were dissolved in water (1 ml) and passed through a cation-exchange resin (H^+ form). The eluate was taken to dryness, the residue dissolved in water (0.2 ml), and the solution heated for 20 min at 100° in a sealed tube. Hydrolysis products were identified by paper chromatography.

B. Samples (5 mg) of the phosphate esters were heated with 2M hydrochloric acid (1 ml) in a sealed tube for 2 h at 100°. Water (15 ml) was added to the hydrolysate,

which was then extracted with tri-*n*-octylamine in chloroform. The neutral, aqueous phase was washed five times with chloroform, concentrated to low volume, and hydrolysis products were identified by paper chromatography.

Hydrolysis of phosphate esters with alkali. — *A.* Samples (5 mg) were dissolved in 0.5M sodium hydroxide (1 ml) and heated for 4 h at 100°. After cooling, the sample was diluted with water (2 ml) and passed through a column of cation-exchange resin (H^+ form). The eluate was neutralised with pyridine and the products separated by paper chromatography.

B. Samples (2–5 mg) of cyclic phosphate diesters were hydrolysed in 0.5M sodium hydroxide (1 ml) for 18 h at 37° and hydrolysis product separated as in *A*.

Reaction of sugar phosphates with DCC. — The sugar phosphate sample (10 mg) in water was converted into a pyridinium salt on a cation-exchange resin in the pyridinium form, and the solution was evaporated to dryness. The residue was dissolved in pyridine (1 ml), and triethylamine (0.1 ml), water (0.1 ml), and DCC (50 mg) were added. The reaction mixture was kept for 18 h at room temperature. Water (5 ml) was added and the crystalline *N,N'*-dicyclohexylurea filtered off. The filtrate was extracted three times with ether, and the reaction products in the aqueous phase were isolated by preparative paper chromatography. Similar conditions were used for checking the formation of *N*-phosphorylureas when sugar phosphates were treated with DCC in aqueous pyridine and in the absence of triethylamine.

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