Carbohydrate Research, 95 (1981) 145–154
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

PURIFICATION OF 2,5-ANHYDRO-D-HEXITOL BIS(PHOSPHATES) AND IDENTIFICATION OF A MAJOR 1,4,6-TRIS(PHOSPHATE) CONTAMINANT BY ^{31}P-, ^{13}C-, AND ^{1}H-N.M.R. SPECTROSCOPY*†

RONALD J. VOLL, THEODORE A. W. KOERNER, JR.‡, PAUL A. BARTLETT**, NORMAN S. BHACCA††, DAVID C. LANKIN††, AND EZZAT S. YOUNATHAN‡‡

Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803 (U.S.A.)

(Received November 7th, 1980; accepted for publication, March 2nd, 1981)

ABSTRACT

The reaction of two equivalents of diphenylchlorophosphate in cold pyridine with 2,5-anhydrohexitols has been assumed to result in only 1,6-bis(diphenylphosphate) products. However, by thin-layer, silica gel dry-column, and DEAE-Sephadex A-25 column chromatography, the products of this reaction have been shown to contain three major components; monophosphates (32 or 30%, by weight), 1,6-bis(phosphates) (40 or 56%), and 1,4,6-tris(phosphates) (28 or 14%): the former percentages for the product from 2,5-anhydro-D-mannitol (**1**) and the latter for the product from 2,5-anhydro-D-glucitol (**10**). The identity of each bis- and tris-(phosphate) of **1** or **10** was established by ^{31}P- and ^{13}C-n.m.r. spectroscopy. Acetylated bis- and tris-(diphenylphosphates) of **1** were also examined by ^{1}H-n.m.r. The significance of these findings on the interpretation of studies of the anomeric specificity of enzymes and on the specificity of the reagent diphenylchlorophosphate are discussed. The formation of only a 1,4,6-tris(phosphate) of **10** suggests that the 1,6-bis(diphenylphosphate) of **10** may undergo formation of a 1,3-cyclic phosphate triester by transesterification with elimination of phenol. A method for the determination of the number of cyclohexylammonium groups crystallizing with a sugar phosphate is proposed that simplifies the elemental analysis of this type of salt.

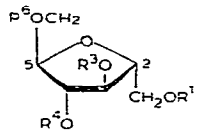
INTRODUCTION

2,5-Anhydro-D-hexitol phosphates have been shown to be excellent substrate analogues in many studies[3–5] on the anomeric specificity of the enzymes that utilize

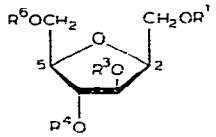
*Part III of a series "New Derivatives of 2,5-Anhydro-D-hexitols". For Parts I and II, see refs. 1 and 2.
†Supported by grant numbers PCM 77-27286 from the National Science Foundation, and AM-21307 from the National Institutes of Health.
‡Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06510, U.S.A.
**Department of Chemistry, University of California, Berkeley, California 94720, U.S.A.
††Department of Chemistry, Louisiana State University.
‡‡To whom inquiries should be addressed.

0008-6215/81/0000–0000/$ 02.50,

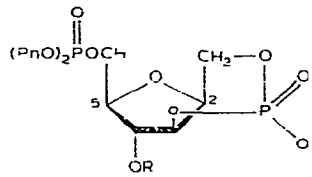
D-fructose phosphates. However, some enzyme studies with 2,5-anhydro-D-mannitol 1,6-bis(phosphate) (**8**) and 2,5-anhydro-D-glucitol 1,6-bis(phosphate) (**15**) have led to ambiguous results. For example, in studies of the anomeric specificity of the activation of yeast pyruvate kinase, compounds **8** and **15** were found either to be ineffective[6] or excellent[7] analogues of D-fructose 1,6-bis(phosphate). More recently, studies[8,9] on the anomeric specificity of D-fructose 1,6-bisphosphatase with the same analogues **8** and **15** have resulted in opposing conclusions.



1: R^1, R^3, R^4, R^6 = H
2: R^6 = $PO(OPh)_2$; R^1, R^3, R^4 = H
3: R^1, R^6 = $PO(OPh)_2$; R^3, R^4 = H
4: R^1, R^6 = $PO(OPh)_2$; R^3, R^4 = Ac
5: R^1, R^4, R^6 = $PO(OPh)_2$; R^3 = H
6: R^1, R^4, R^6 = $PO(OPh)_2$; R^3 = Ac
7: R^6 = $PO(OH)_2$; R^1, R^3, R^4 = H
8: R^1, R^6 = $PO(OH)_2$; R^3, R^4 = H
9: R^1, R^4, R^6 = $PO(OH)_2$; R^3 = H



10: R^1, R^3, R^4, R^6 = H
11: R^6 = $PO(OPh)_2$; R^1, R^3, R^4 = H
12: R^1, R^6 = $PO(OPh)_2$; R^3, R^4 = H
13: R^1, R^4, R^6 = $PO(OPh)_2$; R^3 = H
14: R^6 = $PO(OH)_2$; R^1, R^3, R^4 = H
15: R^1, R^6 = $PO(OH)_2$; R^3, R^4 = H
16: R^1, R^4, R^6 = $PO(OH)_2$; R^3 = H



17: R = H
18: R = $PO(OPh)_2$

The only apparent difference in the two studies on pyruvate kinase was the more rigorous purification of the analogues employed in the second[7] of the two studies. In fact, in all of the other studies in the literature, the analogues (**8** and **15**) that were employed were prepared in cold pyridine according to the original procedure of Hartman and Barker[10] and used without additional purification. We now report that high-resolution column chromatography reveals that these analogues as usually prepared[10,11], contain three major components. The exact structure of these components has been studied by multinuclear n.m.r. spectroscopy. Reasons for the formation of these components is discussed.

A serendipitous finding made during the ^{13}C-n.m.r. studies was the discovery of a method for the determination of the exact number of cyclohexylammonium cations per mole of sugar phosphate. This information provides a reliable basis for comparing experimental data of elemental analysis with theoretically calculated values for cyclohexylammonium salts of sugar phosphates.

RESULTS

Chromatography. —T.l.c. of each 2,5-anhydro-D-hexitol bis(diphenylphosphate) prepared according to the literature[10,11] revealed a mixture of three components. Silica gel, dry-column chromatography of the diphenylphosphate adducts of 2,5-anhydro-D-mannitol (**1**) led to the resolution of three components and isolation of

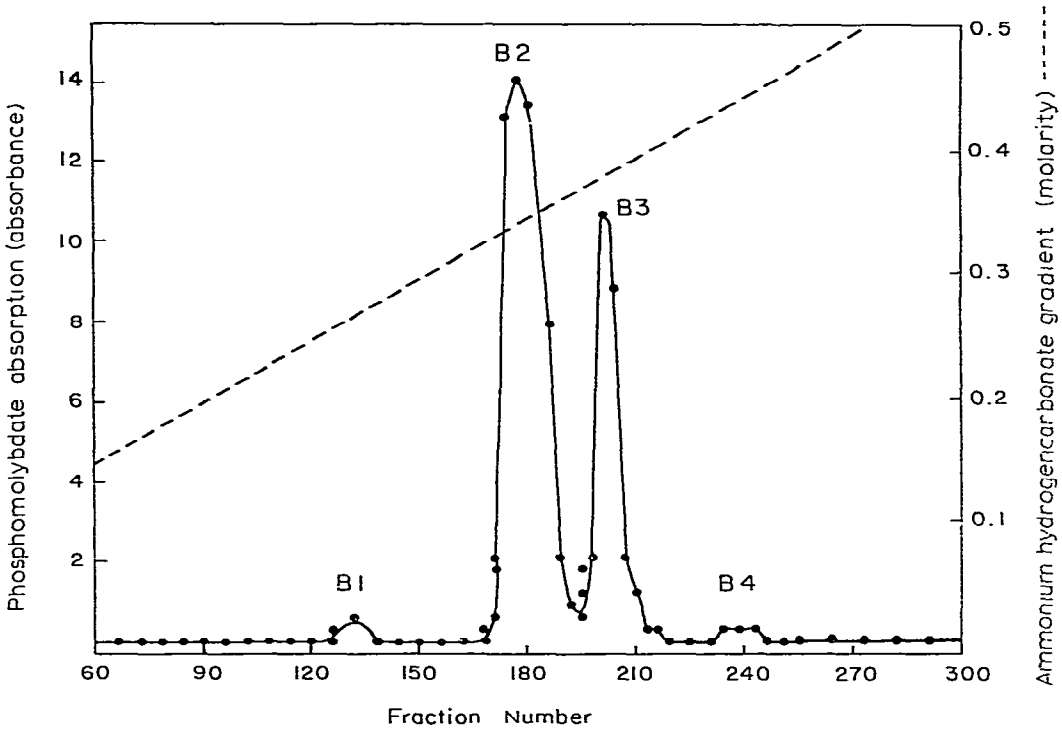


Fig. 1. Column chromatography of 2,5-anhydro-D-glucitol phosphates on DEAE-Sephadex A-25. The four fractions resolved were shown to be: B1: monophosphate; B2: 1,6-bis(phosphate); B3. 1,4,6-tris(phosphate); and B4: tetrakis(phosphate).

the two fastest-moving components in pure form. The fastest moving of the three components (A1) comprised 28% of the total mixture by weight, the next fastest component (A2) comprised 40%, and a broad component at the origin (A3) amounted to 32%.

Dry-column chromatography was attempted for the mixture of diphenylphosphate adducts of 2,5-anhydro-D-glucitol (**10**), but it failed to resolve the mixture. The crude material was then converted by hydrogenolysis into the mixture of free phosphates and the products resolved on a DEAE-Sephadex column (Fig. 1) into four components (B1–B4). The major components, B2 and B3, were isolated in 80 and 20% yields (by weight), respectively. Component B1 was isolated from the column in trace amounts, but it was subsequently observed that the barium salts of the monophosphates of **10** were ethanol soluble and this component, present in substantial amounts (30%), had been removed from the mixture prior to DEAE–Sephadex chromatography. Thus, the overall composition by weight of diphenylphosphorylation products of **10** was 30% monophosphates, 56% bis(phosphates), and 14% tris(phosphates). Component B4 was the tetrakis(phosphate) of **10**, present in trace amounts. Previous attempts[12] to purify the 2,5-anhydro-D-glucitol phosphates failed to resolve the bis- from the tris-(phosphate), apparently because less than half

TABLE I

CARBON-13 CHEMICAL-SHIFT DATA AND CYCLOHEXYLAMMONIUM NUMBERS (C_n) FOR FREE PHOSPHATES **7, 8, 9,** AND **14, 15, 16**[a]

Compound	*Carbon atom*						C_n[b]
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	
7	61.98	83.36	77.20	77.72	82.58	64.70	1.8
8	64.76	82.23	77.07	77.07	82.23	64.76	2.8
9	64.95	82.57	77.16	79.83	82.14	64.82	3.8
14	60.89	81.79	77.72	78.87	84.63	64.94	2.2
15	62.15	81.28	77.55	78.92	85.10	64.80	3.0
16	62.66	81.49	76.75	80.91	85.17	65.08	4.9

[a]Chemical shifts are in p.p.m. downfield from tetramethylsilane and were determined relative to internal 1,4-dioxane (67.40 p.p.m.); spectra were obtained at 50.3 MHz from D_2O–H_2O solutions; accuracy, ±0.05 p.p.m. [b]Cyclohexylammonium number calculated as shown in text (±0.2). All spectra displayed four cyclohexylammonium resonances assigned as follows (±0.15 p.p.m.): C-1, 51.0; C-2 and C-6, 31.1; C-3 and C-5, 24.5; and C-4, 25.0.

the number of fractions were taken, and these two phosphates have very similar retention-times.

Identification of components. — Each major component or its derivative was subjected to multinuclear n.m.r. spectroscopy, from which its structure was deduced. Carbon-13 resonances (Table I) were assigned by off-resonance decoupling and by comparison with the spectra[13] of 2,5-anhydro-D-hexitols and their monophosphates. Phosphorus-31 resonances are assigned only tentatively because of the lack of model compounds in the literature. Proton resonances were assigned by decoupling and by comparison with the spectra[1,2] of closely related systems.

The free phosphate derived from component A2 by hydrogenolysis gave a single resonance in its ^{31}P-n.m.r. spectrum and three resonances of equal area in its ^{13}C-n.m.r. spectrum. Such degenerate spectral patterns indicate that the free phosphate of component A2 is highly symmetrical (C_2 axis) and must have a bis(phosphate) structure. The downfield shift and small $^2J_{CP}$ coupling (~5 Hz) of the C-1, C-6 resonance and large $^3J_{CP}$ coupling (~8 Hz) to C-2, C-5 establish a 1,6-bis(phosphate) structure (**8**) for the free phosphate derivative and a 1,6-bis(diphenylphosphate) structure (**3**) for component A2. The symmetrical (AB)XX'MM'(A'B') pattern observed for the 8-proton anhydrohexitol subspectrum in the ^{1}H-n.m.r. spectrum of the diacetate (**4**) of component A2 confirms the assigned structure (**3**) of the latter.

The free phosphate derivative of component A1 gave three resonances in its ^{31}P-n.m.r. spectrum, indicating that it was a tris(phosphate). Inspection of the ^{13}C-n.m.r. spectrum of this free phosphate revealed that the phosphorylation sites were hydroxyl groups of C-1, -4, and -6 (equivalent to 1, 3, and 6), based on phosphorylation-induced chemical shifts and ^{31}P–^{13}C couplings. Thus, the free phosphate

TABLE II

^{31}P–^{13}C COUPLING-CONSTANTS FOR FREE PHOSPHATES **7**, **8**, **9** AND **14**, **15**, **16**[a]

Compound	*P-1*		*P-4*			*P-6*	
	$^{2}J_{C-1,P-1}$	$^{3}J_{C-2,P-1}$	$^{2}J_{C-4,P-4}$	$^{3}J_{C-3,P-4}$	$^{3}J_{C-5,P-4}$	$^{2}J_{C-6,P-6}$	$^{3}J_{C-5,P-6}$
7						4.3	7.9
8	4.9	8.5				4.9	8.5
9	4.9	7.9	4.9	3.1	8.0	5.2	8.0
14						4.9	7.9
15	4.3	7.3				4.9	7.9
16	4.3	7.9	4.9	3.7	7.9	4.3	7.9

[a]Coupling constants (J) are either through two bonds (^{2}J) or three bonds (^{3}J) and are absolute values in Hz, estimated error, ±0.3 Hz.

derivative has a 1,4,6-tris(phosphate) structure* (**9**) and component A1 a 1,4,6-tris(diphenylphosphate) structure* (**5**). The asymmetric (AB)HMRV(YZ) pattern observed for the 8-proton anhydrohexitol subspectrum in the ^{1}H-n.m.r. spectrum of the acetate derivative (**6**) of component A1 confirms the assigned structure (**5**) of the latter.

Components B2 and B3 displayed two and three resonances in their ^{31}P-n.m.r. spectra, and are thus bis(phosphate) and tris(phosphate) structures, respectively. Through analysis of their ^{31}P–^{13}C coupling-constants (Table II), the phosphorylation sites of these components were determined and B2 was shown to have a 1,6-bis(phosphate) (**15**) and B3 a 1,4,6-tris(phosphate) structure (**16**).

Calculation of salt ratios for elemental analysis. — Cyclohexylammonium salts have long been advocated[14] as the best form in which to isolate sugar phosphates because of their crystallinity and fairly sharp melting-points. However, cyclohexylammonium salts have the disadvantage of often giving elemental analyses that do not agree with the theoretical values[14]. This is the result of the unpredictable number of cyclohexylammonium cations that associate with a given sugar phosphate, or with changes in this number under different crystallization conditions (pH, solvent, etc.).

We have found that this problem of variable cyclohexylammonium number may be completely overcome by inspection of the ^{13}C-n.m.r. spectrum of a sample of each salt prior to microanalysis and calculation of the cyclohexylammonium number (C_n) as follows:

$$C_n = \frac{I_c}{I_s} \times k,$$

*2,5-Anhydro-D-mannitol derivatives have been numbered so as to be comparable with 2,5-anhydro-D-glucitol derivatives.

where, I_c is the integral of the C-1 methine resonance of the cyclohexylammonium cation (51.0 p.p.m.), I_s is the integral of a methine atom of the sugar or sugar derivative, and k is an empirical constant equal to 1.15 for spectra recorded in D_2O–H_2O. Using the nearest integral number of cyclohexylammonium cations (± 0.2) to the calculated value of C_n in the calculation of elemental percentages gives values that accurately predict the found values (see Table I and experimental section).

DISCUSSION

Relevance to enzyme studies. — The presence of large quantities of mono- and tris-(phosphate) contaminants in the 2,5-anhydro-D-hexitol bis(phosphate) preparations[10,11] usually used for enzyme-kinetic studies may be the basis for differences in conclusions concerning the anomeric specificity of enzymes, especially when crude materials are utilized. Differences in the activity of enzymes toward crude and purified materials result from the diminished amount of bis(phosphate) analogue actually present in the crude preparation, and possibly nonspecific or even specific effects of mono- and tris-(phosphate) contaminants on enzyme activity. Studies of enzymic properties of the novel 2,5-anhydro-D-hexitol 1,4,6-tris(phosphates) (**9**, **16**) described herein are underway in this laboratory. It should be clear from the foregoing that future studies of the anomeric specificity of enzymes should employ only sugar phosphate analogues whose purity has been rigorously established, and that studies in the literature performed with crude material are suspect.

Phosphorylation with diphenylchlorophosphate. — When 2,5-anhydro-D-hexitols are treated with two equivalents of diphenylchlorophosphate, considerable amounts of tris(phosphates) are produced, affirming that this phosphorylating reagent is not as specific for primary hydroxyl groups as had been assumed[14]. It is thus essential that sugars or analogues to be specifically phosphorylated for enzyme studies be completely protected at all but the desired point of phosphorylation.

An interesting aspect of the analysis of the diphenylphosphorylation products of 2,5-anhydro-D-glucitol is that the 1,4,6-tris(phosphate) is the only tris(phosphate) formed. This finding suggests that there is a strong steric preference for 4-*O*-diphenylphosphorylation after 1-*O*- and 6-*O*-diphenylphosphorylation or (more appealingly) a 1,3-cyclic phenylphosphate triester intermediate (**17**) is formed from 2,5-anhydro-D-glucitol 1,6-bis(diphenylphosphate) (**4**) and this blocks the HO-3 from esterification. Thus, further diphenylphosphorylation of **17** would result only in formation of **18**. The 1,3-cyclic phosphate of **18** would open up during the hydrogenolysis step, which creates acidic conditions, to give the 1,4,6-tris(phosphate) product that is found. Evidence supporting the formation of the cyclic phosphate intermediate is the detection of large quantities of phenol in the diphenylphosphorylation reaction mixture, and our recent isolation of **18** in crystalline form from the same mixture.

EXPERIMENTAL

General methods. — General analytical and synthetic procedures were as previously published[1,2]. Dry-column silica gel Woelm and u.v.-transparent nylon-film tubing were obtained from ICN. Packed dry columns had the dimensions of 50 (diameter) × 600 mm. Dry columns were monitored by u.v. illumination. DEAE–Sephadex A-25 was obtained from Pharmacia. Inorganic- and total-phosphate were assayed according to the procedure of Bartlett[15]. Fourier-transform n.m.r. spectra were recorded with a Bruker WP200 Electrospin superconducting spectrometer at 50.3 MHz for ^{13}C, 80.8 MHz for ^{31}P, and 200 MHz for ^{1}H studies. All ^{31}P and ^{13}C spectra were proton-decoupled.

Catalytic hydrogenolyses were performed in a Parr, shaker-type hydrogenation apparatus[16] at a pressure of one atmosphere in the presence of 100–300 mg of platinum oxide in abs. methanol. Hydrogenolyses were continued until 100% of the calculated amount of hydrogen had been taken up (0.5–3.0 h). The product of each hydrogenolysis was filtered to remove the catalyst, made neutral (pH 7.0) with cyclohexylamine, and evaporated *in vacuo*. The solution of the resulting viscous syrup in water (10 mL) was adjusted to pH 11.0 with cyclohexylamine and slowly diluted with acetone (100–200 mL). In each case, this procedure afforded a crystalline cyclohexylammonium salt.

*Crude 2,5-anhydro-*D*-hexitol 1,6-bis(diphenylphosphate)s.* — Putative 2,5-anhydro-D-mannitol 1,6-bis(diphenylphosphate) and 2,5-anhydro-D-glucitol 1,6-bis(diphenylphosphate) were prepared from 2,5-anhydro-D-mannitol[17,18] (**1**) or 2,5-anhydro-D-glucitol[1] (**10**) and diphenylchlorophosphate in dry pyridine according to the procedure of Hartman and Barker[10]. The resulting syrupy products were heterogeneous by t.l.c. with ethyl acetate; three components were observed in the product from **1** (R_F 0.0–0.1, 0.35, and 0.58) and in the product from **10** (R_F 0.0–0.1, 0.34, and 0.63).

*Chromatography of crude 2,5-anhydro-*D*-mannitol 1,6-bis(diphenylphosphate).* — The diphenylphosphorylated derivatives of **1** (2.5 g) were applied to a dry column of silica gel in ethyl acetate and the charged column was developed with dry diethyl ether. The resulting three u.v.-positive bands were cut from the column, eluted with ethyl acetate, and pooled. Each dry-column band was identical with one of the three t.l.c. bands. The component at the origin (A3) was 32% of the mixture (by weight) and had a mobility comparable with that of a reference specimen of 2,5-anhydro-D-mannitol 6-diphenylphosphate (**2**), but was not further characterized because of its t.l.c. broadness (heterogeneity). The other components were characterized as follows.

*2,5-Anhydro-*D*-mannitol 1,6-bis(diphenylphosphate)* (**3**). — Component A2 was obtained as a syrup that crystallized after several weeks at room temperature. Recrystallization from ethyl acetate gave fine needles; yield 1.0 g (40% by wt.), m.p. 65–66°.

Anal. Calc. for $C_{30}H_{30}O_{11}P_2$ (628.5); C, 57.33; H, 4.81. Found: C, 57.31; H, 4.91.

3,4-Di-O-acetyl-2,5-anhydro-D-mannitol 1,6-bis(diphenylphosphate) (**4**). — A sample of **3** (100 mg) was conventionally[1] acetylated with an excess of acetic anhydride in dry pyridine for ^{1}H-n.m.r. analysis. ^{1}H-N.m.r. data (chloroform-*d*, Me_4Si; 100 MHz): δ 1.94 (s, 6 H, acetate methyl groups), 4.21 (2 H, XX′ of AA′XX′, X of two equivalent ABX systems, $J_{1,2} = J_{5,6}$ 5.0, $J_{1',2} = J_{5,6'}$ 4.0, $^4J_{2,P} = {}^4J_{5,P}$ = 2.0, $J_{2,3} = J_{4,5}$ 4.0 Hz, H-2 and H-5), 4.37 (2 H, A of two ABX, $J_{1,1'} = J_{6,6'}$ <0.1, $J_{1',2} = J_{5,6'}$ 4.0, $J_{1',P} = J_{6',P}$ 7.0 Hz, H-1′ and H-6′), 4.38 (2 H, B of two ABX, $J_{1,1'} = J_{6,6'}$ <0.1, $J_{1,2} = J_{5,6}$ 5.0, $J_{1,P} = J_{6,P}$ 7.0 Hz, H-1 and H-6), 5.24 (2 H, AA′ of AA′XX′, $J_{2,3} = J_{4,5}$ 4.0, $J_{3,4}$ 2.8 Hz, H-3 and H-4), and 7.0–7.4 (m, 20 H, phenyl protons); composite 8-proton anhydrohexitol pattern: (AB)XX′MM′(A′B′).

2,5-Anhydro-D-mannitol 1,4,6-tris(diphenylphosphate) (**5**). — Component A1 was obtained as a syrup; yield 0.7 g (28% by wt.).

Anal. Calc. for $C_{42}H_{39}O_{14}P_3$ (860.7): C, 58.61; H, 4.57. Found: C, 59.12; H, 4.28.

3-O-Acetyl-2,5-anhydro-D-mannitol 1,4,6-tris(diphenylphosphate) (**6**). — A sample of **5** (100 mg) was conventionally[1] acetylated with an excess of acetic anhydride in dry pyridine for ^{1}H-n.m.r. analysis. ^{1}H-N.m.r. data (chloroform-*d*, Me_4Si; 100 MHz): δ 1.90 (s, 3 H, acetate methyl), 4.08 (1 H, X of first ABX, X of AMRX, $J_{4,5} = J_{5,6} = J_{5,6'}$ 4.0, $J_{5,P\text{-}6}$ 2.0 Hz, H-5), 4.20 (1 H, X of second ABX, A of AMRX, $J_{1,2} = J_{1',2'} = J_{2,3}$ 3.5, $J_{2,P\text{-}1}$ 1.5 Hz, H-2), 4.27 (2 H, AB of second ABX, $J_{1,1'}$ <0.1, $J_{1,2} = J_{1',2}$ 3.5, $J_{1,P\text{-}1} = J_{1',P\text{-}1}$ 7.0 Hz, H-1 and H-1′), 4.35 (2 H, AB of first ABX, $J_{6,6'}$ <0.1, $J_{5,6} = J_{5,6'}$ 4.0, $J_{6,P\text{-}6} = J_{6',P\text{-}6}$ 7.3 Hz, H-6 and H-6′), 5.16 (1 H, R of AMRX, $J_{3,4} = J_{4,5}$ 4.0, $J_{4,P\text{-}4}$ 8.0 Hz, H-4), 5.35 (1 H, M of AMRX, $J_{2,3}$ 3.5, $J_{3,4}$ 4.0 Hz, H-3), and 7.0–7.4 (m, 30 H, phenyl protons); composite 8-proton anhydrohexitol pattern: (AB)HMRV(YZ).

2,5-Anhydro-D-mannitol 1,6-bis(phosphate) tris(cyclohexylammonium) salt (**8**). — A solution of **3** (3.00 g, 4.78 mmol) in methanol was hydrogenolyzed and the product processed (see preceding) to yield fine needles (2.45 g, 83%); m.p. 192–196°; ^{31}P-n.m.r. data (deuterium oxide, external H_3PO_4): δ 2.22 (s, 2P, P-1 and P-6). For ^{13}C-n.m.r. data, see Tables I and II.

Anal. Calc. for $C_{24}H_{53}N_3O_{11}P_2 \cdot H_2O$ (639.7): C, 45.06; H, 8.67; N, 6.57; P, 9.68. Found: C, 45.51; H, 8.86; N, 6.65; P, 9.83.

2,5-Anhydro-D-mannitol 1,4,6-tris(phosphate) tetrakis(cyclohexylammonium) salt (**9**). — A solution of **5** (2.00 g, 2.32 mmol) in methanol was hydrogenolyzed and the product processed (see General Methods) to yield fine needles containing water and acetone of crystallization (1.50 g, 79%); m.p. 196–199°; ^{31}P-n.m.r. data (deuterium oxide, external H_3PO_4): δ 1.13 (s, 1P, P-6), 2.03 (s, 1P, P-1), and 3.27 (s, 1P, P-4).

Anal. Calc. for $C_{30}H_{67}N_4O_{14}P_3 \cdot 2\ H_2O \cdot C_3H_6O$ (894.9): C, 44.29; H, 8.67; N, 6.26; P, 10.38. Found: C, 43.74; H, 8.79; N, 6.72; P, 10.31.

Chromatography of crude 2,5-anhydro-D-glucitol 1,6-bis(phosphate). — Solutions of the diphenylphosphorylated derivatives of **10** (2.70 g) were hydrogenolyzed, filtered, and made neutral with barium methoxide (7.65 mmol). The resulting sus-

pension was filtered and the filtrate precipitated with 4 volumes of abs. ethanol. This procedure removed the ethanol-soluble barium salts (30% by weight) of the monophosphates of **10**. A portion of the resulting precipitate (200 mg) in water was applied to a column (23 × 285 mm) of DEAE–Sephadex A-25 that was eluted with a 0.1–0.5M linear buffer gradient of ammonium hydrogencarbonate. Fractions (5 mL) were collected, and analyzed for total phosphate. This procedure resolved four fractions (Fig. 1, Peaks B1–B4) each of which was pooled, acidified with Dowex-50 resin (H^+ form), filtered, and the product isolated as its cyclohexylammonium salt (see General Methods). Peaks B1 and B4 were present in quantities too small for analysis, but had column retention-times characteristic of mono- and tetrakis-(phosphate)s, respectively. The major peaks (B2 and B3) were characterized as follows.

2,5-Anhydro-D-glucitol 1,6-bis(phosphate) tris(cyclohexylammonium) salt (**15**). — Peak B2 yielded fine needles (160 mg, 80% by wt.); m.p. 168–171°; ^{31}P-n.m.r. data (deuterium oxide, external H_3PO_4): δ 4.38 (s, 1P, P-1) and 4.93 (s, 1P, P-6). For ^{13}C-n.m.r. data, see Tables I and II.

Anal. Calc. for $C_{24}H_{53}N_3O_{11}P_2$ (621.6): C, 46.37; H, 8.59; N, 6.76; P, 9.96. Found: C, 46.28; H, 8.70; N, 6.86; P, 9.60.

2,5-Anhydro-D-glucitol 1,4,6-tris(phosphate) pentakis(cyclohexylammonium) salt (**16**). — Peak B3 yielded fine needles (40 mg, 20% by wt.); m.p. 187–191°; ^{31}P-n.m.r. data (deuterium oxide, external H_3PO_4): δ 3.22 (s, 1P, P-4), 3.41 (s, 1P, P-6), and 4.17 (s, 1P, P-1). For ^{13}C-n.m.r. data, see Tables I and II.

Anal. Calc. for $C_{36}H_{80}N_5O_{14}P_3 \cdot 1.5\ H_2O$ (927.0): C, 46.64; H, 9.02; N, 7.56; P, 10.02. Found: C, 46.50; H, 8.99; N, 7.37; P, 10.52.

ACKNOWLEDGMENTS

The authors thank Mr. R. Seab for performing some of the elemental analyses, and Mr. J. R. Martin for recording the proton-n.m.r. spectra.

REFERENCES

1 T. A. W. KOERNER, JR., R. J. VOLL, AND E. S. YOUNATHAN, *Carbohydr. Res.*, 59 (1977) 403–416.
2 T. A. W. KOERNER, JR., R. J. VOLL, AND E. S. YOUNATHAN, *Carbohydr. Res.*, 70 (1979) 165–168.
3 S. J. BENKOVIC AND K. J. SCHRAY, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 44 (1976) 139–164.
4 B. WURSTER AND B. HESS, *FEBS Lett.*, 40S (1974) 112–118.
5 T. A. W. KOERNER, JR., R. J. VOLL, AND E. S. YOUNATHAN, *FEBS Lett.*, 84 (1977) 207–213.
6 R. FISHBEIN, P. A. BENKOVIC, AND S. J. BENKOVIC, *Biochemistry*, 14 (1975) 4060–4063.
7 B. WURSTER, B. HESS, T. A. W. KOERNER, JR., R. J. VOLL, AND E. S. YOUNATHAN, *FEBS Lett.*, 63 (1976) 17–21.
8 C. J. MARCUS, *J. Biol. Chem.*, 251 (1976) 2963–2966.
9 W. A. FREY, R. FISHBEIN, M. M. DEMAINE, AND S. J. BENKOVIC, *Biochemistry*, 16 (1977) 2479–2484.
10 F. C. HARTMAN AND R. BARKER, *Biochemistry*, 4 (1965) 1068–1075.
11 S. J. BENKOVIC, J. J. KLEINSCHUSTER, M. M. DEMAINE, AND I. J. SIEWERS, *Biochemistry*, 10 (1971) 4881–4887.
12 G. R. GRAY, *Biochemistry*, 10 (1971) 4705–4711.
13 T. A. W. KOERNER, JR., R. J. VOLL, L. W. CARY, AND E. S. YOUNATHAN, *Biochemistry*, 19 (1980) 2795–2801.

14 C. E. BALLOU AND D. L. MACDONALD, *Methods Carbohydr. Chem.*, 2 (1963) 270–272.
15 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466–468.
16 T. A. W. KOERNER, JR., Ph.D. Dissertation (1975), Louisiana State University, Baton Rouge.
17 B. C. BERA, A. B. FOSTER, AND M. STACEY, *J. Chem. Soc.*, (1956) 4531–4535.
18 T. A. W. KOERNER, JR., E. S. YOUNATHAN, A. E. ASHOUR, AND R. J. VOLL, *J. Biol. Chem.*, 249 (1974) 5749–5754.